

Index to U.S. Food and Drug Administration's (FDA) Response to Natural Resources Defense Council's (NRDC) October 11, 2013 Freedom of Information Act (FOIA) Request (FDA FOA Request No 2013-8042) for 20 Selected Generally Recognized as Safe (GRAS) Notices (GRN) Submitted to the Agency between 1998 and 2013.

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¹ Posted at www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing . ² See file labelled "chemicals-in-food-FOIA-Main.pdf" for information on other GRNs.				



NATURAL RESOURCES DEFENSE COUNCIL

October 11, 2013

VIA FAX AND CERTIFIED MAIL

Food and Drug Administration
Division of Freedom of Information
Office of Shared Services
Office of Public Information and Library Services
12420 Parklawn Drive
ELEM-1029
Rockville, MD 20857
FAX: (301) 827-9267

Re: FOIA Request for Records Regarding Generally Recognized as Safe Notices Received by the Agency

Dear FOIA Officer:

I write on behalf of the Natural Resources Defense Council (NRDC) to request disclosure of records pursuant to the Freedom of Information Act ("FOIA"), 5 U.S.C. § 552, and applicable Food and Drug Administration ("FDA") regulations, 21 C.F.R. Part 20.

I. Description of Records Sought

Please produce records¹ in FDA's possession, custody or control on or before September 30, 2013 associated with the Generally Recognized as Safe (GRAS) notices described in Appendix A. We are specifically seeking the following records:

- (a) Communications between FDA and the individual or firm that submitted the GRAS notice to the agency as well as the additive manufacturer or their representative;
- (b) Comments received from other persons outside the agency regarding the GRAS notice;
- (c) Communications between FDA and the European Food Safety Authority regarding the substance or substances described in the GRAS notice; and

¹ "Records" means anything denoted by the use of that word or its singular form in the text of FOIA and includes correspondence, minutes of meetings, memoranda, notes, emails, notices, facsimiles, charts, tables, presentations, orders, filings, and other writings (handwritten, typed, electronic, or otherwise produced, reproduced, or stored). This request seeks responsive records in the custody of any FDA office, including, but not limited to, FDA Headquarters offices, and specifically including FDA offices in possession of records regarding the GRAS notifications described in Appendix A.

- (d) Memo from FDA's scientific staff describing the preliminary or final results of their evaluation of the GRAS notices exposure assessment, toxicity assessment, safety assessment, or environmental impact.

We are not seeking:

- (a) Copies of the notices and agency decisions FDA posted at <http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing> as of October 10, 2013.

II. Request for a Fee Waiver

NRDC requests that FDA waive the fee that it would otherwise charge for search and production of the records described above. FOIA dictates that requested records be provided without charge "if disclosure of the information is in the public interest because it is likely to contribute significantly to public understanding of the operations or activities of the government and is not primarily in the commercial interest of the requester." 5 U.S.C. § 552(a)(4)(A)(iii); *see also* 21 C.F.R. § 20.46. The requested disclosure would meet both of these requirements. In addition, NRDC qualifies as "a representative of the news media" entitled to a reduction of fees under FOIA. 5 U.S.C. § 552(a)(4)(A)(ii)(II).

A. NRDC Satisfies the First Fee Waiver Requirement

The disclosure requested here would be "likely to contribute significantly to public understanding of the operations or activities of the government." 5 U.S.C. § 552(a)(4)(A)(iii); 21 C.F.R. § 20.46(a)(1). Each of the four factors used by FDA to evaluate the first fee waiver requirement indicates that a fee waiver is appropriate for this request. *See* 21 C.F.R. § 20.46(b).

1. Subject of the request

The records requested here were either received by the agency or generated by the agency as it evaluated the GRAS notice. The requested records thus directly concern "the Government's operations or activities." 21 C.F.R. § 20.46(b).

2. Informative value of the information to be disclosed

The requested records are "likely to contribute significantly to public understanding of operations and activities of the Government." 21 C.F.R. § 20.46(a)(1). The public does not currently possess comprehensive information regarding the government's role in addressing public health issues related to the potential use of the listed GRAS substances in food.

We believe that the records requested are not currently in the public domain. Their disclosure would thus meaningfully inform public understanding with respect to food safety, as further discussed below. However, if FDA were to conclude that some of the requested records are publicly available, NRDC would like to discuss that conclusion and might agree to exclude such records from this request.

3. *Contribution to an understanding of the subject by the public is likely to result from disclosure.*

Because NRDC is a “representative of the news media,” as explained in Part II.C below, FDA must presume that this disclosure is likely to contribute to public understanding of its subject. 21 C.F.R. § 20.45(a)(2).

However, even if NRDC were not a media requester, NRDC’s expertise in food safety, extensive communications capabilities, and proven history of dissemination of information of public interest—including information obtained from FOIA records requests—indicate that NRDC has the ability and will to use disclosed records to reach a broad audience of interested persons with any relevant and newsworthy information the records reveal.

NRDC intends to disseminate any newsworthy information in the released records and its analysis of such records to its member base and to the broader public, through one or more of the many communications channels referenced below. NRDC frequently disseminates newsworthy information to the public for free, and does not intend to resell the information requested here. NRDC’s more than one million members and online activists are a broad audience of persons interested in the subject of GRAS notices, and when combined with NRDC’s communications to the public at large, the likely audience of interested persons to be reached is certainly reasonably broad. As NRDC’s long history of incorporating information obtained through FOIA into reports, articles and other communications illustrates, NRDC is well prepared to convey to the public any relevant information it obtains through this records request.

NRDC has the ability to disseminate information on GRAS notices through many channels. As of September 2013, these include, but are not limited to the following:

- NRDC’s website, available at <http://www.nrdc.org>, which is updated daily and draws approximately 1,142,700 page views and 478,000 unique visitors per month.
- *OnEarth* magazine, which is distributed to approximately 130,000 subscribers, for sale at newsstands and bookstores, and available free of charge at <http://www.onearth.org> (a site that itself has about 33,700 email subscribers and receives more than 45,600 unique visitors per month).
- *Nature’s Voice* newsletter on current environmental issues, which is distributed five times a year to NRDC’s more than one million members and online activists, and is available online at <http://www.nrdc.org/naturesvoice/default.asp>.
- *Earth Action* email list which has more than 179,000 subscribers who receive biweekly information on urgent environmental issues. This information is also made available through NRDC’s online Action Center at <http://www.nrdc.org/action/default.asp>.
- *This Green Life*, which is an electronic newsletter on environmentally sustainable living. It is distributed by email to 52,000 subscribers and made available online at <http://www.nrdc.org/thisgreenlife/default.asp>.
- *NRDC Online*, which is a semimonthly electronic environmental newsletter distributed by e-mail to more than 50,400 subscribers, at <http://www.nrdc.org/newsletter>.
- “Switchboard,” available at <http://switchboard.nrdc.org>, which is a staff blogging site that is updated daily and features more than 130 bloggers writing about current environmental issues. The blogs draw approximately 175,00 page views and 109,200 unique visitors per month; Switchboard’s RSS feeds have approximately 7,500 subscribers; and Switchboard posts appear on websites of other major internet media outlets, such as “The Huffington Post,” at <http://www.huffingtonpost.com>.

- NRDC's profiles on "Facebook," at <http://www.facebook.com/nrdc.org>, and "Twitter," at <http://www.twitter.com/nrdc>, are updated daily and have approximately 210,000 fans and 105,900 followers, respectively.

NRDC issues press releases, issue papers, and reports; directs and produces movies, such as *Stories from the Gulf*, narrated by Robert Redford and *Acid Test*, narrated by Sigourney Weaver; participates in press conferences and interviews with reporters and editorial writers; and has approximately thirty staff members dedicated to communications work, *see* list of select communications staff at <http://www.nrdc.org/about/staff.asp>.

NRDC employees provide Congressional testimony; appear on television, radio and web broadcasts and at conferences; and contribute to numerous national newspapers, magazines, academic journals, other periodicals, and books.

NRDC routinely uses FOIA to obtain information from federal agencies that NRDC legal and scientific experts analyze in order to inform the public about a variety of issues, including energy policy, climate change, wildlife protection, nuclear weapons, pesticides, drinking water safety, and air quality. Some specific examples are provided below:

- (1) In October 2008, NRDC issued a report assessing the degree of enforcement of California's environmental and public health laws. This report, *An Uneven Shield: The Record of Enforcement and Violations Under California's Environmental, Health, and Workplace Safety Laws*, examined data on known violations and law enforcement responses under six critical pollution, health, and workplace safety programs. Much of the data analyzed in the study was obtained through formal FOIA requests; some of it was synthesized from other sources. *See id.* at pp. 4, 16.
- (2) NRDC obtained, through a court-enforced FOIA request, records of the operations of the Bush administration's Energy Task Force, headed by Vice President Dick Cheney. It made those records available, along with analysis of selected excerpts and links to the administration's index of withheld documents, on NRDC's website at <http://www.nrdc.org/air/energy/taskforce/tfinx.asp>. NRDC's efforts helped to inform the public about an issue that, even before the records' release, had attracted considerable attention. *See, e.g.,* Elizabeth Shogren, "Bush Gets One-Two Punch on Energy," *L.A. Times*, Mar. 28, 2002, at A22; Bennett Roth, "Houston Energy-Drilling Firm Appears in Documents from Energy Department," *Houston Chronicle*, Apr. 12, 2002.
- (3) NRDC obtained, through a FOIA request, a memorandum by ExxonMobil advocating the replacement of a highly respected atmospheric scientist, Dr. Robert Watson, as the head of the Intergovernmental Panel on Climate Change. NRDC used this memorandum to help inform the public about what may have been behind the decision by the Bush administration to replace Dr. Watson. *See* NRDC Press Release and attached Exxon memorandum, "Confidential Papers Show Exxon Hand in White House Move to Oust Top Scientist from International Global Warming Panel," Apr. 3, 2002; Elizabeth Shogren, "Charges Fly Over Science Panel Pick," *L.A. Times*, Apr. 4, 2002, at A19.
- (4) NRDC incorporated information obtained through FOIA into a 2005 report, published and provided free of charge at NRDC's website, *see* <http://www.nrdc.org/wildlife/marine/sound/contents.asp>, on the impacts of military

sonar and other industrial noise pollution on marine life. *See Sounding the Depths II: The Rising Toll of Sonar, Shipping and Industrial Ocean Noise on Marine Life* (Nov. 2005) (update to 1999 report). The report also relied upon and synthesized information from other sources. Since the report's publication, the sonar issue has continued to attract widespread public attention. *See, e.g.*, "Protest Raised over New Tests of Naval Sonar," National Public Radio, *All Things Considered*, July 24, 2007.

- (5) NRDC scientists have used information obtained through FOIA to publish analyses of the United States' and other nations' nuclear weapons programs. In 2004, for example, NRDC scientists incorporated information obtained through FOIA into a feature article on the United States' plans to deploy a ballistic missile system and the implications for global security. *See* Hans M. Kristensen, Matthew G. McKinzie, and Robert S. Norris, "The Protection Paradox," *Bulletin of Atomic Scientists*, Mar./Apr. 2004.
- (6) NRDC has used White House documents obtained through FOIA and from other sources to inform the public about EPA's failures to protect wildlife and workers from the pesticide atrazine in the face of industry pressure to keep atrazine on the market. *See* <http://www.nrdc.org/health/atrazine/files/atrazine10.pdf>; *see also* William Souder, "It's Not Easy Being Green: Are Weed-Killers Turning Frogs Into Hermaphrodites?" *Harper's Bazaar*, Aug. 1, 2006 (referencing documents obtained and posted online by NRDC).
- (7) NRDC has obtained, through FOIA and other sources, information on the levels of arsenic in drinking water supplies across the country. NRDC synthesized that information into a report, *Arsenic and Old Laws* (2000), printed and made available online through NRDC's website, *see* <http://www.nrdc.org/water/drinking/arsenic/aolinx.asp>, and provided analysis describing its significance and guiding interested members of the public on how to learn more about arsenic in their own drinking water supplies. *Id.*; *see also* Steve LaRue, "EPA Aims to Cut Levels of Arsenic in Well Water," *San Diego Union-Tribune*, June 5, 2000, at B1 (referencing NRDC report).
- (8) In 2000, NRDC used information obtained through FOIA to publish a report analyzing the impacts of manure pollution from large livestock feedlots on human health, fish and wildlife. *See* NRDC, *Spills & Kills*, Aug. 2000.
- (9) In 1999, NRDC obtained, through FOIA, a Defense Department document, *History of the Custody and Deployment of Nuclear Weapons: July 1945 through September 1977*. The document attracted significant press attention once it was disclosed. *See, e.g.*, Walter Pincus, "Study Says U.S. Secretly Placed Bombs; Cold War Deployments Affected Mostly Allies," *Washington Post* (Oct. 20, 1999) at A3. One of NRDC's nuclear scientists, Robert Norris, published a detailed analysis of this document explaining its significance to the public. *See* Robert S. Norris, William M. Arkin, and William Burr, "Where They Were," *Bulletin of Atomic Scientists*, Nov./Dec. 1999.
- (10) In 1996, NRDC obtained, through FOIA, test results regarding lead levels in the District of Columbia's drinking water supplies. NRDC made the test results public along with analysis explaining the significance of the results. *See* D'Vera Cohn, "Tap

Water Safeguards Still Stalled; City Failed to Tell Some Residents of Excess Lead Contamination,” *Washington Post*, Apr. 18, 1996, at J1.

- (11) In 1989, NRDC obtained, through FOIA, testimony, previously suppressed by the first Bush administration, by federal experts who opposed oil drilling off the coasts of California and Florida. *See* Larry Liebert, “Oil Testimony Reportedly Quashed; Environmentalists say Federal Experts Pressured by Bush,” *Orange County Register*, Oct. 5, 1989, at A6.
- (12) In 1988, NRDC obtained, through FOIA, a report by the U.S. Fish and Wildlife Service that declared that the government’s review of offshore oil drilling in Northern California was incomplete and overly optimistic. Reagan administration officials had tried to keep the report secret and then repudiated it upon its release. *See* Eric Lichtblau, “Federal Report Blasts Offshore Oil Studies,” *L.A. Times*, June 4, 1988, at A32.
- (13) In 1982, NRDC obtained, through a FOIA request, an EPA memorandum stating that most air pollution monitors have repeatedly underestimated levels of toxic lead in the air. NRDC used the memorandum to inform the public about the consequences of EPA’s proposal to relax restrictions on lead in gasoline. *See* Sandra Sugawara, “Lead in Air is Undermeasured, EPA Section Chief’s Memo Says,” *Washington Post*, July 11, 1982, at A6.2

As these examples demonstrate, NRDC has a proven ability to digest, synthesize, and quickly disseminate information gleaned from FOIA requests to a broad audience of interested persons. Therefore, the requested records disclosure is likely to contribute to the public’s understanding of the subject.

4. *Significance of the contribution to public understanding*

The records requested shed light on a matter of considerable public interest and concern: GRAS notices for additives use in food.

Public understanding of food safety would be significantly enhanced by disclosure of the requested records concerning GRAS notices. Disclosure would help the public to more effectively evaluate food safety. Disclosure would also help the public to better understand and evaluate FDA’s actions (or inaction) on GRAS notices.

B. NRDC Satisfies the Second Fee Waiver Requirement

Disclosure in this case would also satisfy the second prerequisite of a fee waiver request because NRDC does not have any commercial interest that would be furthered by the requested disclosure. 5 U.S.C. § 552(a)(4)(A)(iii); 21 C.F.R. § 20.46(c). NRDC is a not-for-profit organization and does not act as a middleman to resell information obtained under FOIA. “Congress amended FOIA to ensure that it be ‘liberally construed in favor of waivers for noncommercial requesters.’”

² Information NRDC obtained through FOIA requests resulted in other articles, in addition to those referenced above, *see, e.g.*, Felicity Barringer, “Science Panel Issues Report on Exposure to Pollutant,” *N.Y. Times*, Jan. 11, 2005; Katharine Q. Seelye, “Draft of Air Rule is Said to Exempt Many Old Plants,” *N.Y. Times*, Aug. 22, 2003; Don Van Natta, Jr., “E-Mail Suggests Energy Official Encouraged Lobbyist on Policy,” *N.Y. Times*, Apr. 27, 2002.

Judicial Watch, Inc. v. Rossotti, 326 F.3d 1309, 1312 (D.C. Cir. 2003) (internal citation omitted); *see Natural Res. Def. Council v. United States Env'tl. Prot. Agency*, 581 F. Supp. 2d 491, 498 (S.D.N.Y. 2008). NRDC wishes to serve the public by reviewing, analyzing and disclosing newsworthy and presently non-public information about GRAS notices. As noted at Part II.A, any work done by FDA on GRAS notices relates to a matter of considerable public interest and concern. Disclosure of the requested records will contribute significantly to public understanding of GRAS notices and associated threats to human health and the environment.

C. NRDC Is a Media Requester

Even if FDA denies a public interest waiver of all costs and fees, NRDC is a representative of the news media entitled to a reduction of fees under FOIA, 5 U.S.C. § 552(a)(4)(A)(ii), and FDA's FOIA regulations, 21 C.F.R. § 20.45(a)(2). *See Elec. Privacy Info. Ctr. v. Dep't of Def.*, 241 F. Supp. 2d 5, 6, 11-15 (D.D.C. 2003) (a "non-profit public interest organization" qualifies as a representative of the news media under FOIA where it publishes books and newsletters on issues of current interest to the public); Letter from Alexander C. Morris, FOIA Officer, United States Dep't of Energy, to Joshua Berman, NRDC (Feb. 10, 2011) (granting NRDC media requester status).

NRDC is in part organized and operated to publish or transmit news to the public. As described earlier in this request, NRDC publishes a quarterly magazine, *OnEarth*, which has approximately 150,000 subscribers, is available at newsstands and bookstores, and has won numerous news media awards, including the Independent Press Award for Best Environmental Coverage and for General Excellence, a Gold Eddie Award for editorial excellence among magazines, and the Phillip D. Reed Memorial Award for Outstanding Writing on the Southern Environment. NRDC also publishes a regular newsletter for its more than one million members and online activists; issues other electronic newsletters, action alerts, public reports and analyses; and maintains free online libraries of these publications. NRDC maintains a significant additional communications presence on the internet through its staff blogging site, "Switchboard," which is updated daily and features more than 130 bloggers writing about current environmental issues, and through daily news messaging on "Twitter" and "Facebook." *See* OPEN Government Act of 2007, Pub. L. No. 110-175, § 3, 121 Stat. 2524 (2007) (codified at 5 U.S.C. § 552(a)(4)(A)(ii)) (clarifying that "as methods of news delivery evolve . . . such alternative media shall be considered to be news-media entities"). The aforementioned publications and media sources routinely include information about current events of interest to the readership and the public. To publish and transmit this news content, NRDC employs approximately thirty staff dedicated full-time to communications with the public, including accomplished journalists and editors, *see* list of select communications staff at <http://www.nrdc.org/about/staff.asp>. These staff rely on information acquired under FOIA and through other means. That NRDC is a public interest advocacy organization is inconsequential so long as "its activities qualify as those of a representative of news media," and NRDC's do. *Elec. Privacy Info. Ctr.*, 241 F. Supp. 2d at 12. Public interest organizations meeting the requirements "are regularly granted news representative status." *Serv. Women's Action Network v. Dep't of Def.*, 888 F. Supp. 2d 282, 287-88 (D. Conn. 2012) (according media requester status to the American Civil Liberties Union).³

Information obtained as a result of this request will, if appropriately newsworthy, be synthesized with information from other sources and used by NRDC to create and disseminate unique articles, reports, analyses, blogs, tweets, emails, and/or other distinct informational works through one or more of NRDC's publications or other suitable media channels. NRDC staff gather information

³ To be a representative of the news media, an organization need not *exclusively* perform news gathering functions. If that were required, major news and entertainment entities like the National Broadcasting Company (NBC) would not qualify as representatives of the news media. This country has a long history, dating back to its founding, of news organizations engaging in public advocacy.

from a variety of sources—including documents provided pursuant to FOIA requests—to write original articles and reports that are featured in its *OnEarth* magazine, newsletters, blogs, and other NRDC-operated media outlets. NRDC seeks the requested records to aid its own news-disseminating activities by obtaining, analyzing, and distributing information likely to contribute significantly to public understanding, not to resell the information to other media organizations.

III. Willingness to Pay Fees Under Protest

Please provide the records requested above irrespective of the status and outcome of your evaluation of NRDC's fee category status and fee waiver request. In order to prevent delay in FDA's provision of the requested records, NRDC states that it will, if necessary and under protest, pay fees in accordance with FDA's FOIA regulations at 21 C.F.R. § 20.45 for all or a portion of the requested records. Please consult with NRDC, however, before undertaking any action that would cause the fee to exceed \$500. Such payment will not constitute any waiver of NRDC's right to seek administrative or judicial review of any denial of its fee waiver request and/or rejection of its fee category assertion.

IV. Conclusion

We trust that, in responding to this request, FDA will comply with all relevant deadlines and other obligations set forth in FOIA and FDA's FOIA regulations. *See, e.g.*, 21 C.F.R. Part 20.

Please produce the records above by emailing or mailing them to me at the NRDC office address listed below. Please produce them on a rolling basis; at no point should FDA's search for—or deliberations concerning—certain records delay the production of others that FDA has already retrieved and elected to produce. In the event that FDA concludes that some of the records requested above may already be publicly available, we will be happy to discuss those conclusions. Please do not hesitate to call or email with questions.

Please do not hesitate to call or email with questions. I can be reached at 202-513-6252 and tneltner@nrdc.org.

Thank you for your prompt attention to this request.

Sincerely,



Tom Neltner, Senior Attorney
Natural Resources Defense Council, Inc.
1152 15th Street NW, Suite 300
Washington, DC 20005
202-513-6252
(202) 289-1060 FAX
tneltner@nrdc.org

Appendix A: Generally Recognized as Safe (GRAS) Notices and Agency Actions

APPENDIX A
GENERALLY RECOGNIZED AS SAFE (GRAS) NOTICES AND AGENCY ACTIONS¹

GRN #	Title
1	Soy isoflavone extract
35	Hempseed oil
36	Chromium picolinate; <i>Ginkgo biloba</i> leaf extract; and Ginseng extract
37	Whey protein isolate and dairy product solids
59	Hydrogenated starch hydrolysate
66	Milk thistle extract
150	Glucosamine hydrochloride prepared from chitin obtained from <i>Aspergillus niger</i>
224	trans-Resveratrol
225	Catechins from green tea extract
257	<i>gamma</i> -Amino butyric acid
262	Sweet lupin protein
263	Sweet lupin fiber
264	Sweet lupin flour
295	Aqueous extract of <i>Emblica officinalis</i>
322	Aqueous extract of <i>Emblica officinalis</i>
324	Heat-killed <i>Lactobacillus plantarum</i>
340	Theobromine
362	Levocarnitine
378	Cultured [dairy sources, sugars, wheat, malt, and fruit- and vegetable-based sources] fermented by [<i>Streptococcus thermophilus</i> , <i>Bacillus coagulans</i> , <i>Lactobacillus acidophilus</i> , <i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus sakei</i> , <i>Lactobacillus bulgaricus</i> and <i>Propionibacterium freudenreichii</i> subsp. <i>shermanii</i> or mixtures of these strains]
444	Milk protein concentrate and milk protein isolate

¹ Posted at <http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing>.



DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration
Silver Spring MD 20993

tneltner@nrdc.org

In Reply Refer To: FOI 2013-8042

Dear Requester:

This is in partial response to your October 11, 2013, request to the Food and Drug Administration (FDA) pursuant to the Freedom of Information Act for records regarding:

GRN 1,35,36 ETC

A search of the Office of the Commissioner, Office of the Executive Secretariat files did not reveal any responsive records to your request.

If you wish to appeal from this determination, please submit your appeal within 30 days to Director, News Division, 7700 Wisconsin Avenue, Suite 920, Bethesda, MD 20857 (by U.S. Post), or 7700 Wisconsin Avenue, Suite 920, Bethesda, MD 20814 (by private courier, such as UPS or FedEx). Please mark your envelope FDA FOIA Appeal and please include your control number.

Sincerely,

A handwritten signature in blue ink, which appears to read "Martina H. Varnado", is written over a horizontal line. There is a small "x" mark to the left of the signature.

Martina H. Varnado
Director
Office of the Commissioner
Office of the Executive Secretariat



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD 20740

January 16, 2014

Tom Neltner
Natural Resources Defense Council
1152 15th Street, N.W. Suite 300
Washington, DC 20005

Re: FOI Request No. 2013-8042

Dear Mr. Neltner:

This is in response to your request of October 21, 2013, requesting records related to GRAS Notices 1, 35, 36, 37, 59, 66, 150, 224, 225, 257, 262, 263, 264, 295, 322, 324, 340, 362, 378, and 444. In this response, we have provided responsive records for 19 of the 20 GRAS Notices you requested. We are compiling responsive records for GRAS Notice 1 and will provide these at a later date. Per your request, we do not include copies of the notices and agency letters posted at <http://www.accessdata.fda.gov/scripts/cfn/fcnNavigation.cfm?rpt=grasListing> in our response. We do, however, include in our response documents available in the Federal Register or Docket FDA-1997-N-0020 to ensure completeness of the responsive records. Your request was forwarded to the Office of Food Additive Safety in the Center for Food Safety and Applied Nutrition.

X Enclosed is a partial response of the records you requested (with the exception of responsive records for GRAS Notice 1).

X Certain material has been deleted from the records furnished to you because a preliminary review of the records indicated that the deleted information is not required to be publicly disclosed and that disclosure is not appropriate. FDA has taken this approach to facilitate the process of responding to you. If you dispute FDA's preliminary determination with respect to these records and would like FDA to reconsider any particular deletion, please let us know in writing at the following address: Food and Drug Administration, Division of Freedom of Information, HFI-35, 5600 Fishers Lane, Rockville, MD 20857 within 30 days from the date of this letter. If we do not receive a response in that time period, we will consider the matter closed with respect to these records. If you do request further consideration and if the agency then formally denies your request for any or all of the previously-withheld information, you will have the right to appeal that decision. Any letter of denial will explain how to make this appeal.

The following charges for this request to date may be included in a monthly invoice:

Reproduction \$ 0.00 Search \$0.00 Review \$0.00 Other \$0.00 (CD) Total \$0.00

THE ABOVE TOTAL MAY NOT REFLECT THE FINAL CHARGES FOR THIS REQUEST. **PLEASE DO NOT SEND PAYMENT UNTIL YOU RECEIVE AN INVOICE FOR THE TOTAL MONTHLY FEE.**

Sincerely Yours,

Sharon R. Dodson 5

Sharon R. Dodson
Program Analyst
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

Enclosure



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD 20740

March 19, 2014

Tom Neltner
Natural Resources Defense Council
1152 15th Street, N.W. Suite 300
Washington, DC 20005

Re: FOI Request No. 2013-8042

Dear Mr. Neltner:

This completes our response to your request of October 21, 2013, requesting records related to GRAS Notices 1, 35, 36, 37, 59, 66, 150, 224, 225, 257, 262, 263, 264, 295, 322, 324, 340, 362, 378, and 444. Per your request, we do not include copies of the notice and agency letter posted at <http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing> in our response. We do, however, include in our response documents available in Docket FDA-1997-N-0020 to ensure completeness of the responsive records. Your request was forwarded to the Office of Food Additive Safety in the Center for Food Safety and Applied Nutrition.

In this response, we provide Part 1 of 2 of the responsive records for GRAS Notice 1, thus completing our response for this notice.

X Enclosed is Part 1-GRAS Notice 1 of the records you requested.

X Certain material has been deleted from the records furnished to you because a preliminary review of the records indicated that the deleted information is not required to be publicly disclosed and that disclosure is not appropriate. FDA has taken this approach to facilitate the process of responding to you. If you dispute FDA's preliminary determination with respect to these records and would like FDA to reconsider any particular deletion, please let us know in writing at the following address: Food and Drug Administration, Division of Freedom of Information, HFI-35, 5600 Fishers Lane, Rockville, MD 20857 within 30 days from the date of this letter. If we do not receive a response in that time period, we will consider the matter closed with respect to these records. If you do request further consideration and if the agency then formally denies your request for any or all of the previously-withheld information, you will have the right to appeal that decision. Any letter of denial will explain how to make this appeal.

The following charges for this request to date may be included in a monthly invoice:

Reproduction \$ 0.00 Search \$ 0.00 Review \$ 0.00 Other \$ 0.00 (CD) Total \$ 0.00

THE ABOVE TOTAL MAY NOT REFLECT THE FINAL CHARGES FOR THIS REQUEST. **PLEASE DO NOT SEND PAYMENT UNTIL YOU RECEIVE AN INVOICE FOR THE TOTAL MONTHLY FEE.**

Sincerely Yours,

Sharon R. Dodson

-S

Sharon R. Dodson
Program Analyst
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

Enclosure



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD 20740

March 19, 2014

Tom Neltner
Natural Resources Defense Council
1152 15th Street, N.W. Suite 300
Washington, DC 20005

Re: FOI Request No. 2013-8042

Dear Mr. Neltner:

This completes our response to your request of October 21, 2013, requesting records related to GRAS Notices 1, 35, 36, 37, 59, 66, 150, 224, 225, 257, 262, 263, 264, 295, 322, 324, 340, 362, 378, and 444. Per your request, we do not include copies of the notice and agency letter posted at <http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing> in our response. We do, however, include in our response documents available in Docket FDA-1997-N-0020 to ensure completeness of the responsive records. Your request was forwarded to the Office of Food Additive Safety in the Center for Food Safety and Applied Nutrition.

In this response, we provide Part 2 of 2 of the responsive records for GRAS Notice 1, thus completing our response for this notice.

X Enclosed is Part 1-GRAS Notice 1 of the records you requested.

X Certain material has been deleted from the records furnished to you because a preliminary review of the records indicated that the deleted information is not required to be publicly disclosed and that disclosure is not appropriate. FDA has taken this approach to facilitate the process of responding to you. If you dispute FDA's preliminary determination with respect to these records and would like FDA to reconsider any particular deletion, please let us know in writing at the following address: Food and Drug Administration, Division of Freedom of Information, HFI-35, 5600 Fishers Lane, Rockville, MD 20857 within 30 days from the date of this letter. If we do not receive a response in that time period, we will consider the matter closed with respect to these records. If you do request further consideration and if the agency then formally denies your request for any or all of the previously-withheld information, you will have the right to appeal that decision. Any letter of denial will explain how to make this appeal.

The following charges for this request to date may be included in a monthly invoice:

Reproduction \$ 0.00 Search \$ 0.00 Review \$ 0.00 Other \$ 0.00 (CD) Total \$ 0.00

THE ABOVE TOTAL MAY NOT REFLECT THE FINAL CHARGES FOR THIS REQUEST. **PLEASE DO NOT SEND PAYMENT UNTIL YOU RECEIVE AN INVOICE FOR THE TOTAL MONTHLY FEE.**

Sincerely Yours,

Sharon R.
Dodson -S
Sharon R. Dodson
Program Analyst
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

FOIA request by Tom Neltner
2013-03-19 10:10:00 AM
FOIA request by Tom Neltner
2013-03-19 10:10:00 AM
FOIA request by Tom Neltner
2013-03-19 10:10:00 AM

Enclosure



March 5, 1998

Mr. Mark W. Empie
Director, Regulatory Affairs
Archer Daniels Midland Company
Box 1470
Decatur, IL 62525

Re: GRAS Notice No. GRN 000001

Dear Mr. Empie:

This letter acknowledges receipt on February 10, 1998, of your notice dated February 4, 1998. This notice was submitted to the Food and Drug Administration in accordance with the agency's proposed regulation (proposed 21 CFR 170.36; *Federal Register* of April 17, 1997; 62 FR 18938) regarding a notice of a claim for exemption from the statutory premarket approval requirements based on a GRAS (i.e., generally recognized as safe) determination. In your notice, you state that you have determined that soy isoflavone extract is GRAS for use as a micronutrient in foods such as performance bars, mature adult meal replacements, and certain beverages (which you characterize as "healthy") at a level of 25 mg soy isoflavone extract per serving. The soy isoflavone extract contains a mixture of the aglycone base isoflavone molecules genistein (CAS Reg. No. 446-72-0), daidzein (CAS Reg. No. 486-66-8) and glycitein (CAS Reg. No. 40957-83-3) and their glycone derivatives genistin (CAS Reg. No. 529-59-9), daidzin (CAS Reg. No. 552-66-9) and glycitin (CAS Reg. No. 40246-10-4), respectively.

This notice has been designated GRAS Notice No. GRN 000001. In accordance with proposed § 170.36(f), a copy of your cover letter, which includes the information described in proposed § 170.36(c)(1), is available for public review and copying at the agency's Dockets Management Branch (Docket No. 98S-0103). If you have any questions about your notice, please feel free to call me at (202)418-3101.

Sincerely yours,

Linda Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Division of Product Policy
Center for Food Safety
and Applied Nutrition

(b) (5)

FILE
COPY

OFFICE	SURNAME	DATE	OFFICE	SURNAME	DATE	OFFICE	SURNAME	DATE
(b) (5)								

000149

To: smtp[safetywise@clear.net.nz]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: Soy Isoflavones
Date: Tuesday, March 24, 1998 at 9:43:23 am EST
Attached: None

FD



March 24, 1998

Ms. Yvonne Clapperton
Soy Information Service
P.O. Box 3285
Onerahi
Whangarei, New Zealand

Dear Ms. Clapperton:

This is in response to your electronic mail message, dated March 18, 1998, to the Director of the Center for Food Safety and Applied Nutrition (CFSAN), U.S. Food and Drug Administration (FDA), regarding a report in the publication Food Chemical News that Archer Daniels Midland Company (ADM) had determined that soy isoflavone is generally recognized as safe (GRAS) for use as a micronutrient in food. In your electronic mail message, you ask that we advise you of the appropriate mechanism whereby you can "oppose the petition" of ADM.

Under section 201(s) of the Federal Food, Drug, and Cosmetic Act (the Act), a food ingredient is not subject to premarket approval if the ingredient is generally recognized, among experts qualified by training and experience to evaluate its safety, as having been adequately shown to be safe under the conditions of its intended use. In the Federal Register of April 17, 1997, FDA published a proposed rule that would establish a notification procedure whereby any person may notify FDA of a determination that a particular use of a substance is not subject to the premarket approval requirements of the Act because such use is GRAS. Under the proposed notification procedure, FDA would evaluate whether the submitted notice provides a sufficient basis for a GRAS determination and whether information in the notice or otherwise available to FDA raises issues that lead the agency to question whether use of the substance is GRAS. The endpoint of the proposed notification procedure would be a letter from FDA to the notifier.

Although that proposed rule has not become final, FDA invited interested parties to participate in the notification procedure during the interim between the proposed and final rules. The subject ADM submission is a notice to FDA that ADM has determined that the use of soy isoflavones as a micronutrient in food is GRAS. FDA is evaluating ADM's notice to determine whether it provides a sufficient basis for ADM's GRAS determination. FDA welcomes fact-based information on food safety issues. Accordingly, you may submit such information to the Office of Premarket Approval (HFS-200),

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Center for Food Safety and Applied Nutrition, Food and Drug Administration,
200 C Street SW, Washington, DC 20204 and request that it be considered
with respect to GRAS Notice Number GRN 000001.

The April 17, 1997, proposed rule is available electronically on CFSAN's
home page at <http://vm.cfsan.fda.gov>, within the section regarding Food
Additives and Premarket Approval, Documents for Industry. If you have any
further questions, I am the official contact person for the GRAS
notification program. I am in the office Monday through Friday from 7:15
a.m. to 3:45 p.m. (U.S. Eastern Time).

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C St., SW, Washington DC 20204
Phone: (202)418-3101 Fax: (202)418-3131
Internet: LKAHL@BANGATE.FDA.GOV

000153



Office of Premarket Approval
Center for Food Safety & Applied Nutrition
Food and Drug Administration
200 C Street, S.W. (HFS-206)
Washington, D.C. 20204

TO: Dr. Richard James
c/o Dr. Yvonne Clapperton

FROM: ~~Linda Kell~~

DATE: 3/27/98

PHONE: (202) 418- 3101

FAX: 64-9-434-0567

FAX: (202) 418-3131

MESSAGE

The attached summary of the Amber-Danick Midland GRAS notice is publicly available without going through FOIA, so I am sending it by telefax. I also sent an E-mail to Dr. Clapperton regarding your questions on the format for a submission opposing the GRAS notice.

000167

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To: smtp[safetywise@clear.net.nz]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: Response to telefaxes
Date: Friday, March 27, 1998 at 1:34:49 pm EST
Attached: None

FD



To: Dr. Richard F. James
c/o Dr. Yvonne Clapperton

This is in response to your telefaxes dated 3/25/98 and 3/26/98. We have no prescribed format for a submission that would oppose a GRAS notice; therefore, my only advice is that any information you submit bear on the safety of the whether soy isoflavone extract would be generally recognized as safe under its conditions of intended use - i.e., for use as a micronutrient in foods such as performance bars, mature adult meal replacements, and certain beverages at a level of 25 mg soy isoflavone extract per serving. GRN #000001 does not relate to the use of soy isoflavones in food products such as infant formula or baby foods.

I do, however, recommend that any submission of published articles be supported by your explanation of how each cited article bears on your conclusion regarding whether the subject use of soy isoflavones is GRAS.

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C St., SW, Washington DC 20204
Phone: (202)418-3101 Fax: (202)418-3131
Internet: LKAHL@BANGATE.FDA.GOV

000166

**Memorandum of Telephone Conversations**

Dates: March 31, May 6, May 13, July 9, and September 10, 1998

Between: Linda Kahl, Ph.D. (HFS-206)

and

Gary Yingling, McKenna and Cuneo (202)496-7645
(on behalf of Archer Daniels Midland Company (ADM))

and Mark Empie, ADM

Subject: GRN No 000001; Soy Isoflavones

On March 31, 1998, I telephoned Mr. Yingling and asked whether ADM would be willing to supply an electronic copy of their notice. I made this request so that the electronic copy could be used to respond electronically to Freedom of Information (FOI) requests for copies of the notice. Mr. Yingling contacted Mark Empie of ADM, who arranged for the delivery of the electronic copy. Mr. Empie asked for time to verify that their electronic copy was identical to the hard copy that had been printed for delivery to the agency in the GRAS notice. I received the electronic copy from Mark Matlock of ADM on April 9, 1998, via electronic mail.

On May 6, 1998, I telephoned Mr. Yingling and asked that ADM identify more specifically the food categories that ADM considered to be within the scope of its GRAS determination. I made this request because the notice described the intended food categories as "example use foods," which was inadequate from the perspective of evaluating dietary exposure to soy isoflavones. On May 18, 1998, I received a call from Mark Empie of ADM, who stated that the three "example use foods" listed on p. 24 of the notice (mature adult meal replacements, healthy drinks, and performance bars) were the only food categories that ADM was considering in its GRAS notice for the use of soy isoflavones.

On May 13, 1998, I telephoned Mr. Yingling and informed him that our review scientists had experienced difficulties in finding some references listed in the ADM submission. As examples, we were unable to locate Reference #12 and #40 and we noted that Reference #40a did not include any page numbers. Mr. Yingling called me back for a conference call with Mark Empie of ADM. Mr. Empie suggested that ADM send a copy of its references to Mr. Yingling's office so that any references that we might need would be available for inspection.

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(b) (5)								


Page 2 - Memorandum of Telephone Conference

On July 9, I received a telephone call from Mr. Yingling informing me that he had, in response to a FOIA request, received information that had been incorporated into GRN No. 000001 at the request of other parties. Mr. Yingling also asked whether, while the GRAS notice is pending, it would be possible for ADM to submit to the Office of Special Nutritionals a notice for the use of soy isoflavones as a dietary ingredient of a dietary supplement. I responded that any use of soy isoflavones as a dietary ingredient of a dietary supplement was independent of its use in conventional food and that simultaneous notices could be pending at the agency for both uses.


On September 10, 1998, following a September 9, 1998, meeting between FDA and representatives of ADM, Mr. Yingling and I discussed mechanisms whereby ADM could request that FDA stop its evaluation of the current notice while ADM prepared a submission that would respond to questions raised by outside parties who had submitted information to the GRN No 000001 file. Such a request from ADM would not preclude ADM from subsequently submitting a new notice for the use of soy isoflavones if they chose to do so.

Linda S. Kahl, Ph.D.

(b) (5)



000836

To: smtp[safetywise@clear.net.nz] FD 
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: How to comment on rulemaking (GRN #1 is not rulemaking)
Date: Tuesday, March 31, 1998 at 11:14:55 am EST
Attached: None

Dr. Richard James
c/o Dr. Yvonne Clapperton

This is in response to your request to be informed about GRN 00001, and in particular your reference to information on how to comment on proposed regulations.

The GRAS notice received from Archer Daniels Midland Co. (ADM) is not a proposed rule. Rather, it is notice informing FDA of ADM's view that the use of soy isoflavones as a micronutrient in food is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because ADM has determined that such use is generally recognized as safe. Under FDA's proposed GRAS notification procedure (which was open to public comment for the 90 day period from April 17, 1997 to July 16, 1997), FDA will evaluate ADM's submission and determine whether information in the notice or otherwise available to FDA raises questions that lead the agency to question GRAS status. FDA is still going through that review process. Under the terms of the proposed rule, FDA would respond in writing to the notifier within 90 days. However, until that proposed rule becomes final, FDA is not bound by the 90 day time frame and, considering that this is the first notice that we received, I would not be surprised if our response took longer than 90 days. Importantly, a response letter from FDA that did not question GRAS status would not result in a regulation because the absence of a challenge from FDA would not be equivalent to an agency affirmation of ADM's GRAS determination. I realize that the April 17, 1997 proposed rule is lengthy; the section entitled "Agency Response" (pp. 18950-18951) may be helpful.

It is important to understand the distinction between FDA's proposed rule to establish a GRAS notification procedure (which is "rulemaking" as referenced in your telefax) and the ADM notice (which is not rulemaking). Thus, although FDA welcomes factual-based evidence relating to food safety issues, any submission that you would make would be "information otherwise available to FDA" but would not be a "comment" within the meaning of notice-and-comment rulemaking.

I hope that this answers several of your questions.

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval

000196

To: ISMTP@FDA-OC-TRAINING@FDAOC[<e.fitzpatrick@extra.co.nz>]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: re: Re: GRAS and soy isoflavones
Date: Tuesday, April 7, 1998 at 8:04:45 am EDT
Attached: None

Under sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act (the Act), a food ingredient is not subject to premarket approval if the ingredient is generally recognized, among experts qualified by training and experience to evaluate its safety, as having been adequately shown to be safe under the conditions of its intended use - i.e., to be GRAS. Under these provisions of the act, there is no requirement that a person who determines that a use of a substance is GRAS inform FDA of that determination or obtain FDA's concurrence.

Under the act, a substance may be GRAS through scientific procedures or, for substances used in food before passage of the 1958 Food Additives Amendment, through experience based on common use in food before January 1, 1958. Shortly after the 1958 amendment, FDA assembled a partial list of substances that were known to be commonly used in food prior to 1958. This was the first GRAS list and is now in Part 182 of our regulations. In the 1970's, FDA began a scientific safety review of all substances on this "history of use" GRAS list. If, following completion of its review of a particular substance, FDA affirmed that the use of the substance is GRAS, the agency deleted the listing in Part 182 and added a new listing in Part 184 - for "affirmed GRAS" substances. FDA went through rulemaking to establish the procedures that the agency would use to conduct this review. Because not all substances used in food on the basis of the GRAS exemption were on the agency's GRAS list, FDA also established a voluntary process, the GRAS petition process, whereby persons who had made their own GRAS determination could request that FDA affirm that determination and list the affirmed use in Part 184. That procedure involved "notice and comment rulemaking," in which FDA conducts a pre-filing review to see if the petition meets certain format requirements, publishes a filing notice in the Federal Register, and in that filing notice requests comment on the proposed use. If FDA affirms the proposed use as GRAS, the agency publishes a final rule in the Federal Register and includes in that document a discussion of comments received.

In the Federal Register of April 17, 1997, FDA published a proposed rule that would eliminate the voluntary GRAS petition process. In its place, FDA proposed to establish a voluntary notification procedure whereby any person may notify FDA of a GRAS determination. In so doing, FDA stated that a goal of this process was to increase the agency's knowledge of substances that are being added to food based on the GRAS exemption.

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Under the proposed notification procedure, FDA would not affirm the notifier's GRAS determination and therefore FDA would not itself "classify"

the use of the substance as GRAS. The procedure does not involve notice-and-comment rulemaking because the endpoint of the proposed notification procedure is not a regulation. Instead, the endpoint would be a letter from FDA to the notifier. This letter could identify a problem with the notifier's GRAS determination; however, a letter that does not identify a problem with the notice would not provide an affirmative statement that FDA agreed with the notifier.

Although that proposed rule has not become final, FDA invited interested parties to participate in the notification procedure during the interim between the proposed and final rules. The subject Archer Daniels Midland (ADM) submission is a notice to FDA that ADM has determined that the use of soy isoflavones as a micronutrient in food is GRAS. FDA is evaluating ADM's notice to determine whether it provides a sufficient basis for ADM's GRAS determination. Because the procedure does not involve rulemaking, there is no formal mechanism for submission of "comments" within the meaning of "notice and comment rulemaking." Nonetheless, FDA welcomes fact-based information on food safety issues. Accordingly, you may submit such information to the Office of Premarket Approval (HFS-200), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 200 C Street SW, Washington, DC 20204 and request that it be considered with respect to GRAS Notice Number GRN 000001.

The April 17, 1997, proposed rule is available electronically on CFSAN's home page at <http://vm.cfsan.fda.gov>, within the section regarding Food Additives and Premarket Approval, Documents for Industry. Please note that @,the proposal to establish the notification procedure is in fact notice and comment rulemaking; the comment period closed on July 16, 1997. However, GRAS notices sent to the agency under the auspices of the proposed program are NOT notice-and-comment rulemaking.

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C St., SW, Washington DC 20204
Phone: (202)418-3101 Fax: (202)418-3131
Internet: LKAHL@BANGATE.FDA.GOV

000222



Office of Premarket Approval
Center for Food Safety & Applied Nutrition
Food and Drug Administration
200 C Street, S.W. (HFS-206)
Washington, D.C. 20204

TO: Mr. Richard JamesFROM: ~~Lincoln K. ...~~64-9-434-0567cc: Mr. Bruce Finzen, esq.DATE: 8/7/98612-339-4181PHONE: (202) 418- 3101

FAX: _____

FAX: (202) 418-3131

MESSAGEResponse to telefax from Richard James on April 5,Subject: GRAS Notice on Soy Isoflavones

000223

THIS DOCUMENT IS INTENDED ONLY FOR THE USE OF THE PARTY TO WHOM IT IS ADDRESSED AND MAY CONTAIN INFORMATION THAT IS PRIVILEGED, CONFIDENTIAL, AND PROTECTED FROM DISCLOSURE UNDER APPLICABLE LAW. If you are not the addressee or a person authorized to deliver the document to the addressee, you are hereby notified that any review, disclosure, dissemination, copying, or other action based on the content of this communication is not authorized. If you have received this document in error, please immediately notify us by telephone and return it to us at the above address by mail. Thank you.

Dear Mr. James:

This is in response to your telefax dated April 5, 1998, in which you attached a copy of a 1979 FASEB report prepared under contract to FDA. This report is not part of the current GRAS affirmation petition process. Rather, it was part of a comprehensive, agency-initiated review of the safety of GRAS substances. I have copied the following excerpt from our April 17, 1997 proposed rule because it is a fairly concise description of the GRAS affirmation process, which can be on the agency's own initiative (which in most cases was done under the contract with FASEB) or on the petition of an interested party (the GRAS petition process).

From the April 17, 1997 proposed rule:

In 1969 (34 FR 17063; October 21, 1969), FDA deleted various cyclamate salts, a family of nonnutritive sweeteners, from the GRAS list because they were implicated in the formation of bladder tumors in rats. In response to the concerns raised by the new information on cyclamates, then-President Nixon directed FDA to re-examine the safety of GRAS substances, and FDA announced that the agency was conducting a comprehensive study of substances presumed to be GRAS (35 FR 18623; December 8, 1970). The purpose of the study was to evaluate, by contemporary standards, the available safety information regarding substances presumed to be GRAS and to promulgate each item in a new (i.e., affirmed) GRAS list, a food additive regulation, or an interim food additive regulation pending completion of additional studies.

In the notice announcing the comprehensive agency review of presumed GRAS substances, FDA proposed criteria that could be used to establish whether these substances should be listed as GRAS, become the subject of a food additive regulation, or be listed in an interim food additive regulation pending completion of additional studies (35 FR 18623). These criteria were incorporated into the agency's regulations as § 121.3 (precursor of current § 170.30) (36 FR 12093; June 25, 1971).

FDA made a second announcement that it was conducting a study of presumed GRAS substances (36 FR 20546; October 23, 1971) and subsequently instituted a rulemaking to establish procedures that the agency could use, on its own initiative, to affirm the GRAS status of substances that were the subject of that review and were found to satisfy the criteria established in § 121.3 (proposed rule, 37 FR 6207, March 25, 1972; final rule, 37 FR 25705, December 2, 1972). These procedures were subsequently codified at § 170.35(a) and (b). Because the GRAS review did not cover all GRAS substances (e.g., it did not cover many substances that were marketed based on a manufacturer's independent GRAS determination), that rulemaking included a mechanism (the current GRAS petition process; § 170.35(c)) whereby an individual could petition FDA to review the GRAS status of substances not being considered as part of the agency's GRAS review.

In 1974, the agency proposed to clarify the criteria for GRAS status, the differences between GRAS status and food additive status, and the procedures being used to conduct the current review of food substances (39 FR 34194; September 23, 1974). The final regulations based on this proposal amended § 121.3 (current § 170.30) to distinguish a determination of GRAS status through scientific procedures (scientific procedures GRAS determination; current § 170.30(b)) from a determination of GRAS status through experience based on common use in food (common use GRAS determination; current § 170.30(c)) (41 FR 53600; December 7, 1976). Those final regulations also established definitions for "common use in food" (current § 170.3(f)) and "scientific procedures" (current § 170.3(h)). FDA subsequently added criteria (§ 170.30(c)(2)) for the determination of GRAS status through experience based on common use in food when that use occurred exclusively or primarily outside of the United States (53 FR 16544; May 10, 1988).

I hope that this answers your question.

To: smtp[safetywise@clear.net.nz]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: Response to telefax of April 5
Date: Tuesday, April 7, 1998 at 9:04:15 am EDT
Attached: None

The following was faxed to 64-9-434-0567 at approximately 9:00 am Eastern Daylight Time on Tuesday, April 7.

Dear Mr. James:

This is in response to your telefax dated April 5, 1998, in which you attached a copy of a 1979 FASEB report prepared under contract to FDA. This report is not part of the current GRAS affirmation petition process. Rather, it was part of a comprehensive, agency-initiated review of the safety of GRAS substances. I have copied the following excerpt from our April 17, 1997 proposed rule because it is a fairly concise description of the GRAS affirmation process, which can be on the agency's own initiative (which in most cases was done under the contract with FASEB) or on the petition of an interested party (the GRAS petition process).

From the April 17, 1997 proposed rule:

In 1969 (34 FR 17063; October 21, 1969), FDA deleted various cyclamate salts, a family of nonnutritive sweeteners, from the GRAS list because they were implicated in the formation of bladder tumors in rats. In response to the concerns raised by the new information on cyclamates, then-President Nixon directed FDA to re-examine the safety of GRAS substances, and FDA announced that the agency was conducting a comprehensive study of substances presumed to be GRAS (35 FR 18623; December 8, 1970). The purpose of the study was to evaluate, by contemporary standards, the available safety information regarding substances presumed to be GRAS and to promulgate each item in a new (i.e., affirmed) GRAS list, a food additive regulation, or an interim food additive regulation pending completion of additional studies.

In the notice announcing the comprehensive agency review of presumed GRAS substances, FDA proposed criteria that could be used to establish whether these substances should be listed as GRAS, become the subject of a food additive regulation, or be listed in an interim food additive regulation pending completion of additional studies (35 FR 18623). These criteria were incorporated into the agency's regulations as § 121.3 (precursor of current § 170.30) (36 FR 12093; June 25, 1971).

FDA made a second announcement that it was conducting a study of presumed GRAS substances (36 FR 20546; October 23, 1971) and subsequently instituted a rulemaking to establish procedures that the agency could use, on its own initiative, to affirm the GRAS status of substances that were the subject of that review and were found to satisfy the criteria established in § 121.3 (proposed rule, 37 FR 6207, March 25, 1972; final rule, 37 FR 25705, December 2, 1972). These procedures were subsequently modified at § 170.35(a) and (b). Because the GRAS review did not cover all

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GRAS substances (e.g., it did not cover many substances that were marketed based on a manufacturer's independent GRAS determination), that rulemaking included a mechanism (the current GRAS petition process; § 170.35(c)) whereby an individual could petition FDA to review the GRAS status of substances not being considered as part of the agency's GRAS review.

In 1974, the agency proposed to clarify the criteria for GRAS status, the differences between GRAS status and food additive status, and the procedures being used to conduct the current review of food substances (39 FR 34194; September 23, 1974). The final regulations based on this proposal amended § 121.3 (current § 170.30) to distinguish a determination of GRAS status through scientific procedures (scientific procedures GRAS determination; current § 170.30(b)) from a determination of GRAS status through experience based on common use in food (common use GRAS determination; current § 170.30(c)) (41 FR 53600; December 7, 1976). Those final regulations also established definitions for "common use in food" (current § 170.3(f)) and "scientific procedures" (current § 170.3(h)). FDA subsequently added criteria (§ 170.30(c)(2)) for the determination of GRAS status through experience based on common use in food when that use occurred exclusively or primarily outside of the United States (53 FR 16544; May 10, 1988).

I hope that this answers your question.

000226

FD



FOOD AND DRUG ADMINISTRATION

CENTER FOR FOOD SAFETY AND APPLIED NUTRITION

OFFICE OF PLANT AND DAIRY FOODS AND BEVERAGES

CONTAMINANTS STANDARDS MONITORING AND PROGRAMS BRANCH

200 "C" ST. S.W. • WASHINGTON, D.C. • 20204

FAX 202/260-0498

PHONE NO. 202/205- 5234

FACSIMILE COVER SHEET

DATE: 4/9/88

TO: Dr. Linda Kahl

COMPANY: _____

PHONE NO: _____

FAX NO: _____

TOTAL NO. OF PAGES, INCLUDING COVER PAGE: 6

REMARKS: As promised

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THANK YOU

10/28/96 16:26

NCTR, JEFFERSON, ARKANSAS 72079 → B2022055295

NO.247 P001/02

DEPARTMENT OF HEALTH & HUMAN SERVICES**Public Health Service****MEMORANDUM**

Date: October 17, 1996

From: Daniel M. Sheehan, Ph.D.
Reproductive & Developmental Toxicology, HFT-130

Subject: Soy Infant Formula

To: Beth Yetley, M.D., Director, Office of Special Nutritionals, CFSAN

Through: John F. Young, Ph.D., Director, DRDT, NCTR ~~10/28/96~~
Bernard A. Schwetz, D.V.M., Ph.D., Director, NCTR ~~10/28/96~~

I have just arrived back from an international meeting on soy, including a satellite symposium on soy and infant health. New data presented at the meeting have increased my level of concern regarding the phytoestrogen (isoflavone) content of infant formula. In particular, Ken Setchell, one of the two best clinical and analytical chemists in this area, showed that serum concentrations of isoflavones in infants consuming soy infant formula exceeded 10 µM. Even with a low estrogenic potency, estrogen receptors will be occupied and estrogenic responses are predicted. Ken explicitly stated that this infant serum level will lead to biological responses, but it is unclear whether these would be harmful or beneficial.

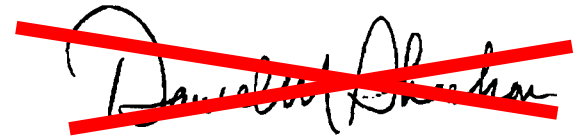
It is my opinion that such a high exposure level to estrogens during early human postnatal development is supraphysiological and, therefore, of concern. By comparison, infant exposure levels to phytoestrogens in milk of mothers consuming soy is greatly lower than from soy formula, so the Asian experience cannot be used for comparison. While it is possible that beneficial effects may be conferred by the soy infant formula estrogens, no human evidence exists to my knowledge. Likewise, as you know, there is so little clinical evidence relating estrogen exposures to human infants with premature breast development and vaginal bleeding. Benefits and risks may also coexist in the same individual at the same doses as they do for a number of human estrogen exposures. Knowledge of benefits and risks is important for informed decisions across varying circumstances ranging from convenience to necessity.

Since neither benefits nor risks have been well-defined, and exposure exceeds physiological levels, I believe we are allowing a huge human exposure with no monitoring for health effects, when biological responses are virtually certain. The situation warrants a close examination; if we fail to take any action, and significant adverse health effects are subsequently reported, the FDA will be blamed for not responding to an anticipated problem. (b) (5)

000254

I suggest that we at the FDA develop a strategy for dealing with this exposure as soon as possible. Additionally, based on what I now know, I can no longer be a signatory to the memo we drafted about eighteen months ago, as it does not reflect our current knowledge.

Thank you for your attention to this matter.



Daniel M. Sheehan, Ph.D.

cc: Janice Oliver, Deputy Director for Systems and Support, CFSAN

000255

Isolflavone Content of Breast Milk and Soy Formulas: Benefits and Risks

To the Editor:

A recent editorial [1] properly credited Franke and Custer [2] for an important study of urinary and breast milk concentrations of isolflavones in women consuming soybeans. This editorial cited literature concerning potential health benefits of phytoestrogens and, to a much lesser extent, potential toxicity. In the accompanying letter below, Franke points out that infants consuming soy-based formula are exposed to high concentrations of phytoestrogens.

Estrogens are two-edged swords in humans; both risks and benefits can be demonstrated in the same person. Two examples are oral contraceptives (benefit: fertility control; risk: increased incidence of breast cancer [3]) and unopposed estrogen replacement therapy (benefit: reduction in mortality due to heart disease and osteoporosis and relief of menopause symptoms; risk: increased incidence of endometrial cancer [4]). Given this characteristic of estrogens generally, what do we know of risks from phytoestrogens?

Adverse effects of phytoestrogens on reproduction and development in wildlife [5], livestock [6], and experimental animals [7] have been documented. Developmental exposure to phytoestrogens results in toxicities similar or identical to those of other estrogens. Neonatal rodents have long been used as a model of human prenatal diethylstilbestrol (DES) exposure on the basis of developmental staging and similar outcomes from exposure [8]. However, the neonatal rodent and postnatal human are not at equivalent morphological stages of development [9] and the neonatal rodent does not model the infant human. In addition to lacking a rodent estrogen model of the human infant, we also have little clinical experience with human infant exposure to estrogens generally. Although the data are limited for developmental effects of phytoestrogens, the similarity of DES and

phytoestrogen effects in newborn rodents should be considered a cautionary note for the developmentally later exposure that occurs with soy infant formula. As the editorial points out, the beneficial effects of soy-based formulas or of milk from mothers consuming phytoestrogens is speculative. The same is true for potential risks.

Phytoestrogen exposure is quite high; ~20% of American infants receive an isolflavonoid dose from soy formula (expressed as mg/kg) that is about five times higher than the dose that lengthened the follicular phase of the menstrual cycle and lowered lutropin and follitropin concentrations in adult women [10]. To my knowledge, only one study is underway in soy formula-exposed infants, despite our great uncertainty concerning benefits and risks of isolflavone exposure.

While metabolism and disposition data are important in both animals and humans, another crucial need is to define appropriate animal models and to explore phytoestrogen effects in these models; to characterize biological effects of phytoestrogens in infants, particularly those consuming soy-based infant formulas; and to be able to compare results across animals and humans. These studies need to define effects as either beneficial or adverse, and to explore a large variety of effects. They should also consider dose response, age at exposure, and length of exposure. Only after completion of such studies can we know the benefits and risks of infant phytoestrogen exposure and thus be able to provide the best advice to parents concerning infant exposures from breast milk and soy-based formulas.

In the meantime, this large, uncontrolled, and basically unmonitored human infant experiment continues unabated.

References

1. Slavin JL. Phytoestrogens in breast milk—another advantage of breast-feeding? [Editorial]. *Clin Chem* 1996;42:841-2.
2. Franke AA, Custer LJ. Daidzein and genistein concentrations in human milk after soy consumption. *Clin Chem* 1996;42:955-64.
3. Collaborative Group on Hormonal Factors in Breast Cancer. Breast cancer and hormonal

contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. *Lancet* 1996;347:1713-27.

4. Goddard MK. Hormone replacement therapy and breast cancer, endometrial cancer and cardiovascular disease: risks and benefits. *Br J Gen Pract* 1992;42:120-5.
5. Leopold AS, Ervin M, Browning B. Phytoestrogens: adverse effects on reproduction in California quail. *Science* 1976;191:98-100.
6. Shutt DA. The effects of plant oestrogens on animal reproduction. *Endeavour* 1974;35:110-3.
7. Medlock KL, Branham WS, Sheehan DM. The effects of phytoestrogens on neonatal rat uterine growth and development. *Proc Soc Exp Biol Med* 1995;208:307-13.
8. Bern HA, Talamantes F. Neonatal mouse models and their relation to disease in the human female. In: Herbst AL, Bern HA, eds. Developmental effects of diethylstilbestrol (DES) in pregnancy. New York: Thieme-Stratton, 1981: 129-47.
9. Ojeda SR, Andrews WW, Advis J, Smith-White S. Recent advances in the endocrinology of puberty. *Endocr Rev* 1980;1:228-57.
10. Irvine CHG, Fitzpatrick M, Robertson I, Woodhams D. The potential adverse effects of soybean phytoestrogens in infant feeding. *N Z Med J* 1995;108:208-9.

Daniel M. Sheehan

National Center for Toxicol. Res.

USFDA

3900 NCTR Rd, HFT-130

Jefferson, AR 72079

To the Editor:

The editorial by Slavin [1] summarizes the effects of isolflavones and recent research on human isolflavone exposure, including our studies on breast milk concentrations after soy consumption [2]. Some brief comments may be helpful to add to the current knowledge in the area of isolflavonoid research.

Extensive analyses of isolflavone concentrations in legumes showed that exclusively soy foods contained considerable amounts of these agents, whereas other legumes such as lentils, beans, and chickpeas contained trace or nondetectable amounts [3]. A recent study confirmed these results by reporting isolflavone concentrations in lentils, beans, and chickpeas to be lower by a factor of 50 to 5000 relative to soybeans [4].

The higher urinary isolflavone recovery after consumption of fermented vs nonfermented soy foods [5] needs to be verified because the protocol applied resulted in differen-

SIGNIFICANCE OF PHYTOESTROGENS IN INFANT SOY FORMULA
Conference Room 2A52, Building 31, NIH Campus

May 15, 1997
9:00 AM - 4:30 PM

- I. Welcome and Charge to the Group Ephraim Y. Levin, M.D.
- II. Introduction by the Chair Frederick Naftolin, M.D.
- III. FDA Presentation Elizabeth A. Yetley, Ph.D.
- IV. Overview and History Samuel J. Fomon, M.D.
- V. Presentations
- ✓ Mindy S. Kurzer, Ph.D., University of Minnesota - Effect of Phytoestrogens on Reproductive Hormones in Adult Women
 - ✓ Patricia L. Whitten, Ph.D., Emory University - Developmental Actions of Isoflavonoids in Comparison to Endocrine Disrupters
 - ✓ Claude Hughes, M.D., Ph.D., Duke University - Developmental Effects of Dietary Soy Phytoestrogens
 - ✓ Doyle Waggle, Protein Technologies - Soy Proteins for Infant Formula: Production and Composition
 - ✓ Kenneth D.R. Setchell, Ph.D., Children's Hospital Medical Center, Cincinnati - Phytoestrogen Exposure in Early Life
 - ✓ Karen O. Klein, M.D., A.I. Dupont Institute - Phytoestrogens, Soy Infant Formula, and Relevance to Endocrine Function
 - ✓ *Pat Murphy* Steven Arnold, Ph.D., Tulane University - Agonistic and Antagonistic Activities of Phytochemicals in Estrogen Response Systems
 - ✓ Daniel M. Sheehan, Ph.D., Food and Drug Administration (FDA) - Potential Toxicity of Soy Infant Formula

VI. Discussion of the Issues (including RFA/RFP/other mechanisms and activities)

Gilman D. Grave, M.D., NICHHD

VII. Other Industry and Government Representatives and Consultants

Jeffrey Baron, M.D., NICHHD
 Dennis M. Bier, M.D., Baylor College of Medicine
 Michael Bolger, Ph.D., FDA
 Robert Burns, Ph.D., Mead Johnson Research Center
 Charlotte Catz, M.D., NICHHD

000257

Darla Danford, D.Sc., R.D., FDA
Daniel Doerge, Ph.D., FDA
Mr. Robert C. Gelardi, Infant Formula Council
George P. Giacoia, M.D., NICHD
Linda Golden, M.D., FDA
Clare M. Hasler, Ph.D., University of Illinois
Marc S. Jacobson, M.D., Committee on Nutrition, American Academy of Pediatrics
Susan M. Kaup, Ph.D., Wyeth-Ayerst Research
Tony Huggett, Ph.D., Nestle Food Company
Eileen Madden, Ph.D., Nestle Food Company
Charles E. Mize, M.D., Ph.D., FDA
Ms. Mardi Mountford, Infant Formula Council
William R. Obermeyer, Jr., Ph.D., FDA
Marge Ostrom, Ph.D., Ross Products Division, Abbott Laboratories
Jean Temeck, M.D., FDA
John E. Vanderveen, Ph.D., FDA
Linda Wright, M.D., NICHD
Sumner J. Yaffe, M.D., NICHD
Ekhard Ziegler, M.D., University of Iowa

000258

SIGNIFICANCE OF PHYTOESTROGENS IN INFANT SOY FORMULA**List of Participants****Conference Room 2A52, Building 31****May 15, 1997**

Steven Arnold, Ph.D.
 Professor, Department of
 Environmental Health Sciences
 Tulane University
 1430 Tulane Avenue
 New Orleans, LA 70112-2699

Charlotte Catz, M.D.
 Chief, Pregnancy and Perinatology
 Branch, CRMC, NICHD, NIH
 Room 4B03, Executive Bldg. 6100
 6100 Executive Blvd. MSC 7510
 Bethesda, MD 20892-7510

Jeffrey Baron, M.D.
 Senior Investigator
 Developmental Endocrinology Branch
 National Institute of Child Health
 and Human Development, NIH
 Room 10N262, Building 10
 9000 Rockville Pike
 Bethesda, MD 20892-1862

Darla Danford, D.Sc., R.D.
 Director
 Division of Science and Applied
 Technology
 Food and Drug Administration
 200 C Street, SW
 Washington, DC 20204

Dennis M. Bier, M.D.
 Professor of Pediatrics
 Director, Children's Nutrition Research Ctr.
 Baylor College of Medicine
 1100 Bates Street
 Houston, TX 77030-2600

Daniel Doerge, Ph.D.
 National Center for Toxicology Research
 3900 NCTR Road, HFT-1
 Room 122, Bldg. 13
 Food and Drug Administration
 Jefferson, AR 72079-9502

Michael Bolger, Ph.D.
 Chief, Contaminant Branch
 Office of Plant and Dairy Foods,
 and Beverages
 Food and Drug Administration, HFS-308
 200 C Street, SW
 Washington, DC 20204

Samuel J. Fomon, M.D.
 415 Windsor Drive
 New Braunfels, TX 78132

Mr. Robert Gelardi
 Infant Formula Council
 Suite 500G
 5775 Peachtree-Dunwoody Road
 Atlanta, GA 30342

Robert Burns, Ph.D.
 Div. of Neonatology and Pharmacology
 Dept. of Medicine and Clinical Affairs
 Mead Johnson Research Center
 2404 W Lloyd Expressway
 Evansville, IN 47721-0001

000259

George P. Giacoia, M.D.
Special Expert
Endocrinology, Nutrition and Growth
Branch, CRMC, NICHD, NIH
Room 4B11, Executive Bldg. 6100
6100 Executive Blvd. MSC 7510
Bethesda, MD 20892-7510

Linda Golden, M.D.
Medical Reviewer
Division of Reproductive Urological
Drug Products
Food and Drug Administration, HFD-580
5600 Fishers Lane
Rockville, MD 20857

Gilman D. Grave, M.D.
Chief
Endocrinology, Nutrition and Growth
Branch, CRMC, NICHD, NIH
Room 4B11, Executive Bldg. 6100
6100 Executive Blvd. MSC 7510
Bethesda, MD 20892-7510

Clare M. Hasler, Ph.D.
Professor
Department of Food Science
and Human Nutrition
University of Illinois
1302 West Pennsylvania Avenue
Urbana, IL 61801

Anthony Huggett, Ph.D.
Nestec S.A. Research Center
Vers-Chez-Les-Blanc Case Postale 44
CH-1000
Lausanne 26 Switzerland

Claude Hughes, M.D., Ph.D.
Associate Professor
Dept. of Obstetrics and Gynecology
Box 3084
Duke University Medical Center
Durham, NC 27710

Marc S. Jacobson, M.D.
Schneider Children's Hospital
Room 187
Long Island Jewish Medical Center
New Hyde Park, NY 11042

Susan M. Kaup, Ph.D.
Principal Scientist
Nutrition Research
Wyeth Nutritionals International
145 King of Prussia Road
Radnor, PA 19087

Karen O. Klein, M.D.
Pediatric Endocrinologist
A.I. Dupont Institute
P.O. Box 269
Wilmington, DE 19899

Mindy S. Kurzer, Ph.D.
Associate Professor
Department of Food Science
and Nutrition
University of Minnesota
1334 Eckles Avenue
St. Paul, MN 55108

Ephraim Y. Levin, M.D.
Medical Officer
Endocrinology, Nutrition and Growth
Branch, CRMC, NICHD, NIH
Room 4B11, Executive Bldg. 6100
6100 Executive Blvd. MSC 7510
Bethesda, MD 20892-7510

Eileen Madden, Ph.D.
Carnation Nutrition Product Division
Nestle Food Company
800 N Brand Blvd.
Glendale, CA 91203

Charles E. Mize, M.D., Ph.D.
Food and Drug Administration
200 C Street, SW
Washington, DC 20204

000260

Ms. Mardi Mountford
Infant Formula Council, Suite 500G
5775 Peachtree-Dunwoody Road
Atlanta, GA 30342

Patricia A. Murphy, Ph.D.
2312 Food Sciences Building
Iowa State University
Ames, IA 50011

Frederick Naftolin, M.D.
Professor and Chairman
Dept. of Obstetrics and Gynecology
Yale University
333 Cedar Street 339FMB
New Haven, CT 06520

William R. Obermeyer, Jr., Ph.D.
Food and Drug Administration
200 C Street, SW
Washington, DC 20204

Marge Ostrom, Ph.D.
Ross Products Division
Abbott Laboratories
625 Cleveland Avenue
Columbus, OH 43215-1724

Kenneth D.R. Setchell, Ph.D.
Professor of Pediatrics
Children's Hospital Medical Center
Dept. of Clinical Mass Spectrometry
333 Burnett Avenue
Cincinnati, OH 45229

Daniel M. Sheehan, Ph.D.
Research Biologist
National Center for Toxicological Research
Food and Drug Administration
3900 NCTR Road, HFT-130
Jefferson, AR 72079

Jean Temeck, M.D.
Medical Officer
Division of Metabolic and Endocrine
Drug Products
Food and Drug Administration, HFD-510
5600 Fishers Lane
Rockville, MD 20857

Louis E. Underwood, M.D.
Chief, Div. of Pediatric Endocrinology
Dept. of Pediatrics
Univ. of North Carolina School of Med.
CB #7220
509 Burnett-Womack Bldg.
Chapel Hill, NC 27599-7220

John E. Vanderveen, Ph.D.
Director
Office of Plant and Dairy Foods,
and Beverages
Food and Drug Administration, HFD-510
200 C Street, SW
Washington, DC 20204

Doyle H. Waggle, Ph.D.
Vice President and Director of Technology
Protein Technologies International, Inc.
Checkerboard Square
St. Louis, MO 63164

Patricia L. Whitten, Ph.D.
Associate Professor
Department of Anthropology
Emory University
Atlanta, GA 30322

Linda Wright, M.D.
Special Assistant to the Director
Center for Research for Mothers
and Children, NICHD, NIH
Room 4B03, Executive Bldg. 6100
6100 Executive Blvd. MSC 7510
Bethesda, MD 20892-7510

000261

Sumner J. Yaffe, M.D.
Director
Center for Research for Mothers
and Children, NICHD, NIH
Room 4B05, Executive Bldg. 6100
6100 Executive Blvd. MSC 7510
Bethesda, MD 20892-7510

Elizabeth Yetley, Ph.D.
Director
Office of Special Nutritionals
Food and Drug Administration
CFSAN HFS-450
200 C Street, SW
Washington, DC 20204

Ekhard E. Ziegler, M.D.
Professor, Dept. of Pediatrics
University of Iowa
200 Hawkins Drive, #W115 GH
Iowa City, IA 52242-1083

000262

To: smtp[safetywise@clear.net.nz]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: Electronic copy of ADM GRAS notice
Date: Thursday, April 9, 1998 at 3:06:04 pm EDT
Attached: h:\grasnoti\soyisofl\gras.wpf

FD



Valerie A. James
Rural Delivery 4
Whangarei
New Zealand

Dear Ms. James:


This is in response to your request under the Freedom of Information Act (FOIA) for the submission of Archer Daniels Midland (ADM) regarding ADM's notice to the Food and Drug Administration (FDA) of ADM's view that the use of soy isoflavones as a micronutrient in food is generally recognized as safe (GRAS). In order to expedite the multiple FOIA requests that FDA has received regarding this notice, ADM has graciously provided an electronic copy of its submission. The electronic copy is in Word Perfect 5.1. Because I myself was able to retrieve this attachment using its .wpf extension, I am transmitting this electronic copy to you in exactly the format that I received. If you are unable to retrieve it, I will attempt to resend it as a Word document, because the first electronic mail message that I received from your E-mail address included a Word attachment.

ADM was unable to include the following figures in the electronic copy: Figure 1 (which shows chemical structures) and Figure 2 (which is a schematic of the manufacturing process). However, a hard copy of the entire ADM submission, including these two figures, is being sent to you in the mail by FDA's FOIA office. The hard copy also includes a copy of a published review article, "Phyto-oestrogens and Western Diseases," by Herman Adlercreutz and Witold Mazur, which was published in The Finnish Medical Society DUODECIM, Ann. Med. 29, pp. 95-120, 1997.

Please let me know if you are able to retrieve this copy.

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C St., SW, Washington DC 20204
Phone: (202)418-3101 Fax: (202)418-3131
Internet: LKAHL@BANGATE.FDA.GOV

000228

To: ISMTP@FDA-OC-TRAINING@FDAOC [<safetywize@clear.net.nz>]
From: Linda Kahl@OPA@FDA.CFSAN FD 
Certify: N
Subject: re: RE: Electronic copy of ADM GRAS notice
Date: Friday, April 10, 1998 at 8:26:46 am EDT
Attached: h:\grasnoti\soyisofl\wordgras.doc,
h:\grasnoti\soyisofl\wp51gras.doc

Well, let's try this again.

The original ADM Word Perfect file came to me with the usual .wp extension but I was unable to retrieve it; they resent it with a .wpf extension, which worked for me but is not the usual extension. So, I made an exact copy of that file in Word Perfect 5.1 and renamed it WP51GRAS.DOC, which is another extension for a WP51 document. I was able to retrieve that in Word by telling Word to retrieve only Word Perfect 5.1 documents, and the .doc extension seems to be a happy one for Word (my first attempt at converting to Word caused my machine to crash, so I am looking for a way to keep Word happy!).

My version of Word is Office 97 and it was only installed a week ago, so I have never used it. I saved the ADM document that I was able to retrieve into Word as a Word 6.0 for Windows 95, because that seemed to be more reasonable than the Office 97 version. That file is named WORDGRAS.DOC.

Hopefully, one of these will work.

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C St., SW, Washington DC 20204
Phone: (202)418-3101 Fax: (202)418-3131
Internet: LKAHL@BANGATE.FDA.GOV

000230

To: smtp[safetywise@clear.net.nz]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: Response to your submissions
Date: Monday, April 27, 1998 at 10:24:33 am EDT
Attached: None

FD



Thank you for the information that you have provided regarding soy isoflavones. I am passing it along to the scientists who are reviewing GRAS Notice GRN #000001 from Archer Daniels Midland.

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C St., SW, Washington DC 20204
Phone: (202)418-3101 Fax: (202)418-3131
Internet: LKAHL@BANGATE.FDA.GOV

000422

Apr 27, 1998

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Computer Technology Services, Inc.

000423

To: ISMTP@FDA-OC-TRAINING@FDAOC[<safetywise@clear.net.nz>]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: re: RE: Response to your submissions
Date: Tuesday, April 28, 1998 at 8:33:53 am EDT
Attached: None

FD



All requests made under the Freedom of Information Act (FOIA) must be sent directly to the FOI office. For your information, FOIA provides that certain existing records of the Food and Drug Administration are available for public disclosure. The identity of review scientists who are working on a particular project is not an existing record. Therefore, in the event that you choose to file a request for this information under FOIA, the likely response would be "no responsive records."

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C St., SW, Washington DC 20204
Phone: (202)418-3101 Fax: (202)418-3131
Internet: LKAHL@BANGATE.FDA.GOV

000464

Apr 28, 1998

To: ISMTP@FDA-OC-TRAINING@FDAOC[<woodhams@iprolink.co.nz>]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: re: re: GRAS Notice GRN 000001
Date: Wednesday, April 29, 1998 at 8:01:29 am EDT
Attached: None

FD



We are aware of Dr. Sheehan's work.

000427

Apr 29, 1998

To: smtp[safetywise@clear.net.nz]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: Defect Action Levels
Date: Wednesday, May 6, 1998 at 9:06:04 am EDT
Attached: None

FD



Richard James
RD 4
Whangarei, NZ

Dear Mr. James:

In response to your telefax dated 5/5/98, the most current version of CFSAN's Defect Action Levels is available on our home page at

<http://vm.cfsan.fda.gov/~lrd/fdaact.html>

According to this document, none of the substances that you mentioned (i.e., isoflavones, genistein, and daidzein) are the subject of a Defect Action Level.

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
100 C St., SW, Washington DC 20204
Phone: (202)418-3101 Fax: (202)418-3131
Internet: LKAHL@BANGATE.FDA.GOV

May 06, 1998

000469



MEMORANDUM OF TELEPHONE CONVERSATION

June 18, 1998

Between: Laura Tarantino, HFS-200

And

Gary Yingling, McKenna and Cuneo

Re: GRN 00001, soy isoflavones

Mr. Yingling called to inquire about the status of the review of the Archer Daniels Midland (ADM) GRAS Notification for soy isoflavones. I told him that, as he was aware, we had received several comments to the notification, some of which made reference to controversy in the scientific community about the safety of these compounds. I noted that the notification had not explicitly addressed this controversy. Mr. Yingling acknowledged that ADM was aware that there was debate about the safety of soy isoflavones.

I asked if he had received a response to his Freedom of Information Act request for the comments. He said he had not, and stated that he thought that ADM would likely be very anxious to respond to the assertions in the comments, and might wish to withdraw the notification while they considered their response. I conveyed my regrets that there had been problems getting the FOIA request referred here for response, and for the consequent delay in getting the information to him. I promised him that we would send him a copy of the comments as soon as possible, and suggested that after he had a chance to consult with ADM, they might wish call us about the best way to proceed administratively.

Laura Tarantino, Ph.D.

(b) (5)

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(b) (5)								

000725

**Memorandum of Meeting**

Date, September 9, 1998

Time 11 00 a.m. - 12:30 p.m.

Place: 1110 Vermont Avenue, 12th floor conference room

Visitors

Joseph Borzelleca	Medical College of Virginia
Charles Capen	Ohio State University
Mark Empie	Archer Daniels Midland
Eric Gugger	Archer Daniels Midland
Susan Onel	McKenna and Cuneo
Gary Yingling	McKenna and Cuneo

FDA

Michael Bolger	HFS-308
Michael DiNovi	HFS-246
Linda Kahl	HFS-206
Helen Lee	HFS-225
Antonia Mattia	HFS-207
George Pauli	HFS-205

Subject: GRN # 000001 (Soy Isoflavones)

The visitors presented an update on GRN #000001 (agenda and slides attached) and discussed with FDA representatives administrative options for Archer Daniels Midland Company to address the submissions made to the administrative file of GRN #000001 by outside parties.

Linda S. Kahl, Ph.D

(b) (5)



000736

FILE
COPY

OFFICE	SURNAME	DATE	OFFICE	SURNAME	DATE	OFFICE	SURNAME	DATE
(b) (5)								

Meeting Agenda
Center for Food Safety And Applied Nutrition
Office of Premarket Approval
1110 Vermont Avenue, N.W.
September 9, 1998 11:00 A.M.

I. AGENDA SOY ISOFLAVONES

1. Introduction
2. Dietary Consumption of Soy Isoflavones
 - a. Animal Safety Data
 - b. Human Safety Data
 - i. Notification
 - ii. Health Claim Petition
 - iii. Other
3. End Point Studies
 - a. Gonadal
 - b. Thyroid
4. Status of Notification
5. Conclusion

II. ARCHER DANIELS MIDLAND COMPANY REPRESENTATIVES

1. Mark Empie, Ph.D., Director of Regulatory Affairs, ADM
2. Eric Gugger, Ph.D., Nutrition Research Scientist, ADM
3. Joseph Borzelleca, Ph.D., Pharmacology & Toxicology, Inc.
4. Charles Capen, Ph.D., Ohio State University, Professor, Department of Veterinary Biosciences
5. Gary L. Yingling, Esq., McKenna & Cuneo, L.L.P.

000737

000738

Update of GRAS Notification File GRN 000001

A Presentation made to the FDA

September 9, 1998

by

The Archer Daniels Midland Company

000738

Update of ADM GRAS Notification Data

1. Dietary consumption of soy isoflavones

a) Animal safety data

b) Human safety data

i) Notification

ii) Health Claim Petition

2. Feeding Studies

a) Gonadal endpoints

b) Thyroid endpoints

Dietary Consumption of Soy Isoflavones
Animal Data

- 60-90 million metric tons of soy meal is produced which will contain (at 2-3 g/kg meal) about 120 - 270 thousand tons of isoflavones.
- Soy meal is commonly used in production animal feed to raise protein quality.
- Incorporation rates up to 30% of feed provide isoflavones in the diet ranging from 3 to 45 mg/kg-bw/day for pigs, cattle, dogs, cats, chickens and sheep.
- These daily animal consumption values are 4 to 64 times the proposed 50 mg value for humans.

Table 1
Isoflavone Content of Diets

Animal	Typical Time on Feed	Typical Soy Meal Content*	Typical Daily Consumption	Daily Isoflavone Dose mg/kg-bw
Pigs (100 kg)	120 days	15-30%	3 kg/da	9-18
Sheep (50 kg lamb) (70 kg gestating ewe)	60-90 da several yrs	0-5% 0-5%	1.5 kg/da 1.7 kg/da	0-3 0-2
Cattle (600 kg)	8-9 mo	5-10%	20 kg/da	3-6
Chickens (2 kg broiler)	7 wk	20-30%	0.15 kg/da	30-45
Chicken (1.5 kg layer)	1-2 yr	15-20%	0.1 kg/da	20-27
Dog (23 kg)	many yrs	(260 ppm isoflavones) direct measurement	0.35 kg/da (recommended)	4
Cat (4 kg)	many yrs	(250 ppm isoflavones) direct measurement	0.065 kg/da (recommended)	4

* assume soy meal contains ~ 2g/kg isoflavones

Dietary Consumption of Soy Isoflavones
Human Data - Notification

- Human daily consumption estimates for Far Eastern soy diet indicate isoflavone ingestion levels to be between 25 to 200 mg/person/da (0.4 - 3 mg/kg-bw/da).
- Isoflavones are metabolized in the G.I. tract, absorbed in the G.I. tract, conjugated in enterohepatic circulation and excreted within 1 to 2 days. Absorption is similar in humans and animals.
- Isoflavone concentrations (e.g. genistein and daidzein) reach maximum levels of up to 4 μ M in plasma after 7 -8 hours post-ingestion and have a half - life of 5 - 6 hours (King, 1998).

Consumption of Soy Isoflavones
Human Data - Health Claim Petition

- Health Claim Petition cites 50 human feeding studies with soy protein containing isoflavones. Protein sources were whole bean, soy flour, soy isolate, soy protein concentrate.

- The feeding studies are summarized below by grouping and combining the individual studies according to soy protein range consumed:

<u>No. Subjects</u>	<u>No. of Studies</u>	<u>Soy Protein (g/day)</u>	<u>Length of Study (weeks)</u>
162	7	10-19	4-8
225	12	20-39	2-4
392	12	40-59	4-8
168	9	60-100	3-6
21	2	> 100	4
57	2	31	16
66	1	40	24
228	2	25	9

- **Summary:** The principle endpoints studied were blood lipid chemistry. Significant positive benefits found included total cholesterol reduction and reductions in LDL cholesterol. HDL levels remained constant.

- No adverse effects were reported in these 50 human studies.

Animal Feeding Studies Directed Toward
Endocrine Endpoints

1. Gonadal endpoints:

- Genistein, daidzein are known to cause gonadal changes at very high dose levels. Many of these studies apply isoflavone agents via non-dietary injection routes (Table 2, appended)
- Isoflavones exhibit weak estrogenic activity, as do many phytochemical compounds in the environment.
- 13 animal feeding studies with soy isoflavones are summarized on the appended Table 3.
- NOEL for isoflavones in these 13 feeding studies ranged from 9 mg/kg-bw/da to about 100 mg/kg-bw/da in the diet for various gonadal endpoints.
- For comparison, 50 mg of isoflavones would provide about 0.7 mg/kg-bw/da for an adult human.
- Thus, the animal feeding study NOEL's are 12 to 1000 times the proposed soy isoflavone use level in humans.

Thyroid Function - Endpoints

1. General Observations

- Historically soy has been associated with goiter formation involving iodine deficient diets. U.S. diets are usually sufficient in iodine to counter the effect, and consumption of soy products has not resulted in an increased incidence of goiter.
- 50 human feeding studies of Health Claim Petition do not note adverse thyroid effects upon feeding soy protein containing isoflavones.

Thyroid Function - Endpoints

- Thyroid peroxidase enzyme (TPO) inhibition has been proposed to be a factor in the development of thyroid disease (Divi, 1996, 1997).
- TPO enzyme inhibition has been hypothesized to lead to TPO autoantibody formation. However, autoantibodies to Tg and TPO are common in the general population and low levels of autoantibodies to TPO are of uncertain significance in the presence of normal thyroid functions (Williams, 1998).
- In model in vitro assays, TPO enzymes can be inhibited by isoflavones in the presence of hydrogen peroxide and low iodide. It is not known if these purified enzyme assays are representative of the enzyme system as found in the thyroid follicular cell membranes in vivo.
- Soy protein and/or isoflavones increase T_4 in animals and humans; T_3 and free T_3 are not altered (Forsyth, 1995; Potter, 1996; Balmir, 1996; Ham, 1993). This finding is inconsistent with TPO inhibition, as suggested by Divi (1996, 1997).
- Despite the potential implications of isoflavones as inhibitors of TPO, the extensive experience of soy consumption, together with the thyroid hormone data from human and animal studies, do not support the proposed physiologic effect.

Conclusions

1. Soy products have been consumed safely for many years. Studies in humans demonstrate no adverse effects of consuming soy isoflavones at dietary levels typically found in Far Eastern soy diets. These consumption levels are consistent with a value of about 50 mg/day.
2. Studies in animals demonstrate no adverse effects on gonadal function for isoflavone consumption at human dietary levels.
3. Studies in animals and humans relating to thyroid function do not support the proposed physiological effects of isoflavones acting to inhibit TPO enzyme, as suggested by Divi et.al.
4. Soy isoflavones are a safe ingredient in foods to supplement the diet when used in single meal replacements, power bars, and healthy drinks. Consumption levels are expected to be about 25 to 50 mg/day.

Table 2
Route of Administration and Dose Levels in Frequently-Cited
Isoflavone Animal Toxicology Studies

Species	Dose	Normalized Body Weight Dose (mg/kg-bw/da)	Novasoy Equivalent 70 Kg Human**	Reference
Rat	5, 25mg S.C.* (Genistein)	17-83	8.4g - 42g/da	Levy, 1995
Rat (neonate 6 gr)	1, 100, 1000 µg S.C. (equol calc'd as daidzein equivalent)	0.17-1.67	95mg-93g/da	Medlock, 1995 (4)
Rat (neonate 6 gr)	100 µ S.C. (equol calc'd as daidzein equivalent)	20	1g/da	Medlock 1955 (4)
Rat (ovariectomized female) neonatal (6 gr)	0, 1, 10, 100, 200, 400, 500, 100 µg S.C. (Genistein)	0.17-167 bw/da	95mg-94gr	Faber & Hughes
Mouse	0.1 & 0.2% diet (Genistein)	200-400	509g/da	Carter, 1955
Mouse	9mg- 72mg/da diet (Genistein)	450-7200	250g-4Kg/da	Matrone, 1956
Mouse	5.6; 45mg S.C. (Genistein)	315-4130	176g-2Kg/da	Carter, 1960

* S.C. = subcutaneous

** Calculated as equivalent to the amount of the agent tested.

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Table 3

Oral Feeding Studies Evaluating Isoflavone Effect on Estrogenic Endpoints

Study	Agent	Animal	Dosage mg/kg-bw/da	Duration (days)	Effect								Novasoy Equiv (mg/kg- bw/da)
					Body Wt	Uterus Wt	Ovary Wt	Time to Vag Open	Mat/Vag Smear Cytol	Testes Wt	Prost	Fert/ Sperm Count	
Matrone (1956)	Genistein mech. dif than DES	mouse	360, 720 1440, 2880	42	No (0) Lo 360					No 720 Lo 1440		No 720 Lo 1440	1440- 11,520
	Genistein dec number litter, not size	mouse	.1, .24 (200-400) diet	1 gen	No .24			No (0) Lo .14					1333- 2666
Carter (1955)	Genistein mech. dif from E, DES, not act as estrogen	mouse	315 4130	28	No (0) Lo 315		No (0) Lo 315			No (0) Lo 315			2100- 27,520
East (1955)	Genistein	mouse	- 100 - 250, 500 750	21 14 1 gen.				No (0) Lo 100	No (0) Lo 100 No 250 Lo 500 No (0) Lo 750				666 1666- 5333 1500
	Genistein	mouse CD1	118, 160	4		No 160						No (0) Lo 750	790- 1067 880
Farmakalidis (1984)	Genistein	mouse CD1	220	4		No 220							1050
	Daidzin	mouse CD1	210	4		No (0) Lo 210							106
Farmakalidis (1984)	Genistein	mouse B6D2F	- 160	4		No (0) Lo 160							880
	Genistein	mouse B6D2F	- 220	4		No (0) Lo 220							1100
Farmakalidis (1984)	Daidzin	mouse B6D2F	- 220	4		No 220							
	Daidzin	mouse B6D2F	- 220	4		No 220							

No = NOEL
Lo = LOEL

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Table 3 continued

Oral Feeding Studies Evaluating Isoflavone Effect on Estrogenic Endpoints

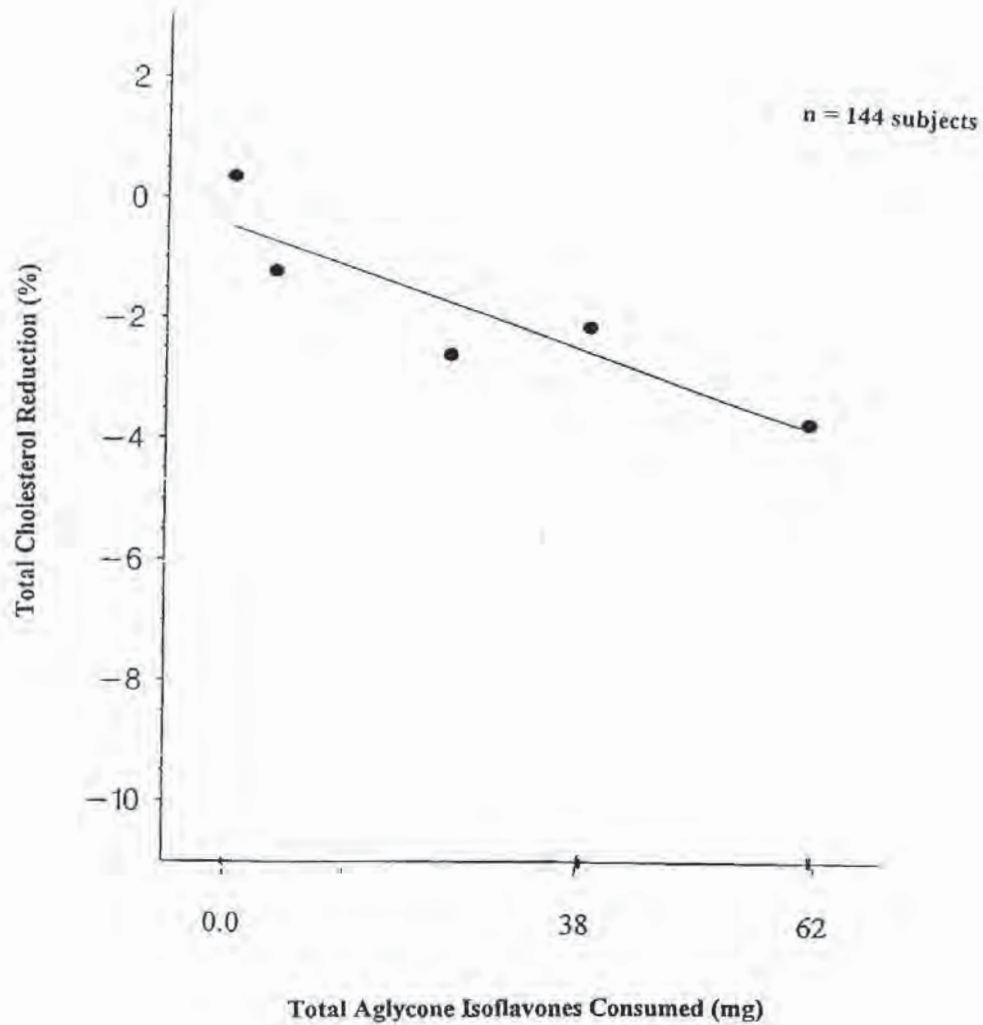
Study	Agent	Animal	Dosage mg/kg-bw/day	Duration (days)	Effect							Novasoy Equiv (mg/kg- bw/day)
					Body Wt	Uterus Wt	Ovary Wt	Time to Vag Open	Mac/Vag Smear Cytol	Testes Wt	Prost	Fert Sperm Count
Ishida (1998)	Genistein	ovx rat	50	28	No 50	No 50						200
	Daidzein	ovx rat	10, 25, 50	28	No 10 Lo 25	No 25 Lo 50						50-250
Magee (1963)	Genistein	rats	60-300	28	No .5%					No .5%		400- 2000
	Genistein	rats	60-300	28	No .1%					No .5%		240- 1200
Yamazaki	Daidzein	rat	6, 28, 100, 400	3		No 100 Lo 400						50-3333
Anthony	Soy isoflavones	monkey	1, 9	159	No 9	No 9				No 9	No 9	2-18
Baird (1995)	Soy isoflavones No clear estrogenic effects in post meno- pausal women	human	~ 2.3	28	No 2.3				No 2.3			4.6
Wilcox (1990)	Soy isoflavones	human post meno- pausal women	(45g/day soy flour) ~ 1.3	14	No 45g				No 10 Lo 45g (p=0.05)			- 2 6
Murkies (1995)	Soy isoflavones	human post meno- pausal women	(45g/day soy flour) ~ 1.3	42 84					No 48g			- 2 6

NO = NOEL
LO = LOEL

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Figure 1

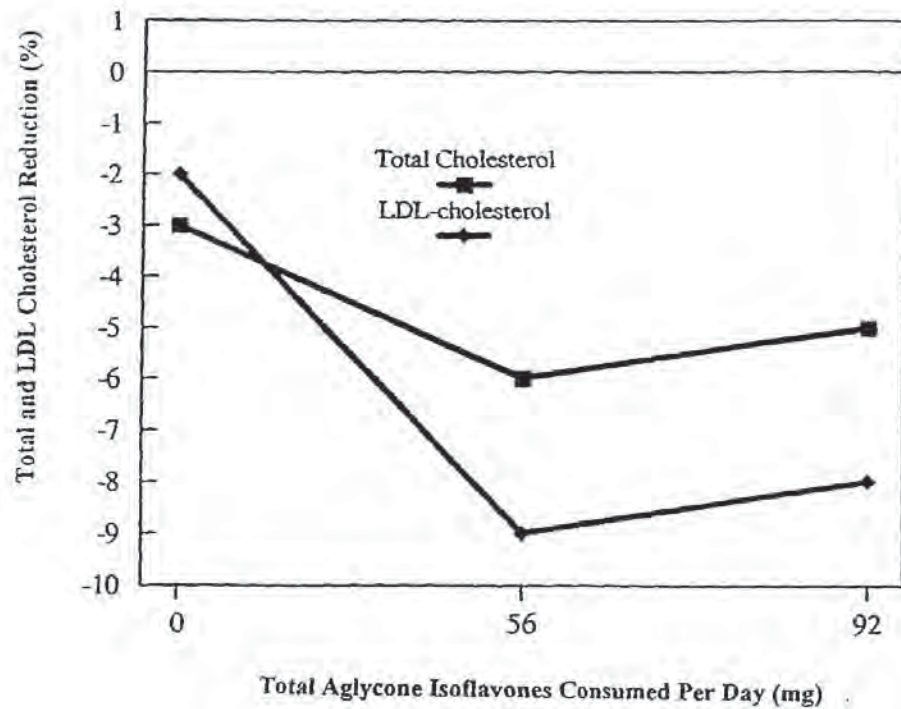
Percentage Reduction in Total Cholesterol as a Function of Isoflavone Content of Soy Protein
(Data from Health Claim Petition)



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Figure 2

**Change in Total and LDL Cholesterol in Subjects Participating in the Study by Baum et.al., in pre
(Data from Health Claim Petition)**



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Recent Publications of Note

McMichael Phillips (1996)	Proliferation rate increase of breast lobular epithelium with isoflavone administration
Verma (1998)	Genistein, equol inc growth of MCF-7 cells alone but inhibit proliferation by other environmental chemicals
Dees (1997)	Isoflavones stimulate human breast tissue to enter cell cycle
Klein (1998)	Review of isoflavones, soy infant formulas and relevance to Endocrine Function
Report to MAFF, U.K. (1997)	Phytoestrogens in the Human Diet
Report to Ministry of Health of New Zealand (1996)	Antinutrients in Soyfoods
Soy Protein Health Claim Petition	Level requested: 25 g protein/day containing 2 mg isoflavones/g protein

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000756

To: ISMTP@FDA-OC-TRAINING@FDAOC [<MFitzpatrick@kma.co.nz>]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: re: RE: RE: Re: GRN 000001 & soy formulas
Date: Wednesday, December 2, 1998 at 12:11:34 pm EST
Attached: None

FD



Dear Dr. Fitzpatrick,

Per your E-mail request of November 1, 1998, to be kept informed of the status of GRN 000001, I have included in this E-mail message the text of FDA's response (dated November 25, 1998) to a recent letter from ADM on this subject. A copy of this letter is in the publicly accessible file in FDA's Dockets Management Branch in Rockville MD. Given your distance from Rockville MD, I am sending the text of this letter as a courtesy

November 25, 1998

Mr. Mark W. Empie
Archer Daniels Midland Company
Box 1470
Decatur, IL 62525

Re: GRAS Notice No. GRN 000001
Docket No. 98S-0104

Dear Mr. Empie:

This is in response to your letter dated November 2, 1998 concerning notice of Archer Daniels Midland Company (ADM) to the Food and Drug Administration (FDA) claiming that the use of soy isoflavones as a micronutrient in performance bars, mature adult meal replacements, and Ahealthy@ beverages at a level of 25 mg soy isoflavone extract per serving is generally recognized as safe (GRAS). FDA had designated this notice as GRAS Notice No. GRN 000001. Your November 2, 1998, letter requests that FDA commit no further resources to the review of GRAS Notice No. GRN 000001 while ADM is in the process of incorporating additional information to update the file.

Given your request, FDA ceased to evaluate GRAS Notice No. GRN 000001, effective November 3, 1998, the date on which we received your letter. If you wish to have FDA consider additional information that you submit regarding soy isoflavones, please be advised that the appropriate mechanism is for ADM to submit, in accordance with proposed ' 170.36, a complete GRAS notice. FDA will assign a new file number to any new notice regarding soy isoflavones.

In accordance with proposed 21 CFR 170.36(f), a copy of this letter has been placed at the agency's Dockets Management Branch (Docket No. 98S-0104). As mentioned in our letter dated March 5, 1998, which

Dec 02, 1998

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acknowledged receipt of your GRAS notice, a copy of the information in your notice that conforms to the information in proposed ' 170.36(c)(1) is likewise available in Docket No. 98S-0103.

(HFS-206)

Approval

cc: Mr. Gary Yingling
McKenna and Cuneo
1900 K Street, N.W.
Washington, DC 20006-1108

Sincerely,
/s/
Linda S. Kahl, Ph.D.
Regulatory Policy Branch

Division of Product Policy
Office of Premarket

Center for Food Safety
and Applied Nutrition

- - - - - Original Message - - - - -
Dear Linda

Thank you for your reply. Are you able to give me any indication when the review process for GRN #1 may be complete?

Also, I thought you might like to read the latest NZ Ministry of Health Statement of Soy-based formulas. I'd be interested in your comments.

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The document was downloaded from the following site

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Mike Fitzpatrick PhD MNZIC
Senior Consultant-Water Quality
Kingett Mitchell & Associates
Environmental Consultants
4 Fred Thomas Drive
Takapuna
North Shore City
NEW ZEALAND

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Dec 02, 1998

ph (09)-486-8068
fax (09)-486-8072

- - - - - End of Original Message - - - - -

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C St., SW, Washington DC 20204
Phone: (202)418-3101 Fax: (202)418-3131
Internet:LKAHL@BANGATE.FDA.GOV

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Dec 02, 1998

To: ISMTP@FDA-OC-TRAINING@FDAOC[<JMcLeod@nzrc.co.nz>]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: re: GRAS notification for isoflavones
Date: Wednesday, December 2, 1998 at 12:16:30 pm EST
Attached: None

Dear Dr. McLeod,

Per your E-mail request of April 13, 1998, to be kept informed of the status of GRN 000001, I have included in this E-mail message the text of FDA's response (dated November 25, 1998) to a recent letter from ADM on this subject. A copy of this letter is in the publicly accessible file in FDA's Dockets Management Branch in Rockville MD. Given your distance from Rockville MD, I am sending the text of this letter as a courtesy

November 25, 1998

Mr. Mark W. Empie
Archer Daniels Midland Company
Box 1470
Decatur, IL 62525

Re: GRAS Notice No. GRN 000001
Docket No. 98S-0104

Dear Mr. Empie:

This is in response to your letter dated November 2, 1998 concerning notice of Archer Daniels Midland Company (ADM) to the Food and Drug Administration (FDA) claiming that the use of soy isoflavones as a micronutrient in performance bars, mature adult meal replacements, and Ahealthy@ beverages at a level of 25 mg soy isoflavone extract per serving is generally recognized as safe (GRAS). FDA had designated this notice as GRAS Notice No. GRN 000001. Your November 2, 1998, letter requests that FDA commit no further resources to the review of GRAS Notice No. GRN 000001 while ADM is in the process of incorporating additional information to update the file.

Given your request, FDA ceased to evaluate GRAS Notice No. GRN 000001, effective November 3, 1998, the date on which we received your letter. If you wish to have FDA consider additional information that you submit regarding soy isoflavones, please be advised that the appropriate mechanism is for ADM to submit, in accordance with proposed ' 170.36, a complete GRAS notice. FDA will assign a new file number to any new notice regarding soy isoflavones.

In accordance with proposed 21 CFR 170.36(f), a copy of this letter has been placed at the agency's Dockets Management Branch (Docket No. 98S-0104). As mentioned in our letter dated March 5, 1998, which

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Dec 02, 1998

acknowledged receipt of your GRAS notice, a copy of the information in your notice that conforms to the information in proposed ' 170.36(c)(1) is likewise available in Docket No. 98S-0103.

Sincerely,

/s/

Linda S. Kahl, Ph.D.
Regulatory Policy Branch

(HFS-206)

Division of Product Policy
Office of Premarket

Approval

Center for Food Safety
and Applied Nutrition

cc: Mr. Gary Yingling
McKenna and Cuneo
1900 K Street, N.W.
Washington, DC 20006-1108

- - - - - Original Message - - - - -

Dr Kahl,

I E- mailed you 30/3/98 re the above. I was interested to know what has or is happening with respect to the GRAS notification for soy products.

There are several other issues that I would like to bring to your attention and so I wonder if there is an objection or submission process that I can pursue with respect to these.

I look forward to your reply and the opportunity to discuss this further at as any status such as GRAS applied to soy isoflavones truly concerns me scientific, medical and ethical levels.

Thanking you

Dr Jim McLeod

- - - - - End of Original Message - - - - -

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C St., SW, Washington DC 20204
Phone: (202)418-3101 Fax: (202)418-3131
Internet:LKAHL@BANGATE.FDA.GOV

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Dec 02, 1998

To: ISMTP@FDA-OC-TRAINING@FDAOC[<safetywise@clear.net.nz>]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: re: SOY ISOFLAVONES
Date: Wednesday, December 2, 1998 at 12:23:13 pm EST
Attached: None

Dear Ms. Clapperton:

This is in response to the text of an attachment to an electronic mail message. The date of the letter in the attachment is March 30, 1998. In that attachment, you asked to be advised of the date, time and place of the hearings which will be held on the notice to FDA by Archer Daniels Midland Company regarding the GRAS status of soy isoflavones. In response to your request, I explained that the proposed GRAS notification procedure did not include public hearings.

As a followup to your request, I have included in this E-mail message the text of FDA's response (dated November 25, 1998) to a recent letter from ADM on this subject. A copy of this letter is in the publicly accessible file in FDA's Dockets Management Branch in Rockville MD. Given your distance from Rockville MD, I am sending the text of this letter as a courtesy.

November 25, 1998

Mr. Mark W. Empie
Archer Daniels Midland Company
Box 1470
Decatur, IL 62525

Re: GRAS Notice No. GRN 000001
Docket No. 98S-0104

Dear Mr. Empie:

This is in response to your letter dated November 2, 1998 concerning notice of Archer Daniels Midland Company (ADM) to the Food and Drug Administration (FDA) claiming that the use of soy isoflavones as a micronutrient in performance bars, mature adult meal replacements, and Ahealthy@ beverages at a level of 25 mg soy isoflavone extract per serving is generally recognized as safe (GRAS). FDA had designated this notice as GRAS Notice No. GRN 000001. Your November 2, 1998, letter requests that FDA commit no further resources to the review of GRAS Notice No. GRN 000001 while ADM is in the process of incorporating additional information to update the file.

Given your request, FDA ceased to evaluate GRAS Notice No. GRN 000001, effective November 3, 1998, the date on which we received your letter. If you wish to have FDA consider additional information that you submit

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Dec 02, 1998

regarding soy isoflavones, please be advised that the appropriate mechanism is for ADM to submit, in accordance with proposed ' 170.36, a complete GRAS notice. FDA will assign a new file number to any new notice regarding soy isoflavones.

✓ In accordance with proposed 21 CFR 170.36(f), a copy of this letter has been placed at the agency's Dockets Management Branch (Docket No. 98S-0104). As mentioned in our letter dated March 5, 1998, which acknowledged receipt of your GRAS notice, a copy of the information in your notice that conforms to the information in proposed ' 170.36(c)(1) is likewise available in Docket No. 98S-0103.

Sincerely,
/s/
Linda S. Kahl, Ph.D.
Regulatory Policy Branch

(HFS-206)

Division of Product Policy
Office of Premarket

Approval

Center for Food Safety
and Applied Nutrition

cc: Mr. Gary Yingling
McKenna and Cuneo
1900 K Street, N.W.
Washington, DC 20006-1108

- - - - - Original Message - - - - -

30 March 1998

Dr Linda S Kahl

Dear Dr Kahl

GRN # 000001

ARCHER DANIELS MIDLAND - SOYBEAN ISOFLAVONES

Soy Information services is astounded that this company would "determine" that soy isoflavones are "safe". It must surely be familiar enough with its own product to know that this is not true.

✓ I have known personally for many years that soy products cause thyroid

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Dec 02, 1998

disease. One research paper we have commences thus: "Thyroid enlargement in rats and humans, especially women and children fed with soybean, has been known for half a century". That is the Kyoto University Medical School at work.

Minimal reading of paediatric journals shows a number of clinical reports reporting that soy baby foods have caused thyroid damage and cretinism. Thyroid damage has also been reported in children and adult men; and other endocrine effects.

Even the parrot keepers' text book "Diseases of Cage Birds" reports goitre, infertility and lung damage associated with goitrogenic diets; eg. soybean.

We believe your own National Centre for Toxicological Research has recently proved that the soybean isoflavones cause thyroid damage.

Soy Information Services believes it is misleading for A.D.M. to deny knowledge of these dangers.

Their letter application for G.R.A.S. status should be denied. The subject should be addressed openly and publicly by the procedures which have been always used in the past. The apparent attempt to circumvent accepted procedures by providing untrue safety certificates is not acceptable.

When these hearings are scheduled, we would like to present all the appropriate medical papers; though I see your own toxicology experts refer to most of them in their research report. We wonder why you did not consult your own specialists.

Please advise us the date, time and place of the hearings which will be held. do this.

Yours sincerely

Yvonne Clapperton
for and on behalf of Soy Information Services.

- - - - - End of Original Message - - - - -

Linda S. Kahl, Ph.D.

000845

Dec 02, 1998



**DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration**

Memorandum of Meeting

Date: July 21, 1999

Time: 2:30 p.m. - 3:30 p.m.

Place: 1110 Vermont Avenue, 12th Floor Conference Room

Subject: Soy Isoflavones

Participants:

<u>NAME</u>	<u>AFFILIATION</u>
Alan Rulis	FDA, HFS-200
George Pauli	FDA, HFS-205
Linda Kahl	FDA, HFS-206
Antonia Mattia	FDA, HFS-207
Michael DiNovi	FDA, HFS-246
Susan Carberry	FDA, HFS-246
Suzan Onel	McKenna and Cuneo on behalf of Archer Daniels Midland
Mark Empie	Archer Daniels Midland
Gary Miller	Archer Daniels Midland
Walter Glinsmann	Consultant to Archer Daniels Midland
Joseph Borzelleca	Medical College of Virginia, Consultant to Archer Daniels Midland

The visitors requested the meeting to discuss the findings of a panel that Archer Daniels Midland convened to address the safety of soy isoflavones, particularly in light of questions raised by individuals at the National Center for Toxicological Research about GRAS Notice No. GRN 000001. The visitors and FDA discussed options for Archer Daniels Midland to engage with the agency regarding the findings of their panel and their determination that the use of soy isoflavones in performance bars, adult meal replacements, and healthy beverages is GRAS.

~~*Linda S. Kahl*~~
Linda S. Kahl

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FILE
COPY

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OFFICE	SURNAME	DATE	OFFICE	SURNAME	DATE	OFFICE	SURNAME	DATE

000965

SOY INFORMATION SERVICE

PO Box 3285
Onerahi
Whangarei, NEW ZEALAND

OU



Phone 09 434 0564
Fax 09 434 0567

1998 MAR 18 A 8: 06

18 March 1998

The Director
U.S.F.D.A.
C.F.S.A.N.

Dear Director

SOY ISOFLAVONES

In the publication "Food Chemical News" issue of March 2 1998 there was a report that Archer Daniel Midland Co. had "determined that soy isoflavone is generally recognised as safe for use as a micronutrient in food".

A further quotation was "Epidemiologies and feeding studies in both animals and humans referenced in the A.D.M. submission "indicate no toxic effects at dietary levels".

Soy Information Service believes these statements are completely untrue. In a November 1997 report by the British Medical Research Council's Institute for Environment Health for the U.K. Ministry of Agriculture Food & Fisheries at page 9, the British expert committee says:

Studies on the developmental effects of adverse effects including those on sexual differentiation of the brain, maturation of neuroendocrine control of ovulation and puberty and development of the female reproductive tract. However, there are also possible beneficial effects relating to antiproliferative actions on mammary tissue which may be linked to this developmental phase (Chapin et al., 1996).

The effects of dietary phytoestrogens during the reproductive period have been investigated in a number of animal species including cattle, cheetahs, mice, quail, rabbits and sheep. The overall effect, which is relatively consistent and more distinct in females than males, is that of depressed fertility. Potential sites of action in females include the genital tract, ovaries, pituitary and the central nervous system, Considering all the available data, Chapin et al. (1996) suggested that

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the effects of phytoestrogens can only be regarded as predominantly adverse with regard to fertility in females of these animal species.

Therefore, Soy Information service wishes to oppose the petition of Archer Daniels Midland Company to have isoflavones designated as Generally regarded as Safe.

Could you please advise us by e-mail how we may do this.

Yours sincerely

Yvonne Clapperton
for and on behalf of Soy Information Services.

000151

OU

~~WHEAREI~~

Dr Linda S. Kahl
Regulatory Policy Board
Office of Pre-Market Approval
200 E St Washington DC
FA# 202 418 3131

R.D. 4
WHEAREI N-2
3/25/98
Gr. 9 to 3 to 0567

Dear Dr Kahl,

The following letter has been airmailed
to the address you gave Dr Yvonne
Clapperton. Thank you for that response.

Could you please also forward a copy
of this fax to the Director, Office of
Pre-Market Approval.

Sincerely

~~Richard J. Evans~~

000154

~~W. E. R. Jones~~
Director,
Office of Premarket Approval (H.E.S. 200)
Food and Drug Administration
200 C St S.W.
Washington D.C. 20204

R.D.H.
W.H. WARE
NEW ZEALAND
MAR 25 1998

Dear Director

ERAS NOTICE GRN 000001
ARCHER-DANIELS-MIDLAND CO

We wish to submit information in opposition to this notice. Could you please advise if there is a prescribed form to do this, and whether we will need to file full research papers, or whether a tabulated list of references would suffice.

The thrust of our submission will be the biological effects demonstrated in the following published papers:

① "ANTI-THYROID ISOFLAVONES FROM SOYBEAN: ISOLATION CHARACTERISATION AND MECHANISMS OF ACTION" DIVI, CHANG & DOERGE in Biochemical Pharmacology 54(10) 1087-1096 Nov 15 1997
and all the previous research referenced therein.

② "INHIBITION OF THYROID PEROXIDASE BY DIETARY FLAVONOIDS" DOERGE ET AL in Chemical Research in Toxicology 9(1) 16-23 1996 January

③ "PHYTOESTROGENS IN SOY BASED INFANT FOODS: CONCENTRATIONS, DAILY INTAKE AND POSSIBLE BIOLOGICAL EFFECTS" CHEE IRVINE ET AL in P. S. E. B. M. 1998 Vol 217 247-253.

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2

- (4) "THE EFFECTS ON THE THYROID GLAND OF SOYBEAN ADMINISTERED EXPERIMENTALLY IN HEALTHY SUBJECTS" ISHIZUKI et al in Nihon Naibungi Gakkai 67 : 622-629 1991.
- (5) "BREAST & SOY FORMULA FEEDINGS IN EARLY INFANCY AND THE PREVALENCE OF AUTOIMMUNE THYROID DISEASE IN CHILDREN"; FORT et al in Jour. Am. College Nutr 1986 56 439-441.
- (6) "PREMATURE THELARCHE IN PUERTO RICO"; Freni-TITUBAER et al in American Journal of Diseases of Children 1986, 160, 1263-1261.
- (7) "BIOLOGICAL EFFECTS OF A DIET RICH OF SOY PROTEIN RICH IN ISOFLAVONES ON THE MENSTRUAL CYCLE OF PRE-MENOPAUSAL WOMEN" A. CASSIDY et al in American Jour Clinical Nutrition 1994 60 333-340.
- (8) "DIETARY ESTROGENS - A PROBABLE CAUSE OF INFERTILITY AND LIVER DISEASE IN CAPTIVE CHEETAHs" K D R SETCHELL et al in Gastroenterology 1987 93 225-33.
- (9) "IDENTIFICATION OF PHYTOESTROGENS IN THE URINE OF MALE DOGS" P.E. JUNIEWICZ et al in Jour Steroid Biochemistry 1988 31, 6, 987-994.
- (10) "REPRODUCTIVE AND GENERAL METABOLIC EFFECTS OF PHYTOESTROGENS IN MAMMALS" R S Kaldas et al in Reproductive Toxicology 1989 3 31-89.

Yam sincerely

~~18/04/98 J. J. Jones~~

000156

OU



Dr Linda S. Kahl
FNU 001(202) 418 3131

3/26/98

Dear Dr Kahl

Thank you for your latest E-Mail to
Dr Clapperton

For general interest, you may wish to obtain
a copy of the Report put out by the Food
Protection Committee of the National Academy
of Sciences

It is titled "Toxicants Occurring Naturally
in Foodstuffs" We have the 1973 2nd Edition.

When we noticed the premature development
of little girls, and little parrots, which were
fed soy-based proprietary foods, Dr M. G.
Fitzpatrick, at the request of a local health
food company, performed an examination of the
estrogenic levels in these foods. That was at
the end of 1993.

A year later, it was apparent that
an institutional attempt was being made
to "divert" a whole generation of knowledge, on
a global basis.

Fortunately, the British and Swiss Governments
were sufficiently impressed by Dr Fitzpatrick's
report to initiate health warnings

Sincerely

~~Richard J. James~~

000157

NOTICE: The project that is the subject of this report was approved by the Governing Board of the National Research Council, acting in behalf of the National Academy of Sciences. Such approval reflects the Board's judgment that the project is of national importance and appropriate with respect to both the purposes and resources of the National Research Council.

The members of the committee selected to undertake this project and prepare this report were chosen for recognized scholarly competence and with due consideration for the balance of disciplines appropriate to the project. Responsibility for the detailed aspects of this report rests with that committee.

Each report issuing from a study committee of the National Research Council is reviewed by an independent group of qualified individuals according to procedures established and monitored by the Report Review Committee of the National Academy of Sciences. Distribution of the report is approved, by the President of the Academy, upon satisfactory completion of the review process.

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PREFACE

Much new information on food safety has become available in the 7 years since the first edition of this report* was published, and significant changes in public attitude have occurred. A number of food components, both naturally occurring and introduced by man, that were "generally recognized as safe" at that time have now come under suspicion. Public apprehension concerning the food supply has reached a high point. At the same time, there is all too little recognition of some of the hazards associated with food.

To the extent feasible, the subject matter of this report has been organized on the basis of the chemical nature of the materials considered. Authors of individual chapters were selected as specialists on the particular topic covered and as recognized authorities in the field. The final compilation was then thoroughly reviewed by the subcommittee. We wish to acknowledge our deep indebtedness to the authors and to thank them for their contributions.

*Committee on Food Protection, NRC. 1966. Toxicants occurring naturally in foods. National Academy of Sciences, Washington, D.C. 301 p.

*60000 were issued to
health professionals in the
U.K.*

PUBLIC HEALTH LINK

EMBARGOED

PLEASE CASCADE TO HEALTH PROFESSIONALS URGENTLY. THIS MESSAGE IS SENT TO YOU IN ADVANCE OF A PRESS BRIEFING AT 10.30AM ON THURSDAY 18 JULY 1996. PLEASE DO NOT DISCLOSE TO THE MEDIA IN ADVANCE OF THE PUBLIC ANNOUNCEMENT

To: All Directors of Public Health
Cc: Consultants in Communicable Disease Control
From: Sir Kenneth Calman, Chief Medical Officer
Department of Health
Date: 17th July 1996
Reference: CEM/CMO/96/8
Category: * URGENT MESSAGE-PLEASE ACTIVATE THE CASCADE*

PHYTOESTROGENS IN SOYA INFANT FORMULA MILK

Dear Colleague

Please forward the attached message urgently to the following Health Professionals to ensure that they have this information by 10.30am Thursday 18 July at the latest:

All General Practitioners and practice nurses
All community pharmacists via the procedure for
Class 1 Drug Alerts

Medical Directors in all NHS hospital units to
cascade to appropriate Health Professionals - in
particular:

Paediatricians
Obstetricians
Dieticians

The Chief Executive of the Health Authority
Chief Executives on community health units

Thank you for your co-operation.

000159

In preliminary studies in premenopausal women normally consuming a Western diet, ingestion of soy protein (60g per day for one month, equivalent to 0.73 mg isoflavones/kg bw/day) has been shown to suppress mid-cycle peaks of LH and FSH and significantly increase the duration of the follicular phase (26, 27). The effects lasted for up to 3 months following the termination of soy consumption. There are reports that phytoestrogens can reduce blood cholesterol (25, 26, 28, 29), and that they may protect against osteoporosis and reduce flushing in postmenopausal women (29, 30). Epidemiological evidence from adult populations which habitually ingest high quantities of soy (eg, Chinese and Japanese) suggest that these individuals have a lower incidence of some types of cancer (4, 15, 18, 28, 29). However, it is difficult to resolve the effects and consequences of other dietary variables such as fibre, vitamins, fruit, vegetables and meat when considering the validity of this observation. The subject of dietary constituents and cancer is presently under review by a Working Group of the Committee on Medical Aspects of Food Policy (COMA).

The potential for phytoestrogens, including isoflavones, to affect adversely infants is of particular concern since it is possible that a hormonal imbalance in early life can permanently affect sexual development and fertility. Such effects have been observed in a number of animal species (2, 4, 9-14). We are not aware of any reports which suggest that populations which habitually ingest high quantities of soy (eg Chinese, Japanese) have impaired fertility or altered sexual development. Limited data indicate that the estimated intake of isoflavones by infants fed soy-based formulae is in the region of 4 mg/kg bw/day (31, 32). This is higher than the intake reported to cause hormonal effects in premenopausal women (approximately 0.73 mg/kg bw/day). Since we do not have data specifically relating to the potential effects of soy phytoestrogens in human infants, particularly in those whose mothers normally consume a western diet, we recommend that research should be undertaken as a matter of high priority to determine whether ingestion of soy based formulae carries any risk for infants. (See Annex A for list of recommended research proposals). As a result of further research, it may be necessary to consider the potential risk of soy products to other sectors of the population. We endorse the advice of the Department of Health that breast milk and cows' milk formulae are the preferred sources of nutrition for infants. However, women who have been advised by their doctor or other health professionals to feed their baby soy-based formulae should continue to do so. The Committee on Medical Aspects of Food Policy has published a more detailed report of the preferred sources of nutrition for infants (Department of Health. Weaning and the Weaning Diet. London: HMSO, 1994. Report of Health and Social Subjects: 45).

July 1996

Mr R. E. Jones
112

Vorkommen und Bedeutung der Isoflavone Daidzein und Genistein in der Säuglingsanfangsnahrung

Occurrence and Significance of the Isoflavones Daidzein and Genistein in Infant Formulas

Key words: Isoflavones, Daidzein, Genistein, Food levels, Metabolism, Biological effects

Bernhard Zimmerli und Josef Schlatter
Bundesamt für Gesundheit, Abteilung Lebensmittelwissenschaft, Bern

Einleitung

Die menschliche Nahrung enthält eine Vielzahl von Stoffen natürlicher Herkunft, deren chemische Struktur, Metabolismus und biologische Wirkung im Organismus bisher nie untersucht wurde. Pflanzen wurden auch in vielen Kulturen seit Jahrhunderten zu therapeutischen Zwecken eingesetzt, ohne das Wirkprinzip zu kennen. So wurden Pflanzen beispielsweise zur Erhöhung oder Verminderung der Fruchtbarkeit verwendet¹. In diesem Zusammenhang wurde Mitte der 20er Jahre entdeckt, dass Pflanzen natürlicherweise Stoffe mit östrogenen Wirkung enthalten können (Phytoöstrogene). Mitte der 40er Jahre wurde dann in Australien eine östrogene Wirkung gewisser Kleearten bei Schafen beschrieben, die bis zur Unfruchtbarkeit der Tiere führte («clover-disease»)². Inzwischen wurden in verschiedenen pflanzlichen Lebensmitteln natürlicherweise enthaltene Stoffe nachgewiesen, die in Modell- oder Tierversuchen eine mehr oder weniger ausgeprägte östrogene Wirkung entfalten. Solche Stoffe kommen beispielsweise in Karotten, Kohl, Erbsen, Linsen, Hopfen, Reis, Weizenkleie und -keime, Leinsamen sowie Soja und daraus hergestellten Produkten vor (1-3).

¹ So soll z. B. die Flüssigkeit, die durch Einweichen gemahlener Sojabohnen in wenig Wasser und Abpressen erhalten wird, im alten China zu Abtreibungen benutzt worden sein (S. Wäkelin, Rural News, New Zealand, March 20, 1995).

² Neuerdings stellten offenbar Berner Bauern, welche ihre Schweine seit dem Sommer 1996 vegetarisch (Sojafaschot) ernähren, einen Rückgang der Fruchtbarkeit ihrer Tiere fest (H. Guggenbühl, Berner Zeitung [BZ] vom 17. Februar 1997, Seite 3).

volle Anregungen und Diskussionen. Herrn Prof. Dr. med. O. Tönz, Luzern, sei auch die kritische Durchsicht des Manuskripts verdankt.

Zusammenfassung

Es wird ein kurzer Überblick über die Exposition, Metabolismus, biologische Wirkung und die potentielle Gesundheitsgefährdung von Säuglingen und Kleinkindern durch die Isoflavone Genistein und Daidzein gegeben. Die in der Schweiz 1995 im Handel angebotenen drei Säuglingsanfangsnahrungen auf Sojabasis enthielten 280-980 µg Isoflavone pro g Trockenmasse. Ein Neugeborenes, das ausschliesslich mit einem solchen Produkt ernährt wird, erhält täglich bis zu 20 mg/kg Körpermasse dieser Isoflavone zugeführt. Diese Menge ist bis zu 25mal grösser als jene, die bei Frauen zu einer geringen Verlängerung des Menstruationszyklus führte. In Anbetracht der bestehenden Wissenslücken über allfällige negative Auswirkungen dieser Stoffe wird gefordert, dass isoflavonhaltige Säuglingsanfangsnahrungen nur bei präziser medizinischer Indikation und fehlenden Ersatzprodukten eingesetzt werden.

Résumé

Le présent aperçu informe au sujet de l'ampleur d'exposition des nourrissons et petits enfants au génistéine et au daidzéine ainsi que du métabolisme, des effets biologiques et des risques potentiels de santé de ces isoflavones. La teneur en isoflavones de trois différents aliments pour nourrissons, contenant du soja et provenant du marché suisse en 1995, se situe entre 280-980 µg par g matière sèche. Un nouveau-né exclusivement nourri par ce genre d'aliment peut absorber jusqu'à 20 mg/kg de poids du corps. Il s'agit d'une quantité jusqu'à 25 fois plus importante que celle qui prolonge de manière minime le cycle de menstruation chez les femmes. Vu les lacunes quant aux connaissances des effets négatifs des isoflavones sur les bébés et les petits enfants, il est recommandé que les aliments pour nourrissons contenant ces substances ne soient utilisés que sous indication médicale et lorsque d'autres aliments ne sont pas disponibles.

Summary

The exposure levels of newborns and infants with the isoflavones genistein and daidzein are reviewed as well as their metabolism, biological effects, and potential health hazard. The isoflavones were analysed in three soy-based infant formulas from the Swiss market during 1995: a range of 280-980 µg isoflavones per g dry matter was found. Feeding of newborns exclusively with such formulas would result in a mean daily intake of isoflavones of up to 20 mg/kg body mass. This dose is up to 25 times higher than the dose which was shown to prolong slightly the menstrual cycle in women. Taking into account the very limited knowledge on the possible adverse health effects of an isoflavone exposure in newborns and infants it is demanded that soy-based infant formulas containing isoflavones should be used only under strict medical indications and a lack of alternative products.

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Les phyto-oestrogènes dans l'alimentation des nourrissons à base de protéine de soja

BEST ORIGINAL COPY

Remarque préliminaire

La Commission fédérale de l'alimentation s'est penchée sur le thème des oestrogènes dans l'alimentation lors de sa dernière séance de l'année 1996. Le fait que des substances à action oestrogénique se trouvent naturellement en quantité notable dans les fèves de soja et donc aussi dans les substituts de lait maternel fabriqués à base de protéine de soja, a déclenché dans le monde entier une certaine insécurité. La Commission fédérale de l'alimentation avait décidé de n'émettre aucun avertissement général mais de remettre une information directement aux pédiatres. Le Président d'alors (Otmar Tönz) remplit ici cette obligation.

Le résumé ci-dessous se base sur une mise au point actuelle du problème faite par B. Zimmerli et J. Schlatter, OFSP, denrées alimentaires. «Vorkommen und Bedeutung der Isoflavone Daidzein und Genistein in der Säuglingsnahrung». Mitt. Gebiete Lebensm. Hyg. 88, 219-232 (1997). On trouvera de nombreuses références et des données techniques plus détaillées dans cet article.

Phyto-oestrogènes et xéno-oestrogènes dans l'alimentation et l'environnement

L'alimentation humaine contient un grand nombre de substances naturelles dont l'action biologique est encore insuffisamment connue. On sait toutefois depuis longtemps que les plantes recèlent des substances à action oestrogénique (phyto-oestrogènes) et cela dans des concentrations qui permettent réellement un effet oestrogénique dans des modèles biochimiques et animaux. Ainsi on a mis en évidence en Australie une infertilité de brebis nourries au trèfle contenant des oestrogènes («Clover-

disease»). De telles substances sont présentes dans de nombreux aliments végétaux, principalement dans les légumineuses, donc aussi dans les fèves de soja.

D'autre part, on met en cause aujourd'hui également un grand nombre de substances chimiques de l'environnement qui possèdent une activité oestrogénique, les xéno-oestrogènes. On leur reproche d'être responsables de la baisse continue de la richesse du sperme humain dans les dernières décennies et de différents problèmes de fertilité observés également chez les animaux sauvages. Le fait que la concentration de xéno-oestrogènes dans les aliments soit jusqu'à 10'000 fois plus faible que celle des phyto-oestrogènes, ne doit pas nous amener à banaliser mais plutôt nous pousser à prendre encore plus au sérieux le problème des phyto-oestrogènes.

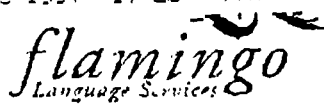
Problèmes de la fève de soja

Le soja est incontestablement une des plantes alimentaires parmi les plus importantes et précieuses car elle offre un bon rapport qualité-prix grâce à son apport énergétique élevé et à son taux de protéines. Malheureusement cette «vache de Chine» souffre toutefois de certains inconvénients.

Le produit brut contient plusieurs substances problématiques pour l'alimentation humaine, particulièrement celle des nourrissons. Tout d'abord, il n'est plus contesté que la protéine de soja soit un puissant allergène. Ensuite, la protéine de soja contient trop peu de méthionine. Lors de la fabrication d'aliments pour nourrissons cette dernière doit être ajoutée au produit fini (en

Chine, c'est depuis fort longtemps que l'on rajoute au lait de soja un jaune d'œuf riche en méthionine). D'autres substances indésirables comme les inhibiteurs de la trypsin ou l'hémagglutinine (lectine) sont éliminées par un traitement de chaleur. L'addition d'une phytase dégrade les acides phytiques. Dans les années 50 et 60, on incrimina la farine de soja dans le développement de goitre mais l'existence de substances goitrogènes n'a jamais pu être identifiée exactement. Toutefois les fabricants rajoutent de l'iode pour se prémunir de ce phénomène. Accessoirement, on remarquera que ces produits doivent également être enrichis en calcium, magnésium et autres oligo-éléments.

Pour conclure, il reste encore à citer les isoflavones génistéine et daidzéine, comme substances indésirables, les deux présentant des propriétés oestrogéniques. Leurs structures chimiques sont peu éloignées de celles des oestrogènes et des autres hormones stéroïdiennes (voir fig. 1). Leurs récepteurs correspondants sont extrêmement anciens dans l'évolution, pas particulièrement spécifiques, et sont capables de lier ces substances et de les rendre endocrinologiquement actives. Certes, elles sont 100 à 150'000 fois moins actives que le β -oestradiol corporel. Les produits qui sont enregistrés en Suisse comme préparations de soja pour nourrissons (SOM de Milupa, Humana SL de Milchwerke Westfalen, Herford, ou Mamina Soja de Wander) contiennent 280-980 μ g d'isoflavone/gramme de substance sèche. Un nourrisson qui est nourri uniquement avec de telles préparations en ingérera donc quotidiennement 6-20 mg/kg de poids corporel. Ceci correspond à 9 jusqu'à 25 fois la quantité qui allonge le cycle chez les femmes.



145 S. 10th St. Auckland City.

Auckland, New Zealand

Telephone: 64-9-308 9264
Facsimile: 64-9-308 9369

Translation

Information Sheet

Phyto-oestrogens in baby food based on soya bean protein

Paediatrica, 8 (5), 1997

Preliminary Remarks

BEST ORIGINAL COPY

At its last meeting for the year 1996 the (Swiss) Federal Commission on Food (German initials EFK) discussed the question of oestrogens in food. The fact that substances with an oestrogenic effect occur naturally and in large quantities in the soya bean and therefore also in mother's milk substitutes based on soya bean protein has led to worldwide concern. The EFK decided not to issue a general warning, but to send out an Information Sheet direct to paediatricians. The then President of the Commission, Omar Tönz, is hereby fulfilling his obligation to do so.

The summary below is based on a current study of the problem by B. Zimmerli and J. Schlatter, BAG, Department of Food Science: "Existence and Development of Isoflavones Daidzein and Genistein in Baby Food". Communication regarding Foodstuffs in Hyg. 88. 219-232 (1997), which includes bibliographical references and other technical details.

Phyto and Xeno-oestrogens in food and the environment

Food consumed by humans contains a large number of natural substances the exact biological effect of which is still insufficiently clear. We have known, however, for some time that plants contain substances with an oestrogenic effect (phyto-oestrogens) and in concentrations which show a recognisable oestrogenic effect in model and animal experiments. In Australia it was established that infertility occurred in sheep fed on clover which contained oestrogens, and had developed clover disease. These substances occur in numerous plant foodstuffs, especially in the leguminous plants and also in the soya bean.

In addition, we are frequently warned against a number of environmental chemicals which likewise produce an oestrogenic effect - the xeno-oestrogens. They are blamed for possibly being responsible for the continual lowering of the sperm count in men and for the fact that various fertility problems are also observable in wild animals. The fact that the concentrations of the xeno-oestrogens in foodstuffs is up to 10,000 times lower than that of the phyto-oestrogens must not lead us into minimising the importance of this retrograde situation but rather urge us to regard the problem of the phyto-oestrogens as at least equally serious.

Soya bean problems

The soya bean is indisputably regarded worldwide as one of the most important and profitable food plants which, because of its high albumen and total energy content, has an excellent cost-effective ratio. Unfortunately this "cow of China" has certain disadvantages.

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flamingo
Language Services

Translation

The raw product contains several substances which are not completely without problems for food consumed by humans, particularly baby food. First of all, there is no doubt that the soya bean is a very powerful allergen. Next, it contains too little methionine, which therefore has to be added during the production of baby food. (For some time now in China, egg yolk rich in methionine, has been added to soya milk). Undesirable substances such as trypsin inhibitors or haemagglutinine (lectin) are eliminated by heat treatment. Phytase is used to eliminate phytic acid. In the 50-60s when it was necessary to determine whether goitre was caused by soya bean flour, the presence of goitrous substances was postulated, but could never be precisely identified. For all that, manufacturers feel bound to add iodine to these products to guard against this occurrence. It may be mentioned, as a marginal note, that finally calcium, magnesium and other trace elements have had to be added as well.

Finally there remain to include as undesirable substances the isoflavones **Genistein** and **Daidzein**, both of which exhibit oestrogenic properties. Their chemical structure reminds one faintly of oestrogens or the steroidal hormones (see Fig. 1). Since the relevant receptors are extremely old in the development of evolution, they are not particularly specific, but can bind these substances and make them endocrinologically active. They are naturally 100-150,000 times less active than the corporal β -oestradiol. The soya bean products registered in Switzerland for infants - SOM (Milupa), Humana SL (Milchwerke Westfalen, Herford) and Mamina Soya (Wander) contain 280-980 μg per gram of dry weight of the above mentioned isoflavones. A baby which is fed solely on such products receives a daily amount of 6-20 mg/kg of such substance. This corresponds to 8-25 times the amount which extends the cycle in women.

Fig. 1: Chemical structure of isoflavone, compared with that of oestradiol. The phylogenetically old, but not very specific receptor for oestrogen officially also accepts genistein and daidzein as well as many environmental chemicals having a structure similar to that of oestrogen.

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Metabolism and biological effect of isoflavone

After separating from the glycosides with which they are bound, genistein and daidzein are absorbed and further metabolised in the liver (glucuronisation) and finally excreted in the urine. Like bilirubin or similar sex hormones, they have an enterohepatic cycle. Their plasmic half-life is 7 to 8 hours in adults and considerably longer in infants.

Isoflavones also occur in milk, so that the breast fed baby also receives small amounts if the mother consumes soya bean products. Similarly these substances may also be found in cow's milk in quantities which vary with the type of fodder used. According to an American study of a group of 4-month old male infants who were either breast fed, or fed with a cow's milk or a soya bean based preparation (isomil), approximately 3.25 or 180 μg of isoflavone were found to have been excreted in the urine each day. More recent measurements on infants fed entirely on soya bean preparations showed plasma concentrations of 0.5 $\mu\text{g/ml}$. Compared with the concentrations of 30-60 $\mu\text{g/ml}$ of oestradiol this corresponds roughly to 20,000 times

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Level 2, Patten Centre
140 Symonds St, Auckland City

PO Box 8567 Symonds St
Auckland, New Zealand

Telephone 64 9 308 9284
Facsimile 64 9 308 7358

Translation

the amount of isoflavone. By comparison, the endogenous oestradiol in women is between 30 and 200 µg/ml.

Other effects of isoflavone include inhibiting peroxidation of lipids (anti-oxidative effect), and inhibiting angiogenesis. According to their concentration in vitro genistein and daidzein can slow up or stimulate the growth of tumorous cells. Other effects are stimulation of the synthesis of protein which binds the sex hormones, and inhibition of the enzyme aromatase.

Evaluation

In animal experiments the administration of isoflavone to foetuses and new-born animals resulted in negative effects as regards feminisation. Of course the doses given were distinctly higher than those for a baby fed on soya bean products.

On the other hand soya bean has been used as a baby food in China for about 70 years and in the USA for at least 40 years to a relatively large extent. No negative effects have so far been established. However, targeted studies were never made, such as are now to be undertaken in the USA. In the Asiatic countries it was speculated that perhaps genetic adaptation may have taken place during the 5,000 years of normal consumption of the soya bean. In those parts of the world consumption of the soya bean is associated with a decrease in the prevalence of cancer and cardio-vascular diseases.

It is clear that chronic effects of high doses of isoflavone on the human being have been poorly or never researched. Experiments with the synthetic hormone di-ethylstilboestrol, which had been used for dozens of years to prevent abortion, and led to an increase in cervical and vaginal carcinoma only in the offspring of the women concerned should give cause for thought in this connection.

Conclusion

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As a baby food, and particularly as a substitute for mother's milk, very restrictive use should be made of soya bean products, especially if potentially harmful effects for babies and small children have not been investigated scientifically or if isoflavone has not been successfully eliminated during preparation of the product. (That the, in any case, already expensive production has to be further complicated by yet another biotechnological concept should be mentioned only in brackets!). In New Zealand, some people called for an absolute ban, while in the USA they officially ask for restrictions. In our country, soya bean products should not be used routinely in food prepared for healthy babies, and are subject only to a few medical indications (intolerance of lactose, galactocaemia, possibly intolerance of cow's milk or allergy); and in no case for ecological, ideological or ethical reasons! But in any case hydrolysed or lactose-free products based on cow's milk are probably better than those based on the soya bean!

O. Tönz, Lucerne
B. Zimmerli, Bern

000165

To: Linda Kahl@OPA@FDA.CFSAN
From: "McLEOD, JIM (HS4)" <JMcLeod@nzrc.co.nz>
Certify: N
Subject: GRAS classification of isoflavones.
Date: Sunday, March 29, 1998 at 6:11:21 pm EST
Attached: None

OU



Dr Kahl,

It has come to my attention that an application has been made to the FDA to declare isoflavones Generally Regarded as Safe (GRAS) when used as a micronutrient.

I would ask that this application be very carefully reviewed and all information and research be reviewed. I have grave concerns about the use of isoflavones as a dietary supplement even in adult foods given the possibility of thyroid function disorders and fertility disorders that have been discovered in some animal species (1,2).

It is imperative that further research is carried out on humans already exposed to these dietary supplements to establish any increased risk of thyroid/fertility problems (or any other issues) prior to such and application being approved.

Thank you for your time in this,

Yours faithfully

Dr J.B. McLeod.

Company Medical Adviser

1. Divi RL. et al Anti- thyroid isoflavones from soy bean- Isolation, characterisation, and mechanisms of action. Biochemical Pharmacology. 54(10):1087-1096, 1997 Nov 15.
2. Chapin RE. et al Endocrine modulation of reproduction. Fundam Appl Toxicol, 29, 1-17 1996.

000168

Dr Linda S. Kahl

FAX # 202 418 3131

~~RECEIVED E-MAILS~~

R.D. 4

WITANCARET N.2

3/29/98

Dear Dr Kahl E.R.N. 000001

Thank you for your E-Mail of 3/28/98 to Yvonne Clapperton at 6:34 pm.

It seems that a subtle shift has occurred in that objectors are being asked to prove an additive unsafe; the obligation still should only be on the applicant to prove safety. After all, this is a Generally Regarded as Safe application, and it is not an application for restricted use.

[Experience here has shown that bread and breakfast cereals containing soy and linseed to relieve menopause problems are being promoted as "good for all the family" and feature pictures of children on swings]

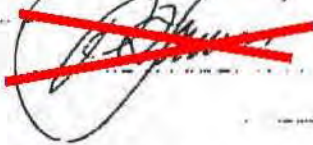
It all is rather academic, since factors of body size, daily dose, frequency of dose, age and sex all should be considered. At the moment the F.D.A. is asking for a discussion of the length of a piece of string.

However, Archer Daniels Midlands' application is clearly untrue, as there are many examples in the literature of dose levels which cause adverse effects in humans, and animals, at modest levels.

It will take us some time to collate them. I should appreciate their explanation how each article they cited bears on their determination that isoflavones are safe.

Respectfully, since this is ultimately a question of environmental toxicology, I suggest

you obtain detailed input from your own
Environment Protection Agency and your
National Center for Toxicological Research.

Yours sincerely
~~~~

000170

Dr Linda S Kahl
Regulatory Policy Branch
Office of Pre Market Approval
Center for Food Safety
200 C St SW Washington D.C.
FAx (202) 418 3131

~~R. F. James~~
R.D. &
WIMANGAREI
NEW ZEALAND
MARCH 29 1998

Dear Dr Kahl, ~~Boy Isflavones~~

Thanks you for your most helpful advice. I see nothing in Archer Daniel's Midland's submission which limits use of these chemicals to energy bars or pep up drinks. It is a request to bypass normal procedures to obtain G.R.A.S. designation, and obtain fast track approval, based on incorrect representations that no adverse effects have been reported in humans or animals.

I would not like to think the goal posts are on the move while the ball is in flight; I doubt whether a Federal District Court judge would look kindly on a regulatory agency re-interpreting a supplicants application of its own volition. There is nothing in A.D.M.'s material which excludes any age group. However, to the substance, Toxic levels are demonstrated at specified normal dietary doses.

Cheetahs (1) Under 50 mg of isoflavones, over time, were associated with, endometriosis, and fatal liver disease in zoo cheetahs; and resulted in few offspring living to reach breeding age

Female Pigs (2) 20% soy meal for 14 weeks caused precocious sexual development in infant female pigs.

Laboratory Mice (3) 10% soy in the diet caused significant uterotropic activity over a few months.

Laboratory Mice (4) A decrease in reproductive. 000171

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performance was observed in female mice fed a soy based diet or a diet supplemented at .02% with genistein.

There are numerous other studies, but some require extrapolations. For instance, in male beagles prostate tumors resulted from feeding a commercial diet containing soy protein (5). One would have to assume a percentage, and calculate a daily dose level and also assume no other dietary inputs.

Infant foods The daily intake of isoflavones has been well reported in the literature; as has been their absorption rate; and their possible biological effects. Adverse effects on thyroid function have been demonstrated (6)

Adult Japanese men (7) Again, the isoflavone content of the soaked and pickled beans would have to be assumed, but 30 gr per day for less than 90 days caused a pituitary depression, altered T.S.H., and caused goiters.

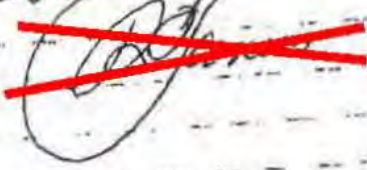
Adult English women (8) 45 mg of isoflavones in soy protein depressed ovulatory hormone release in under 30 days

Adult American women (9) 38 mg of genistein caused abnormal breast development, absorbed from 38 gr of soy protein per day.

The front abstract sheets of each of those papers follow (numbered in sequence) this fax. This is not an exhaustive list, but in my opinion it is sufficient to prove Archer Daniels Midland's petition to be based on untrue

(3)

statements. Therefore the whole matter should be submitted to full and open public hearings, as required by law, and full scientific presentations should be solicited.

Sincerely,


GASTROENTEROLOGY 106:703:225-73

LIVER AND BILIARY TRACT

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Dietary Estrogens—A Probable Cause of Infertility and Liver Disease in Captive Cheetahs

K. D. R. SETCHELL, S. J. GOSSELIN, M. B. WELSH, J. O. JOHNSTON, W. F. BALISTRERI, L. W. KRAMER, B. L. DRESSER, and M. J. TARR
 Clinical Mass Spectrometry Laboratories, Department of Pediatric Gastroenterology and Nutrition, Children's Hospital Medical Center, Cincinnati; Department of Pathology/Toxicology and Department of Enzyme Chemistry, Merrell-Dow Research Institute, Cincinnati; Cincinnati Zoo, Department of Obstetrics/Gynecology, University of Cincinnati, Cincinnati, Ohio; Kings Island Wild Animal Habitat, Kings Island, Ohio; and Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio

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The cheetah in the wild is "racing towards extinction" mostly due to habitat destruction. Its survival will probably depend on accelerated captive breeding. At this time, however, reproductive failure and liver disease threaten the future of the captive cheetah population. Histopathological evaluation of more than 100 cheetah livers identified venoocclusive disease as the main hepatic lesion responsible

for the withdrawal of this feline diet by substitution with a chicken diet resulted in an improvement in conventional liver function tests and a normalization in the appearance of hepatic mitochondria. We conclude that the relatively high concentrations of phytoestrogens from soybean protein present in the commercial diet fed to captive cheetahs in North American

...pathological evaluation of more than 100 cheetah livers identified veno-occlusive disease as the main hepatic lesion responsible for liver disease in this species. Analysis of the commercial feline diet by high-performance liquid chromatography and gas-liquid chromatography-mass spectrometry revealed large amounts of two phytoestrogens identified as daidzein and genistein. These compounds were found to be derived from a soybean product that was a component of the cheetah diet, and their concentrations both ranged from 9 to 35 µg/g diet. The adult cheetah consequently consumes ~50 mg/day of these weak estrogens. When extracts of the diet were tested for estrogenicity using a bioassay, a dose-related increase in uterine weight was observed. In 4 cheetahs studied,

estrogens from soybean protein present in the commercial diet fed to captive cheetahs in North American zoos may be one of the major factors in the decline of fertility and in the etiology of liver disease in this species. The survival of the captive cheetah population could depend upon a simple change of diet by excluding exogenous estrogen.

Cheetah populations are diminishing in the wild as a result of poaching and habitat destruction (1). Another factor that may be contributing to their decline is the lack of genetic variation within the species (2). To quote cheetah researcher Randall Eaton, the cheetah is "racing towards extinction" (3). The survival of the cheetah, as a species, will probably depend on accelerated captive breeding. At this time, however, reproductive failure, as well as shortened life spans, threaten the future of the captive cheetah population. The situation has become alarming for this already endangered species. North American zoos cannot maintain their cheetah populations because deaths have outnumbered births during the last few years. In 1985, North American zoos reported 29 deaths and only 18 births of which 7 died before reaching adulthood (4).

The average life span of the captive cheetah in North American zoos is 8.9 yr (5), a much shorter

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Address requests for reprints to: Kenneth D. R. Sutchell, Ph.D., Children's Hospital Medical Center, Division of Pediatric Gastroenterology and Nutrition, Elland and Bethesda Avenues, Cincinnati, Ohio 45229-2899.

The authors acknowledge the technical assistance of C. L. Wright, L. C. Meyer, D. E. Loudy, and W. A. Rogers. They also thank the following zoological institutions for their contribution to this project: Baltimore, Baton Rouge, Cleveland, Cheyenne, Mountain, Cleveland, Columbus, Detroit, Wayne, Cedar Point, Henry Doorly, Houston, Kansas City, Knoxville, Norfolk, Philadelphia, St. Louis, San Antonio, San Diego, Toledo, Front Royal, Rim Ranch, Wildlife Safari, DeWaldt Cheetah Research and Breeding Centre, South Africa, and the manufacturer of the commercial feline diet, Animal Sciences Inc., Nelapaka.

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0010-5085/87/53 53

Abbreviations used in this paper: GLC, gas-liquid chromatography; HPLC, high-pressure liquid chromatography; MS, mass spectrometry.

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Research in Veterinary

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POSSIBLE OESTROGENIC EFFECTS OF FEEDING SOYAMEAL TO PREPUBERAL GILTS

By H. M. DRANE, A. E. WRATHALL, D. S. P. PATTERSON AND C. N. HEDERT

Central Veterinary Laboratory, Weybridge, Surrey

SUMMARY

Six ten-week old gilts were fed a diet containing 20% soyameal for a period of 14 weeks. Their body weights and vulval measurements were compared with those of six similar gilts fed a soya-free diet. After five weeks, the vulvae of the soya-fed group were slightly larger than those of the controls. The difference was maintained until the end of the experiment and was highly significant over the last five weeks. All 12 gilts were slaughtered after 14 weeks on the test diets and a general post-mortem examination carried out. The reproductive tracts were weighed and samples taken for histological examination; however no significant differences between the two groups were observed.

INTRODUCTION

Soyameal products are now widely used in compound animal feeds and, following the reported uterotrophic activity of soyameals in mice (Drane, *et al.*, 1980), it was decided to investigate possible oestrogenic effects in farm animals. Accordingly, prepuberal gilts were fed a diet high in soyameal and compared with those fed a soya-free ration. The main criterion adopted to assess whether an oestrogenic response had occurred was measurement of vulval size because this organ appears to be particularly sensitive to oestrogens (Wrathall, 1975).

MATERIALS AND METHODS

Animals. Twelve eight-week-old Landrace cross gilts weighing 11 to 18.5 kg were divided into two groups and housed six to a pen.

Diet. For the first two weeks, all gilts were fed proprietary pig creep pellets. From 10 weeks of age the two groups of gilts were fed an experimental diet that contained either soyameal or an alternative protein. These were provided by Labsure Animal Feeds. The composition and analysis of the two diets are given in Table I. Each group of six pigs received 3 kg of slightly moistened feed twice daily, the ration being increased

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Smith, T. H. F. & Thompson, G. R. (1980a). Hydroxycoumarins: A survey of consumer patch-test sensitization. *Food Cosmet. Toxicol.* 18, 407.

Smith, T. H. F. & Thompson, G. R. (1980b). Citral: A survey of consumer patch-test sensitization. *Food Cosmet. Toxicol.* 18, 413.

Van Kester, W. G. (1978). Dermatitis from an afterhave. *Contact Dermatitis* 4, 117.

Food Cosmet. Toxicol. Vol. 18, pp. 405-417
Pergamon Press Ltd 1980. Printed in Great Britain

SHORT PAPER

OESTROGENIC ACTIVITY OF SOYA-BEAN PRODUCTS

H. M. DRANE, D. S. P. PATTERSON, B. A. ROBERTS and N. SABA
Central Veterinary Laboratory, Weybridge, Surrey, KT15 2NB, England

(Received 22 October 1979)

Abstract—Normal rat cake containing soya meal was found to be oestrogenic. Sixteen samples of soya meal were examined in the mouse uterine weight bioassay and all were found to have oestrogenic activity. Ethyl-acetate extracts of the meals also had oestrogenic activity. Genistein and daidzein were present in the extracts.

Introduction

It has previously been reported from this laboratory (Drane, Patterson, Roberts & Saba, 1975) that rat cake used as a control feed in routine mouse bioassays for oestrogens had developed significant uterotrophic activity over a period of a few months. We have recently encountered another oestrogenic control feed, higher basal uterine weights than expected being found in mice fed this ration. Investigation of the components of the feed showed that the oestrogenic activity was due to soya meal, which made up 10% of the rat cubes.

Little attention seems to have been paid to soya meal as a possible source of oestrogenicity although daidzein and genistein were isolated from soya beans nearly 50 yr ago (Walt, 1931). The oestrogenic activity of these and other isoflavones is well documented (Bickoff, Livingston, Hendrickson & Booth, 1962; Carter, Smart & Maitone, 1953; Cheng, Story, Yoder, Hale & Burroughs, 1953). A new isoflavone, glycitein, has been isolated from soya beans (Naim, Gestelner, Kinson, Burk & Bondi, 1973) and recently coumestrol was also shown to be present at levels ranging from 0.05–30 µg/g (Lockhart, Jones & Finney, 1978). The present report provides bioassay data showing that oestrogenic activity was present in all sixteen samples of soya meal examined.

Experimental

Materials. Sample 1 was the extracted soya-bean meal that had been used in the manufacture of the control feed (Porton Rat Diet) associated with the original problem. Samples 2–14 were soya-bean meals of various origins destined for the manufacture of farm-animal feeds. Sample 15 was a pelleted form of feed. Samples 15 and 16 were soya-bean products intended for human consumption. Semi-synthetic (SS) feed supplied by RHM Labware Ltd. was used as a soya-free control.

Three extracts were prepared. For the first, 90 g soya-bean meal was exhaustively extracted with ethyl acetate in a Soxhlet apparatus. The solvent was evaporated to dryness and the residue was re-dissolved in

a convenient volume of ethanol-ethyl acetate (1:1, v/v). A second, 70% ethanol extract was prepared as described for the extraction of oestrogens from white clover (Saba, Drane, Hebert & Holdsworth, 1974). A third extract in aqueous acetonitrile was also prepared (Drane *et al.* 1975).

Oestrogen bioassay. Eighteen-day-old MFI weanling female mice weighing 3–9 g were supplied by OLAC 1976 Ltd, Bicester, Oxon. They were housed in groups of six to a cage and each group was given 40 g of feed over a period of 35 days. On the following day the mice were killed and the uteri were dissected out, blotted on filter paper and weighed. Each assay included a control group given only the SS feed and three or four groups given SS feed containing known amounts of diethylstilboestrol (DES). The test soya meal samples were fed alone, or mixed with SS diet, or as an extract mixed into SS diet and air dried.

Mycology and mycotoxin screening. Samples of soya-bean meal were screened for possible mycotoxin contamination by the method described by Roberts & Patterson (1975) as modified by Patterson & Roberts (1979). The mycological examination of six samples was carried out by the methods described by Sturme, Patterson & Roberts (1975).

Thin-layer chromatography (TLC). Biologically-active ethyl-acetate extracts were examined for phyto-oestrogens by TLC using Polygram Sil G/UV₂₅₄ sheets and methanol-chloroform (7:93, v/v) as the developing solvent. Genistein, daidzein and formononetin (minimum detectable levels 10 µg/g) and coumestrol (minimum detectable level 1 µg/g) were run as reference compounds. The developed chromatogram was examined under long- (360 nm) and shortwave (250 nm) ultra-violet light for fluorescing and absorbing spots both before and after exposure to ammonia vapour. These active extracts were also analysed for zearalenone using the mycotoxin method cited above (analytical limit 20 µg/kg).

Results and Discussion

No mould growth was evident in any of the six soya meals sampled and *Fusarium* species were not

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EFFECT OF GENISTIN ON REPRODUCTION OF THE MOUSE^{1,2}

M. W. CARTER, GERRARD MATRONE AND W. W. G. SMART, JR.
*Animal Nutrition Section of Department of Animal Industry, North Carolina
Agricultural Experiment Station, Raleigh*

(Received for publication November 1, 1971)

Reproductive disturbances have been reported to occur in sheep and in rabbits fed the soybean plant as a large part of the diet (Harr, '35; Kendall et al, '50; Matrone, '52). That these reproductive disturbances might have been caused in part by the presence of an estrogenic-like substance can be inferred from data in the literature. The evidence is as follows: first, these reproductive disturbances are similar to those reported to occur in sheep grazing on the estrogenically active subterranean clover pastures in Australia (Bennetts and Underwood, '49; Underwood and Shier, '51); and second, the compound, genistein (4', 5, 7-trihydroxyisoflavone), responsible for the estrogenic activity of subterranean clover (Bradbury and White, '51) also is present in soybean oil meal as the glucoside of genistein, genistin (Walz, '31; Walter, '41).

N.B. | Injections of genistin have shown it to be estrogenically active (Cheng et al, '53). By means of the mouse uterine weight assay, Carter and associates ('53) have shown that commercial soybean oil meal also is estrogenically active but that commercial soybean oil meal residue from which genistin has been extracted is inactive. The present report, a part of

¹Published with the approval of the Director of Research as Paper no. 600 of the Journal Series. The data were taken from a thesis submitted by the senior author in partial fulfillment of the requirements for the Master of Science degree.

²Supported in part by Tennessee Valley Authority, Knoxville, Tennessee.

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IDENTIFICATION OF PHYTOESTROGENS IN THE URINE OF MALE DOGS

P. E. JUNEVICZ¹*, S. PALLANTE MORSILLI, A. MOSER² and L. L. EWING³

¹Department of Population Dynamics, Division of Reproductive Biology, Johns Hopkins School of Hygiene and Public Health, Baltimore, MD 21205, ²Middle Atlantic Mass Spectrometry Facility, Department of Pharmacology, Johns Hopkins School of Medicine, Baltimore, MD 21205 and ³Biochemistry Laboratory, Kennedy Institute, 707 North Broadway, Baltimore, MD 21203, U.S.A.

(Received 18 April 1988)

Summary—It is becoming increasingly apparent that dietary factors may play a role in the etiology of hormone dependent neoplasias. It has been hypothesized that estrogens play some role in the etiology of benign prostatic hyperplasia (BPH) in the canine. The presence of estrogen receptor binding activity in a fraction of canine urine purified by high performance liquid chromatography (HPLC) that did not correspond to estrinol, estradiol, estrone or any of their primary metabolites was observed in the present study. We used thermospray-mass spectrometry and GC-MS to identify the phytoestrogens daidzein, equol, formononetin and genistein in HPLC purified fractions of urine obtained from male beagles. Using the same techniques we also confirmed the presence of daidzein and genistein in the commercial diet fed to these same dogs. Using the immature rat uterine cytosol estrogen receptor assay, relative binding affinities of 0.03, 1.1, <0.01 and 3.9% were obtained for daidzein, equol, formononetin and genistein, respectively when compared to estradiol (100%). In conclusion, phytoestrogens are present in urine of male beagles. Moreover, the commercial diet fed to these dogs contains isoflavones which can be converted to equol by intestinal microflora. These results suggest the need for investigations of phytoestrogens (e.g. equol) excreted into the urine daily and its relationship to the incidence and severity of BPH in the dog.

INTRODUCTION

We [1] recently completed a multivariate analysis of 82 biologic variables in beagles to gain insight into factors which cause benign prostatic hyperplasia (BPH). We concluded that ageing increases the sensitivity of prostatic growth to constant or slowly diminishing endogenous testosterone production.

It has been hypothesized that estrogens play some role in this age-related increase in prostate sensitivity to androgen [2] in part because the estradiol/testosterone ratio increases in old dogs [3] and in part because exogenous estrogen treatment of castrate dogs sensitizes prostatic growth response to sex-reduced androgens [4]. However, the role of estrogen(s) in the etiology of BPH remains enigmatic.

During the course of our studies into the etiology of BPH in beagles, we observed the presence of an estrogen receptor binding activity in an HPLC fraction obtained from an organic extract of dog urine. This compound did not correspond in elution volume to estrinol, estradiol, estrone or any of their primary metabolites. During our attempts to identify this compound, we became aware of the idea that dietary factors may play an important role in the etiology of neoplastic disease in general [5] and particularly in

hormone dependent neoplasias such as breast and prostate cancer [6].

We learned that a variety of naturally occurring and synthetic non-steroidal compounds can exhibit estrogenic activity [7]. Our attention was drawn to the isoflavones including daidzein, formononetin, genistein and biochanin A for several reasons. First, the isoflavones are present in relatively high levels in soy bean and soy bean-based products which are widely used in human and animal food products [8,9]. Second, equol is formed by intestinal degradation of isoflavones in several species [10-13]. Third, equol has both uterotropic and anti-estrogenic activities [14,15]. Fourth, dietary equol has been associated with hyperestrogenism and infertility in sheep [16] and endemic hepatotoxicity and infertility in captive cheetahs [17].

In the present report, we used HPLC, thermospray-mass spectrometry and GC-MS to identify the presence of daidzein, equol, formononetin, and genistein in the urine of male beagles. Also, we verified the presence of daidzein and genistein in the commercial dog chow fed to these dogs. Finally, we confirmed that these phytoestrogens bind to the rat uterine cytosol estrogen receptor.

EXPERIMENTAL

Animals and urine collection

Fifteen healthy intact male beagles (Laboratory Research Enterprises, Inc., Kalamazoo, MI) that

*Present address: Department for Endocrine Pharmacology, Sterling Winthrop Research Institute, Kenilworth, NY 12141, U.S.A.

To whom correspondence should be addressed.

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Authors
DML RL. Chang HC. Doerge DR.
Title
ANTI-THYROID ISOFLAVONES FROM SOYBEAN • ISOLATION,
CHARACTERIZATION, AND
MECHANISMS OF ACTION

Source
gastrointestinal Pharmacology. 54(10):1087-1096, 1997 Nov 15.

← PAGE 6

Abstract

The soybean has been implicated in diet-induced goiter by many studies. The extensive consumption of soy products in infant formulas and in Vegetarian diets makes it essential to define the goitrogenic potential. In this report, it was observed that an acidic methanolic extract of soybeans contains compounds that inhibit thyroid peroxidase- (TPO) catalyzed reactions essential to thyroid hormone synthesis. Analysis of the soybean extract using HPLC, UV-VIS spectrophotometry, and LC-MS led to identification of the isoflavones genistein and daidzein as major components by direct comparison with authentic standard reference isoflavones. HPLC fractionation and enzymatic assay of the soybean extract showed that the components responsible for inhibition of TPO-catalyzed reactions coeluted with daidzein and genistein. In the presence of iodide ion, genistein and daidzein blocked TPO-catalyzed tyrosine iodination by acting as alternate substrates, yielding mono-, di-, and triiodoisoflavones. Genistein also inhibited thyroxine synthesis using iodinated casein or human goiter thyroglobulin as substrates for the coupling reaction. Incubation of either isoflavone with TPO in the presence of H₂O₂ caused irreversible inactivation of the enzyme; however, the presence of iodide ion in the incubations completely abolished the inactivation. The IC₅₀ values for inhibition of TPO-catalyzed reactions by genistein and daidzein were ca. 1-10 μ M, concentrations that approach the total isoflavone levels (ca. 1 μ M) previously measured in plasma from humans consuming soy products. Because inhibition of thyroid hormone synthesis can induce goiter and thyroid neoplasia in rodents, delineation of anti-thyroid mechanisms for soy isoflavones may be important for extrapolating goitrogenic hazards identified in chronic rodent bioassays to humans consuming soy products. (C) 1997 Elsevier Science Inc.

[References:
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PAGE 6(a)

Phytoestrogens In Soy-Based Infant Foods: Concentrations, Daily Intake, and Possible Biological Effects (44229)

C. H. G. IRVINE,*¹ M. G. FITZPATRICK† AND S. L. ALEXANDER*

Animal and Veterinary Sciences Group,* Lincoln University, New Zealand, and Chemistry Department,† University of Auckland, Auckland, New Zealand

Abstract. Exposure to estrogenic compounds may pose a developmental hazard to infants. Soy products, which contain the phytoestrogens, genistein and daidzein, are becoming increasingly popular as infant foods. To begin to evaluate the potential of the phytoestrogens in these products to affect infants, we measured total genistein and daidzein contents of commercially available soy-based infant formulas, infant cereals, dinners, and rusks. We also assayed phytoestrogens in dairy-based formulas and in breast milk from omnivorous or vegetarian mothers. In most cases, the glucoside forms of the phytoestrogens were hydrolyzed before separation by HPLC.

Mean (\pm SEM) total genistein and daidzein contents in four soy infant formulas were 87 ± 3 and 49 ± 2 μ g/g, respectively. The phytoestrogen content of cereals varied with brand, with genistein ranging from 3–287 μ g/g and daidzein from 2–276 μ g/g. By contrast, no phytoestrogens were detected in dairy-based infant formulas or in human breast milk, irrespective of the mother's diet (detection limit = 0.05 μ g/ml). When fed according to the manufacturer's instruction, soy formulas provide the infant with a daily dose rate of total isoflavones (i.e., genistein + daidzein) of approximately 3 mg/kg body weight, which is maintained at a fairly constant level between 0–4 months of age. Supplementing the diet of 4-month-old infants with a single daily serving of cereal can increase their isoflavone intake by over 85%, depending on the brand chosen. This rate of isoflavone intake is much greater than that shown in adult humans to alter reproductive hormones. Since the available evidence suggests that infants can digest and absorb dietary phytoestrogens in active forms and since neonates are generally more susceptible than adults to perturbations of the sex steroid milieu, we suggest that it would be highly desirable to study the effects of soy isoflavones on steroid-dependent developmental processes in human babies. (P.S.E.B.M. 1998, Vol 217)

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The use of soy-containing infant foods is increasing as the public has been made aware of the health promoting properties of soy. Even in 1986, approximately 75% of the liquid infant formulas sold in North America were soy-based (1). Although soy also contains antinutritive factors (2), these are largely eliminated by processing or counteracted by supplementation for infant feeding (3). By contrast, the isoflavone phytoestrogens, genis-

tein and daidzein, present in raw beans primarily as the glucosides, genistein and daidzin (4), are heat stable and show substantial carryover through regular processing methods (5). Recently, concern has been expressed that exposure to soy isoflavones may pose a developmental hazard to infants, particularly to the reproductive system (6–9). This is because the isoflavones may cause perturbations of the sex steroid milieu, which are poorly tolerated by neonates (7). *In vitro* experiments have shown that the soy isoflavones can bind to estrogen receptors and act as competitive agonists or antagonists to endogenous estrogens depending on relative concentrations and affinities (10–12). Moreover, they can influence endogenous steroid metabolism by inhibiting 17 β -hydroxysteroid oxidoreductase type 1, which is the enzyme responsible for converting relatively impotent estrone to the more potent estradiol and, to a lesser

* For reprint requests for reprints should be addressed to Animal & Veterinary Sciences Group, PO Box 84, Lincoln University, Canterbury, New Zealand. E-mail: rsm@h6whi.lucoln.ac.nz

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Source: Nihon Naibun
 Gakkaï Zasshi, 67(15)
 日本内分泌学会, (Folia endocrinol.) 67, 622-629, 1981

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健康者甲状腺機能への大豆の効果 —大豆投与実験—

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名古屋大学環境医学研究所

村 田 善 晴

SMI ブリストル (株) 北里バイオケミカルラボラトリー

富 樫 和 美

The Effects on the Thyroid Gland of Soybeans Administered Experimentally in Healthy Subjects

Ishizuki Thyroid Clinic

Yoshimochi ISHIZUKI

The Fourth Department of Internal Medicine, Aichi Medical University

Yoshifumi HIROOKA

The Research Institute of Environmental Medicine, Nagoya University

Yoshiharu MURATA

SMI-Bristol, Kitazato Biochemical Laboratories

Kazuyoshi TOGASHI

To elucidate whether soybeans would suppress the thyroid function in healthy adults, we selected 37 subjects who had never had goiters or serum antithyroid antibodies. They were given 30g of soybeans everyday and were divided into 3 groups subject to age and duration of soybean administration.

In group 1, 20 subjects were given soybeans for 1 month. Groups 2 and 3 were composed of 7 younger subjects (mean 29 y.o.) and 10 elder subjects (mean 61 y.o.) respectively, and the subjects belonging to these groups received soybeans for 3 months. The Wilcoxon-test and t-test were used in the statistical analyses. In all groups, the various parameters of serum thyroid hormones remained unchanged by taking soybeans, however TSH levels rose significantly although they stayed within normal ranges. The TSH response after TRH stimulation in group 3 revealed a more significant increase than that in

$\frac{1}{2}$ the usually
 dose - over
 twice as long

group 2, although inorganic iodide levels were lowered during the administration of the soybeans. We have not obtained any significant correlation between serum inorganic iodide and TSH.

Hypometabolic symptoms (malaise, constipation, sleepiness) and goiters appeared in half the subjects in groups 2 and 3 after taking soybeans for 3 months, but they disappeared 1 month after the cessation of soybean ingestion.

These findings suggested that excessive soybean ingestion for a certain duration might suppress thyroid function and cause goiters in healthy people, especially elderly subjects.

結 言

大豆には甲状腺腫瘍発物質¹⁾が含まれており、実験ラットに与えると、短期間でも血中T₄値が低下²⁾し、I¹³¹摂取率が抑制³⁾されるといわれている。人では豆乳で育てた乳児に甲状腺腫、甲状腺機能低下症が発症した数症例が報告⁴⁾されている。吾々は大豆が慢性甲状腺炎の甲状腺機能を巨量に抑制する事を示し、成人甲状腺にも影響を及ぼす事を報告した。しかし常食の大豆が健康成人の甲状腺機能を抑制するかどうかについて、系統的に検討した報告がない。

そこでこの研究では健康者に大豆を投与し、成人甲状腺機能に変化を与えるかどうかを検討した。そして甲状腺機能検査値を判断する時、食事の影響を無視して判定して良いかどうかを考察する事を研究目的とした。

対象並びに方法

対象には、甲状腺疾患の既往や甲状腺腫がなく、血中抗甲状腺抗体陰性で、TBG変動を招く薬剤服用がなく、通常の仕事に従事している人を健康者とし、22~76才の男8、女45計53例を選んだ。

5群に分けた。第1群は大豆1ヵ月投与の短期投与群で、22~60才の男7、女13例計20例である。大豆3ヵ月投与の長期投与群を年齢により更に2群に分けた。第2群は22~39才平均29才の女性若年群7例で、第3群は46~76才平均61才の男1、女9計10例の高年群である。第2、3群と夫々に同年令分布、同平均年齢、同数例を、対照群に選んだ。第4群が7例、第5群が10例である。

酢大豆は炒った大豆(高山産)を米酢に漬け、香えたもので、30g/日を2分割し毎日経口投与した。三稀、味噌、醤油摂取には制限を加えず、通常生活を行わせた。

大豆投与実験は1989年8月から1年間に行い毎日摂取を怠ったものは除外した。

検査は大豆投与前、投与最終日、及び大豆中止3ヵ月以降日の午前中に施行し、問診、甲状腺触診、採血を行った。-80℃で保存した血清を用い大豆前後をペアで同時測定した。甲状腺腫を認めた例は超音波検査を行った。自覚症状は期間中の再三の間診から先ず全ての症状を取り上げた。その中から持続した症状のみを取り上げ、他病態によると判断される症状は除外した。大豆中止後に消失した症状を大豆効果と見なして採択した。対象例中4例は投与前検査が、また2例は大豆中止後の検査が出来なかった。

血清T₄、T₃、FT₄、FT₃、rT₃、TBGはRIAで、TSHは高感度法で測定した。NEFAは酵素法で、CPK、LDH、GOT、GPTはUV法で測定した。TRHテストは500ug静注法を用い、30分後の

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Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women¹⁻³

Aedin Cassidy, Sheila Bingham, and Kenneth DR Setchell

ABSTRACT The influence of a diet containing soy protein on the hormonal status and regulation of the menstrual cycle was examined in six premenopausal women with regular ovulatory cycles. Soy protein (60 g containing 45 mg isoflavones) given daily for 1 mo significantly ($P < 0.01$) increased follicular phase length and/or delayed menstruation. Midcycle surges of luteinizing hormone and follicle-stimulating hormone were significantly suppressed during dietary intervention with soy protein. Plasma estradiol concentrations increased in the follicular phase and cholesterol concentrations decreased 9.6%. Similar responses occur with tamoxifen, an antiestrogen undergoing clinical trial as a prophylactic agent in women at high risk for breast cancer. These effects are presumed to be due to nonsteroidal estrogens of the isoflavone class, which behave as partial estrogen agonists/antagonists. The responses to soy protein are potentially beneficial with respect to risk factors for breast cancer and may in part explain the low incidence of breast cancer and its correlation with a high soy intake in Japanese and Chinese women. *Am J Clin Nutr* 1994;60:333-40.

KEY WORDS Soy, hormonal status, menstrual cycle, tamoxifen, breast cancer, isoflavones

Introduction

Breast cancer is the second most common cancer in Western countries but its incidence is significantly less in Third World and Asian populations. Age-specific rates for breast cancer in England and Wales are 199.4 per 100 000 in women 60-65 y of age compared with 52.3 per 100 000 for women of similar age in Japan (1). Epidemiological data from migrant studies suggest that in most cases the susceptibility to breast cancer is the result of environmental rather than genetic differences between these populations and that diet is a major contributing factor (2). The largest and most carefully controlled prospective study of diet and breast cancer, however, failed to provide conclusive evidence to support an association between relative risk and a diet high in fat (3). Furthermore, although elevated free estrogen concentrations have been associated with increased risk (4), there have been few adequately controlled studies of the influence of fat on hormonal status.

Apart from fat, there are many other differences between the typical diets of Far Eastern and Western populations (5). In the East a significant quantity of soybean protein is consumed in many different forms, including beans, miso, tofu, and soy milk,

Soy is a rich source of nonsteroidal estrogens of the isoflavone class (6). These compounds, which are structurally similar to estrogens, bind to the estrogen receptor and behave as partial estrogen antagonists (7). For this reason we previously suggested that a diet of soy protein may be beneficial in the protection against and/or treatment of breast cancer (8). This hypothesis is strengthened by recent studies, which have shown that a diet of soy protein leads to a significant dose-dependent reduction in mammary tumor growth in two animal models of chemically induced mammary carcinoma (9). In these studies, tumor formation was negatively correlated with total dietary isoflavone concentration, and in particular with the dietary intake of genistein and the urinary isoflavone excretion (9).

Because little is known about the biological and physiological effects of dietary estrogens in humans and in view of well-documented examples of the biological potency of isoflavone ingestion by animals (10-12), we investigated in a controlled dietary-intervention study the influence of soy-protein intake on hormonal status of premenopausal women. We hypothesized that a constant diet of soy protein containing isoflavones would lead to significant modifications to the hormonal status of the menstrual cycle and that these changes would be beneficial with regard to risk factors for breast cancer.

Subjects, materials, and methods

Study protocol

Six healthy nonvegetarian women (21-29 y of age) were enrolled in the study. The protocol was approved by the Dunn Nutrition Unit Ethical Committee. All women had normal menstrual cycles and had taken no medication for ≥ 6 mo before starting

¹ From the Dunn Clinical Nutrition Centre, Cambridge, UK, and the Division of Clinical Mass Spectrometry, Department of Pediatrics, Children's Hospital Medical Center, Cincinnati.

² Supported in part by the Ministry of Agriculture, Fisheries and Foods and the National Institutes of Health, National Cancer Institute, grant RO1-CA56302-01.

³ Address reprint requests to KDR Setchell, Division of Clinical Mass Spectrometry, Department of Pediatrics, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229, or A Cassidy, Dunn Nutrition Center, 100 Tennis Court Road, Cambridge, UK, CB2 1QL.

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Stimulatory influence of soy protein isolate on breast secretion in pre- and postmenopausal women.Petrakis NL, Barnes S, King EB, Lowenstein J, Wiencke J, Lee MM, Miike R, Kirk M, Coward L*Cancer Epidemiol Biomarkers Prev* 1996 Oct 5;10:785-94**Abstract**

Soy foods have been reported to have protective effects against premenopausal breast cancer in Asian women. No studies have been reported on potential physiological effects of dietary soy consumption on breast gland function. We evaluated the influence of the long-term ingestion of a commercial soy protein isolate on breast secretory activity. We hypothesized that the features of nipple aspirate fluid (NAF) of non-Asian women would be altered so as to resemble those previously found in Asian women. At monthly intervals for 1 year, 24 normal pre- and postmenopausal white women, ages 30 to 58, underwent nipple aspiration of breast fluid and gave blood and 24-h urine samples for biochemical studies. No soy was administered in months 1-3 and 10-12. Between months 4-9 the women ingested daily 38 g of soy protein isolate containing 38 mg of genistein. NAF volume, gross cystic disease fluid protein (GCDFP-15) concentration, and NAF cytology were used as biomarkers of possible effects of soy protein isolate on the breast. In addition, plasma concentrations of estradiol, progesterone, sex hormone binding globulin, prolactin, cholesterol, high density lipoprotein-cholesterol, and triglycerides were measured. Compliance was assessed by measurements of genistein and daidzein and their metabolites in 24 h urine samples. Excellent compliance with the study protocol was obtained. Compared with NAF volumes obtained in months 1-3, a 2-6-fold increase in NAF volume ensued during months 4-9 in all premenopausal women. A minimal increase or no response was found in postmenopausal women. No changes were found in plasma prolactin, sex hormone binding globulin, cholesterol, high density lipoprotein cholesterol, and triglyceride concentrations. Compared with concentrations found in months 1-3 (no soy), plasma estradiol concentrations were elevated erratically throughout a "composite" menstrual cycle during the months of soy consumption. No significant changes were seen in plasma progesterone concentrations. No significant changes were found in plasma

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throughout a "composite" menstrual cycle during the months of soy consumption. No significant changes were seen in plasma progesterone concentrations. No significant changes were found in plasma estrone levels in postmenopausal women. A moderate decrease occurred in the mean concentration of GUDFP-15 in NAF in premenopausal women during the months of soy ingestion. Of potential concern ← was the cytological detection of epithelial hyperplasia in 7 of 24 women (29.2%) during the months they were consuming soy protein isolate. The findings did not support our a priori hypothesis. Instead, this pilot study indicates that prolonged consumption of soy protein isolate has a stimulatory effect on the

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premenopausal female breast, characterized by increased secretion of breast fluid, the appearance of hyperplastic epithelial cells, and elevated levels of plasma estradiol. These findings are suggestive of an estrogenic stimulus from the isoflavones genistein and daidzein contained in soy protein isolate.

Me: A

Adult ; Biological Markers ; Breast ; Carrier Proteins ; Chromatography ; High Pressure Liquid ; Estrogens ; Exudates and Transudates ; Female ; Human ; Hyperplasia ; Isoflavones ; Middle Age ; Nipples ; Pilot Projects ; Postmenopause ; Premenopause ; Soy Proteins ; Spectrum Analysis ; Mass ; Support, Non U.S. Gov't ; Support, U.S. Gov't, P.H.S. ;

Author Address

Department of Epidemiology and Biostatistics, University of California, San Francisco 94143-0560, USA.

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To: Linda Kahl@OPA@FDA.CFSAN
From: "Dave Woodhams" <woodhams@iprolink.co.nz>
Certify: N
Subject: GRAS Notice GRN 000001
Date: Tuesday, March 31, 1998 at 6:26:24 am EST
Attached: None

Dear Dr Kahl

I am given to understand that Archer Daniels Midland has provided notice in accordance with a proposed regulation at 62 Fed. Reg. 18938 (April 17, 1997) that the substance "soy isoflavone" is generally recognised as safe for use as a micronutrient in food.

Would you please advise which substances are in fact included in the term "soy isoflavone" as this is a generic term covering different forms of several isoflavones and it is not a single "substance".

In a recent publication (Cassidy A, Bingham S, Setchell K D R (1994), "Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women" Am J Clin Nutr. 60: 333-340) evidence was presented on the physiological effects of 45mg/day of "soy isoflavones" from 60g/day soy protein isolate in the diet on the menstrual cycle of premenopausal women. Principal among these were the suppression of the midcycle surge of luteinising hormone (LH) and follicle stimulating hormone (FSH) by 67% and 50% respectively. These are the hormones that trigger ovulation and the data were measured for a soy diet during only one cycle for each woman.

In my professional opinion, there is a danger that providing GRAS classification for these substances could result in temporary infertility at dietary doses on prolonged exposure. The use of greater doses as dietary supplements or through direct addition to conventional foods, in addition to doses received from both soy foods and processed foods having soy protein isolate as a constituent, could have more lasting effects. To the best of my knowledge the effects of higher doses and longer exposure than 30 days on fertility have not been assessed in humans.

I am also concerned that there appears to have been little examination of the potential immune suppressant action of genistein in the diet. This action of genistein was reported by S. and D. Atluru ("Evidence that Genistein, a Protein-Tyrosine Kinase Inhibitor, Inhibits CD28 Monoclonal-Antibody-Stimulated Human T-Cell Proliferation", Transplantation 51 448-50 [1991]).

There is also the fascinating study of adult Japanese males (Y. Ishizuki, Y. Hirooka, Y. Murata and K. Togashi, "The effects on the thyroid gland of Soybeans administered experimentally in healthy subjects" Nippon Naibunpi

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gakkai Zasshi, 67, 622-29 [1991])). This study showed that in the groups of subjects fed 30g soybeans per day for 3 months, half the subjects exhibited hypometabolic symptoms (malaise, constipation, sleepiness) and goiters. These symptoms disappeared one month after cessation of soybean ingestion. I have a parallel Japanese/English translation of this paper which I would be happy to copy and send you, should you wish to see it, translated by the Japanese Communication Service in Wellington, New Zealand.

For the above reasons I would oppose the granting of GRAS status to "soy isoflavone".

Yours sincerely

Dr David J Woodhams CEng
Dairy Process Consultant
PO Box 32 236
2/47 Church Street
Devonport 1309
New Zealand
Phone: +64 9 445 8721
Fax: +64 9 445 9834
Email: woodhams@iprolink.co.nz

000188

Dr Linda Kahl

OU

~~R.F. Jones~~

R.Dh

Whangwee

N. 2

3/31/98

Dear Dr Kahl

G.R.N. 000001

The abstracts below are for your personal information. From anecdotes I have about 8 or 9 year old girls who drink a cup of soy milk (based on soy protein isolate) a day, it is my opinion that all consumers of specially soy products should be afforded health warnings.

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Stimulatory influence of soy protein isolate on breast secretion in pre- and postmenopausal women

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Petakis NI., Barnes S., King F.B., Lowenstein J., Wiencke J., Lee MM, Muike R., Kirk M.

postmenopausal women.

Petrakis NI., Barnes S., King FB., Lowenstein J., Wiencke J., Lee MM, Miike R., Kirk M., Coward L.

Cancer Epidemiol Biomarkers Prev 1996 Oct 5:10 785-94

Abstract

Soy foods have been reported to have protective effects against premenopausal breast cancer in Asian women. No studies have been reported on potential physiological effects of dietary soy consumption on breast gland function. We evaluated the influence of the long-term ingestion of a commercial soy protein isolate on breast secretory activity. We hypothesized that the features of nipple aspirate fluid (NAF) of non-Asian women would be altered so as to resemble those previously found in Asian women. At monthly intervals for 1 year, 24 normal pre- and postmenopausal white women, ages 30 to 58, underwent nipple aspiration of breast fluid and gave blood and 24-h urine samples for biochemical studies. No soy was administered in months 1-3 and 10-12. Between months 4-9 the women ingested daily 38 g of soy protein isolate containing 38 mg of genistein. NAF volume, gross cystic disease fluid protein (GCDFP-15) concentration, and NAF cytology were used as biomarkers of possible effects of soy protein isolate on the breast. In addition, plasma concentrations of estradiol, progesterone, sex hormone binding globulin, prolactin, cholesterol, high density lipoprotein-cholesterol, and triglycerides were measured. Compliance was assessed by measurements of genistein and daidzein and their metabolites in 24-h urine samples. Excellent compliance with the study protocol was obtained. Compared with NAF volumes obtained in months 1-3, a 2-6-fold increase in NAF volume ensued during months 4-9 in all premenopausal women. A minimal increase or no response was found in postmenopausal women. No changes were found in plasma prolactin, sex hormone binding globulin, cholesterol, high density lipoprotein cholesterol, and triglyceride concentrations. Compared with concentrations found in months 1-3 (no soy), plasma estradiol concentrations were elevated erratically throughout a "composite" menstrual cycle during the months of soy consumption. No significant changes were seen in plasma progesterone concentrations. No significant changes were found in plasma estrogen levels in postmenopausal women. A moderate decrease occurred in the mean concentration of GCDFP-15 in NAF in premenopausal women during the months of soy ingestion. Of potential concern ← was the cytological detection of epithelial hyperplasia in 7 of 24 women (29.2%) during the months they were consuming soy protein isolate. The findings did not support our a priori hypothesis. Instead, this pilot study indicates that prolonged consumption of soy protein isolate has a stimulatory effect on the

Clinical Changes in Ovariectomized Ewes Exposed to Phytoestrogens and 17β -Estradiol Implants (43838)

AGNES I. NWANNENNA,^{*1} T. J.-O. LUNDH,[†] A. MADEJ,^{‡2} G. FREDRIKSSON,^{*3} AND G. BJÖRNHAG[§]
Departments of Obstetrics and Gynaecology, Animal Nutrition and Management,† Physiology,‡ and Animal Physiology,§ Swedish University of Agricultural Sciences S-75007 Uppsala, Sweden*

Abstract Eight Swedish Finewool Landrace ewes, ovariectomized 5 months earlier and kept on nonestrogenic hay, were each fed 3.5 kg red clover silage, corresponding to 6.1 g phytoestrogens (of which 3.5 g was formononetin) per day, for 14 days in November (short days). In January (short days), two groups (3 each) of these ewes received one or two 17β -estradiol sc implants. In May (long days), one of two new groups (4 each) of these ewes was reexposed to phytoestrogens for another 14 days while the other served as a control. Physical examination of ewes for changes in reproductive organs was carried out two or three times per week during each feeding/treatment, and continued until observed changes disappeared. Clinically significant changes occurred in the reproductive organs of ewes fed red clover. Vulva color changed from pale to pink and red, and there were enlargements of the vulva, uterus, and udder. In addition, teat length and circumference increased, and secretion of milky fluid began. These changes were similar, but more pronounced during treatment with 17β -estradiol, particularly teat circumference. The changes in vulva were more dramatic in May than in November and resembled those observed in ewes treated with estradiol. Our data show that a daily intake of 3.5 g formononetin for 14 days caused the increase of teat size and changes in the color of the vulva and in uterus weight in ovariectomized ewes.

[P S F B.M. 1995, Vol 208]

The presence of estrogenic substances in plants was first demonstrated in the 1920s (1, 2). Later, such substances were reported to cause reproductive disorders in sheep grazing subterranean clover in Australia (3). Genistein and other isoflavones, daidzein, biochanin-A, and formononetin were isolated from clover and proved to be the cause of the disorders (1). The estrogenic activities of forage plants have been reported from various parts of the world (4, 5, 6, 7). It is known that the relative activity of individual isoflavones may vary depending on the strain as well as the species of animals (8) owing to inter- (2) and

intraspecific variation in the metabolism of isoflavones (9). Formononetin is the most important isoflavone causing reproductive disorders in sheep (10) through its main stable metabolite, equol.

This paper presents the clinical responses of ovariectomized Swedish Finewool Landrace ewes exposed either to red clover silage or to 17β -estradiol.

Materials and Methods

Experimental Animals. Eight Swedish Finewool Landrace ewes, all of which had lambed in January, were ovariectomized in May through a mid-ventral laparotomy under general anaesthesia. The ewes were kept on pasture until September. They were then moved indoors and kept in individual, adjacent boxes under natural light conditions until the end of the experiment. The ewes were given *ad libitum* access to nonestrogenic hay (mainly Timothy grass) and 100 g of concentrate (39% barley, 39% oats, 11% soya, 7% rapeseed, and 4% other additions) per day except during experimental feeding periods. They had free access to mineral licks and good drinking water at all times.

¹ Permanent address: Ahmadu Bello University, Faculty of Veterinary Medicine, Department of Surgery and Medicine, Zaria, Nigeria.

² To whom requests for reprints should be addressed at Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, Department of Physiology, P.O. Box 7045, S-750 07 Uppsala, Sweden.

³ Deceased.

OU



Dr Linda S. Kahl
Food and Drug Administration
FPO (202) 418 3131

~~RF James~~
R.D.H.
WHANGAREI N:
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FAX 64.9 4340567

Dear Dr Kahl

GRN 000001

The item below appeared in our favorite
humor magazine. Could you please advise
me if the E.P.A. knows what the F.D.A
is trying to achieve.

Cheers

~~RF James~~

6E 6 V 1E HAM 8961
MAR 31 4 9 39

000192

March 16, 1998

FOOD CHEMICAL NEWS 5

synergistically, enabling use of lower intensities of each factor than would be necessary if each were used alone." Marth observed.

Certain ingredients that are barriers to microbial growth can be used in the formulation of extended shelf-life refrigerated products, Marth suggested. Organic acids, particularly acetic but also lactic and citric, can inhibit bacterial growth; sorbate, propionate and benzoate have both antibacterial and antifungal properties.

With regard to storage temperature and shelf life, Marth said that product temperature should be maintained just slightly above freezing. Furthermore, he said, acceptable product shelf life at specified temperature limits should be established and monitored to help manage food safety and quality. "Because the potential exists for temperature abuse at some point during handling or for storage past the intended shelf life, time-temperature indicators or integrators can be useful in determining when refrigeration temperatures or intended storage times have been exceeded," he added.

— Adrienne Dern



and phytoestrogens in soy-based infant formula on their list of six "high-priority" mixtures for initial screening under the new program.

Figuring out how to prioritize chemical mixtures was one of the thorniest issues that EDSTAC faced, Thomas Osimitz of S.C. Johnson and Son told a Society of Toxicology workshop in Seattle March 4. "Dealing with mixtures has plagued toxicologists for years and years, and it always will."

The Food Quality Protection Act and the Safe Drinking Water Act Amendments, both passed in August 1996, require that the U.S. Environmental Protection Agency develop a testing system for screening thousands of chemicals for possible

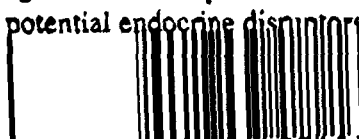
estrogenic effects. EPA convened EDSTAC, a large stakeholder committee, to develop a conceptual framework for a massive new endocrine disruptor screening program.

EDSTAC's Priority Setting Working Group recommended the following mixtures for the first round of endocrine disruptor screening. Osimitz said:

- Contaminants in human breast milk
- Phytoestrogens in soy-based infant formula
- Chemicals commonly found at hazardous waste sites
- Disinfection by-products
- Gasoline
- Pesticide and fertilizer mixtures found in groundwater

"We consider these to be a high priority," said Osimitz, a working group member.

Osimitz told *Food Chemical News* that the high-priority status for contaminants in human breast milk "should not be used to recommend for or against breast feeding," while the working group simply agreed on the importance of screening infant milk for potential endocrine disruptors.



James Schmitt, chief of EPA's Office of Prevention, Pesticides and Toxic Substances said the Scientific Advisory Board is likely to consult with the agency on the endocrine disruptor program at its May meeting. The final EDSTAC report will be published in the *Federal Register* in late July, she said.

Peter deFur of the Center for Environmental Studies at Virginia Commonwealth University reminded the workshop that EDSTAC's recommendations are just that. "Nothing is final as of yet," deFur said.

Testing schemes outlined

In addition to estrogenic effects, EDSTAC recommended that chemicals be screened for androgenic and thyroid effects. The current scheme

000193

Dr Linda S Kahl
Food and Drug Administration

~~P.D. 4~~
Whangarei N.Z.

FAX 202 418 3131

MAR 31 1998

Dear Dr Kahl,

Your E Mail to Dr Blaffer has been
passed to me

Please keep me informed daily
We will be consulting Washington
Lawyers

I follow with a page from the
Internet "Making your voice heard at
the F.D.A.

Sincerely

~~P.D. 4~~

1998 MAR 31 A 9:10

000194

BEST ORIGINAL COPY

Making Your Voice Heard at FDA: How to Comment on Proposed Regulations and Submit Petitions

January 19, 1996

As a regulatory agency, FDA publishes rules that establish or modify the way it regulates foods, drugs, biologics, cosmetics, radiation-emitting electronic products, and medical devices--commodities close to the daily lives of all Americans. FDA rules have considerable impact on the nation's health, industries and economy. These rules are not created arbitrarily or in a vacuum. They are formed with the public's help.

By law, anyone can participate in the rule-making process by commenting in writing on rules FDA proposes. FDA allows plenty of time for public input and carefully considers these comments when it draws up a final rule.

FDA gathers public comments mainly through two channels: proposed rules and petitions.

1. Proposed Rules

When FDA plans to issue a new regulation or revise an existing one, it places an announcement in the Federal Register on the day the public comment period begins. Published every weekday, the Federal Register is available at many public libraries and colleges, and even on the Internet. Issues open to public comment often are reported by the news media and may frequently be found on FDA's Internet home page. (See "Using the Internet.")

In the Federal Register, the "notice of proposed rulemaking" describes the planned regulation and provides background on the issue. It also gives the address for submitting written comments and the name of the person to contact for more information.

Also noted is the "comment period," which specifies how long the agency will accept public comments. Usually, the file--or docket--stays open for comments at least 60 days, though some comment periods have been as short as 10 days or as long as nine months. Weekends and holidays are included in the comment period.

There is no special form to fill out for comments, nor do submitters have to follow a certain style. But FDA can process comments more effectively if they are presented--either written legibly or typed--on 8-1/2-inch by 11-inch paper.

Here are some other suggestions for making sure your comment has the greatest possible impact:

- Clearly indicate if you are for or against the proposed rule or some part of it and why. FDA regulatory decisions are based largely on law and science, and agency reviewers look for reasoning, logic, and good science in comments they evaluate.
- Refer to the docket number, listed in Federal Register notice.
- Include a copy of articles or other references that support your comments. Only relevant material should be submitted.

000195



PUBLISHED BY
THE AMERICAN CHEMICAL SOCIETY

Department of Biochemistry
College of Biological Sciences
University of Minnesota
1479 Gortner Avenue
St. Paul, Minnesota 55108
Phone: (612) 625-8244
Fax: (612) 624-5358
E-mail: jafc@tc.umn.edu

Irvin E. Liener, *Editor*

Dr. Linda S. Kahl
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C Street, SW
Washington, DC 20204

April 1, 1998

Dear Dr. Kahl:

It has come to my attention that the FDA has received from ADM a submission that the use of soy isoflavones as a micronutrient in food is GRAS, and that this request is now under evaluation by the FDA as to whether ADM's notice provides a sufficient basis for GRAS approval.

In this connection I believe it is relevant to call your attention to a letter which I, as editor of a highly respected journal, have received which seriously questions the conclusion that soy isoflavones can be regarded as an ingredient that can be safely used in the human diet. In addition I would call your attention to the enclosed article entitled "New Research Questions Safety of Soya Baby Milks". I trust you will take this information seriously in your deliberations as to whether soy isoflavones can indeed be considered as GRAS.

Sincerely yours,

Irvin E. Liener
Editor-in-Chief

1998 APR - 1 A 8:35

R & V. JAMES
RD 4
WHANGAREI
NEW ZEALAND

28 February 1998

Kathleen E Duffy
Journals Editing Manager
Journal of Agricultural and Food Chemistry
PO Box 3330
Columbus OH 43210
U S A

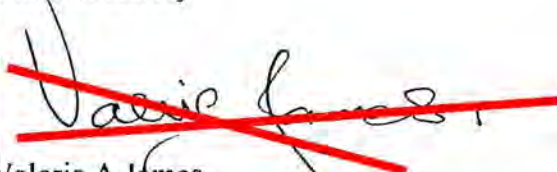
Dear Editor,

Isoflavones in Soy Based Formula

Recent research published in your journal (1) confirmed previous reports (2,3) that isoflavone intakes for infants on soy based infant formulae, are greater than those typically consumed by adult Japanese soy food consumers. Not able to be substantiated however, is the claim that soy¹ formulas have been fed to millions of infants with no evidence of harmful effects (1). The American Academy of Paediatrics (4) cited a number of clinical reports of acute allergic and hypersensitivity reactions as a result of soy formula use and an examination of published literature reveals others including severe gastro-intestinal damage (5,6,7) and epidemiologies (8,9,10) which associate soy feedings in infancy with subsequent hormonal disturbances.

Because isoflavones have historically been regarded as toxicants (11) and have been implicated in causing reproductive system damage and infertility in animals (12,13,14;) and as having hormonal effects in women (15) two governmental agencies have issued cautions. The U.K. Government's statement included information that, "The potential for phytoestrogens, including isoflavones, to ~~o~~ffect adversely infants is of particular concern, since it is possible that a hormonal imbalance in early life can permanently affect sexual development and fertility". (16); and the Swiss Federal Health Service advised that, "taking into account the very limited knowledge on the possible adverse health effects of an isoflavone exposure in new-borns and infants, it is demanded that soy-based infant formulas containing isoflavones should be used only under strict medical indications and a lack of alternative products (3).

Yours sincerely


Valerie A. James.

000198

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2. The Potential Adverse Effects of Soybean Isoflavones in Infant Feeding: Irvine C U et al: NZ Med J 1995 108: 218.
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14. Naturally Occurring Non-Steroidal Estrogens of Dietary Origin: K D R Setchell in Estrogens in the Environment: ed J A McLachlan 1985.
15. Biological Effects of a Diet of Soy Protein Rich in Isoflavones on the Menstrual Cycle of Pre Menopausal women: A Cassidy, S Bingham, K D R Setchell; Am. Jour Clin Nutr 1994: 60 , 333 - 40.
16. U.K. Dept of Health Document 96/244. Advice on Soya Based Infant Formula July 19 1996 from a Statement by the Committee on the Toxicity of Chemicals in Food, Consumer Products, and the Environment.

000199

New research questions safety of soya baby milks

Research published in *The Lancet* this summer shows that babies fed soya formula milks are getting even higher doses of isoflavones (phytoestrogens) than was previously thought. One of the world's leading researchers into phytoestrogens, Prof Kenneth Setchell, has found that infants fed soya baby milks get 6 to 11 times greater amounts of phytoestrogens on a body weight basis than the dose that has hormonal effects in adults consuming soya foods. The researchers found that blood levels of phytoestrogens in babies fed soya formula, which they measured from birth to 4 months, were 13,000 to 22,000 times higher than normal. This, says Prof Setchell, may be sufficient to exert biological effects, whereas the contribution of phytoestrogens from cows milk or from breastmilk, even from mothers consuming soya foods, was negligible.

Soya formula manufacturers, seeking to play down concerns about the suitability of soya formula, have claimed that breast milk does contain phytoestrogens. Cow and Gate, manufacturers of InfaSoy, in its

briefing document, *Phytoestrogens in Soya Infant Formulas*, circulated widely to health professionals, categorically states this. The Food Commission will be asking the company to correct its error and inform all those it has previously circulated.

As well as being oestrogen mimics, phytoestrogens, can inhibit certain enzymes and interfere with cell signal transduction pathways, according to Prof Setchell. The ingestion of high concentrations of phytoestrogens has adversely affected reproduction in several animal species. In pre-menopausal women, soya protein affects reproductive hormone levels. Much research is now looking at the role that phytoestrogens may play in preventing hormone-dependent diseases, including some cancers, osteoporosis and cardiovascular disease but little money is going into investigating the risks to infants.

Last summer the Department of Health issued advice that soya formula milks should only be given to babies on the advice of a health professional and called for high

priority research to determine the risks to infants. Swiss health authorities advise that in early infancy soya formula should only be used for precise medical conditions, where there is a proven inability to use formulas based on cows or goats milk, and it should not be used for 'ecological' reasons such as the avoidance of animal proteins.

The New Zealand Soy Information Network, whose scientists first raised concerns about soya infant formulas, is calling for soya formula milks to be restricted to sales in pharmacies says 'There is no excuse for permitting normal children to be subjected to these unknown risks with no compensating medical benefits.'

The research also raises questions about over-the-counter supplements containing isoflavones. The potential dangerous effects from self-induced mega-dosing are a concern say the authors. Last year the Food Commission reported that men undergoing sex changes were developing breasts after consuming supplements containing large doses of phytoestrogens.

Now there are also warnings that a new bread, Burgen Bread, made by Allied Bakeries which contains phytoestrogens from soya and linseed, could lead to lowered sperm counts in men. The company, which claims the bread may help stave off menopausal hot flushes and protect against osteoarthritis in women, was asked to tone down its claims that it could protect against some cancers heart disease and hormone related disorders by the Ministry of Health in New Zealand following its launch there earlier in the year.

■ Setchell, K et al, Exposure of infant to phyto-estrogens from soy-based infant formula, *The Lancet*, 350: 23 27, July 5, 1997

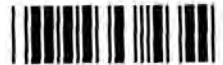
■ The Soy Information Network can be contacted at PO Box 32236, Devonport, New Zealand Tel: +64 445 8721 Fax: +64 9 445 9834

Congratulations..
to the Food Commission's Sue Dill who has been appointed as a consumer representative to MAF Working Party on Chemical Contaminants in Food. The remit the Working Party covers naturally occurring toxicants including phytoestrogens and organic and inorganic contaminants in food. If you wish to raise any such concerns please contact Sue at Food Commission.

BEST ORIGINAL COPY

000200

OU

~~R. F. Jones~~

B D 4

WISN/GARE1 N. 2

APRIL 2 1998

Dr Linda A. Kahl
Office of Pre Market Approval
U.S. F. D. A.

FAX (202) 418 3131

C. R. N #000001ARCHER DANIELS MIDLAND CO

Dear Dr Kahl,

Yvonne Clapperton passed your latest
E-MAIL along.

I am very glad to see that normal
procedures are in place at last.

As I intimated, I instructed U.S.
attorneys to adopt a watching brief, but I'm
sure now that they won't need to be
overly active.

The firm is Robbins Kaplan Miller and
Cirisi, by way of their Minneapolis office.

Sincerely

~~Richard F. Jones~~

000201



Ross H. Hall, Ph.D

1998 APR -7 A 7:17

Mount Tabor Road
Danby, VT 05739-0239
Tel 802-293-5149
Fax 802-293-5717
April 2, 1998

Dr. Linda S. Kahl
Regulatory Policy Branch HFS-206
Office of Premarket Approval, Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, S.W.
Washington, D.C. 20204

Dear Dr. Kahl: Re: GRAS Notice No. GRN 000001

I write regarding the petition of The Archer Daniels Midland Company (ADM) to grant GRAS status to soybean isoflavones as a micronutrient in food. These substances are not as benign as the petition claims. The scientific literature shows that soy isoflavones have estrogenic activity and as such they have the potential to interfere or modify in some way normal hormonal function of both women and men.

It has been suggested that this estrogenic activity benefits adults in helping to ward off breast and prostate cancers, a fact mentioned in the petition. But modifying hormonal function has a down side. Numerous studies identify a number of adverse health effects due to consumption of soy isoflavones. Infants and children, in particular, are at risk.

It's not my intention to review the literature. I understand that Mr. Richard James, Whangerei, New Zealand, sent you a list of 10 recent publications that present evidence casting doubt on the wisdom of exposing humans of any age to added soy isoflavones. I also refer you to a book, Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection, edited by T. Colborn and C. Clement, Princeton Scientific Publishing Co., 1992. This book summarizes dozens of scientific studies concerning the ill effects of exogenous estrogens on animals and humans.

I have a background in biochemistry, nutrition and toxicology and I am troubled by the fact ADM intends to add soy isoflavones to conventional foods. Once added to a food, there is no control over who eats it, what age, and how much a person consumes. Based on the published evidence of adverse effects, no exogenous estrogen, which includes the soy isoflavones, should be deliberately added to foods.

GRAS status should not be granted to the soy isoflavones.

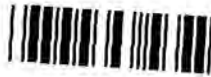
Yours sincerely,

000218

To: Linda S. Kahl, Ph.D.,
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C Street, S.W. Washington, D.C. 20204.

April 8th, 1998.

OU



1998 APR -3 A 9:17

Faxed to: 001 - 202 - 418 - 8131.

From: ~~Walter T. Jones~~ R.D. 4 Rural Delivery, Whangarei, N.Z.
Faxed from: 064 - 9 - 4340567.

Re: Gras Notification for Isoflavones Derived
from Soy Beans; GRN # 000001.

Dear Dr. Kahl,

In respect of the above application, I wish the following to be considered.

1. The National Institute of Environmental Health Sciences has, on a number of occasions released information into the internet on risks and benefits of phytoestrogens. Not once have they indicated that there was a consensus that they were generally regarded as safe.

2. On January 27th 1995, D. Jesse Waytall, D.M., Ph.D., DABUT, Contaminants Standards Monitoring Programs Branch (HFS - 308) Center for Food Safety and Applied Nutrition, USFDA, wrote to the New Zealand Ministry of Health that "we have recently installed in the Internet a large bibliography on poisonous plants, including many records dealing with soybeans and potentially toxic substances in them such as phytoestrogens.

It can be accessed by Anonymous F.T.P. UAFS 8. CFS AN F.D.A. GOO. Another access route is the world wide Web. More specific information about phytoestrogens can be obtained from an Internet file maintained by the National Center for Toxicologic Research (NCTR). It can be accessed through the NCTR Gopher or through World Wide Web. 000202

Since this readily available information contradicts the submission by Archer Davies Midland (A.D.M.) their submission is misleading and must be rejected.

I have a great deal of other relevant information on file if you need it.

Yours sincerely, ~~Walter T. Jones~~

April 4th 1998. OU



To: Linda S. Kahl, PhD,
Regulatory Policy Branch, HFS-206,
Office of Premarket Approval,
Center for Food Safety and Applied Nutrition,
200 C Street, S.W. Washington, D.C. 20204.

1998 APR -6 A 7:19

Faxed to: 001 - 202 - 468 - 3131.

From: ~~Malcolm Turner~~, R.O.4 Rural Delivery, Whangarei, N.Z.

Faxed from: 064 - 9 - 434 - 0567

Re: { Gras Notification for Isoflavones Derived from
Soy Beans; G.R.N. # 000001.

Dear Dr. Kahl,

When I faxed you my submission last night, our machine picked up only the first page, an omission not noticed until this morning. Therefore I shall refax both the message and the relevant documents which are ① Internet perspective as of May, 1996.

② Letter from Health and Human Services to N.Z. Ministry of Health. (Please note Para 5, line 5)

I shall now also include the facing page of the actual thesis by Helen Cassidy, which formed the basis 000203 of "Biological Effects of a Diet of Soy Protein Rich in Isoflavones on the Menstrual Cycle of Premenopausal Women," Cassidy, Bingham, Setchell, Am. Jour. Clin. Nutr. 1996: 60, 333-40. Also I include the figures she selected for her thesis concerning breast cancer rates in "Asian" countries. I urge you to study the source book, Muir et al. I also urge you to study "Soy Bean in Asia" Edited by Narong Chamchadwong, Paisee Lawnsuwan. This is an F.A.O. publication. Then it will be obvious that, ① most "Asian" countries traditionally, do not eat soy, ② most "Asian" countries that do not eat soy have lower rates of breast cancer, or equivalent rates to Japan which has the highest level

of soy consumption. Page 32 states (of "Soy Bean in Asia")
"Although China is a large country in soybean production with a long history in soybean growing, soybean was regarded only as a supplementary food, not indispensable."

The above goes to show that figures obtained by epidemiologists or by statisticians need to be interpreted cautiously when considering not only the facts of the matter but by regulators.

Therefore please keep me informed.

Yours sincerely,

~~Valerie J. [Signature]~~

P.S. I have become involved in this issue because, as a U.S. citizen, I believe not only in the right of free speech but in the obligation of it. That is, that all the facts of the matter, and from more than one perspective, be made available. Because of this, my husband and I have spent more than \$250,000 (N.Z.) of our life's savings on research and communications. We have received no funding from any other source. We have done so because we felt that we had an obligation to become informed and to make that information available.

~~Valerie J. [Signature]~~

000204

To: Linda S. Kahl, Ph.D.,

April 11, 1998.

Regulatory Policy Branch, HFS-206

Office of Premarket Approval

Center for Food Safety and Applied Nutrition
200 C Street, S.W. Washington, D.C. 20204.

Faced to: 001 - 202 - 418-3131.

From: ~~Walter James~~, R.D. 4 Rural Delivery, Whangarei, N.Z.

Faced from: 064-9-4340567.

Re: Gras Notification for Isoflavones Derived
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Dear Dr. Kahl,

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recently installed in the Internet a large bibliography on poisonous
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It can be accessed by Anonymous F.T.P. URS 8. CFS AN
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Since this readily available information contradicts the
submission by Aether Davies Midland (A.D.M) their submission is
misleading and must be rejected.

I have a great deal of other relevant information on file if you need
it.

Internal 9.6.96.

ENVIRONMENTAL HEALTH PERSPECTIVES

PHYTOESTROGENS, BENEFITS AND RISKS

Phytoestrogens are plant chemicals that may act as fungicides, deter herbivores, regulate plant hormones, and protect plants against ultraviolet radiation. Structurally, some phytoestrogens resemble endogenous estrogens of humans and animals, and recent research suggests they may also function as estrogen agonists or antagonists when eaten by humans. Although humans have used phytoestrogens medicinally for thousands of years, only in the last 15 years or so have researchers begun to look beyond the folk remedies to investigate phytoestrogens' possible roles in modern health care. Although the popular media has at times heralded phytoestrogens as panaceas, medical data remain inconclusive. Still, recent epidemiological studies and experiments with animals suggest many varied benefits of phytoestrogens. "Although much in-depth research has been done to identify and characterize the pharmacokinetics of certain phytoestrogens, the whole area of using phytoestrogens for medicinal purposes remains wide open," says Retha Newbold, a research biologist in the NIEHS Reproductive Toxicology Group.

Reports about environmental estrogens, or xenoestrogens, have been widespread in the last few years. There are important distinctions between estrogenic compounds of industrial origin and those that come from plants, however. Compounds such as the insecticide DDT and industrial PCBs have been implicated by some researchers in causing estrogen-dependent cancers in exposed populations. These same compounds have also been suggested as causes of declining sperm counts and other fertility problems in humans, as well as reproductive failures and anatomical abnormalities in wildlife. Unlike some industrial xenoestrogens, which tend to bioaccumulate in adipose tissue and persist in the body for years, phytoestrogens are readily metabolized and spend relatively little time in the body. However, during this time they can have significant effects on body systems. The timing of exposure, repeated exposures, and levels of exposure to phytoestrogens are important. "The issue is a lot more complicated than it looks on the surface," says Newbold. "Just because a substance like phytoestrogens is naturally produced doesn't make it automatically harmless or beneficial."

HOW THEY WORK

Extensive research has been done to identify the types of phytoestrogens that are found in humans and how they are metabolized. The two major classes of phytoestrogens that have captured the most scientific attention are lignans and isoflavones. Kenneth Setchell, associate professor of pediatrics at the University of Cincinnati Children's Hospital, began studying phytoestrogens in the early 1980s. For several years Setchell and his colleagues had been puzzled by unknown steroids in biological fluids. As part of a study on hormonal fluctuations in women, he began investigating urinary steroid hormone metabolites. A closer look at these compounds prompted speculation that they might be previously unidentified endogenous estrogens. Therefore, it came as a surprise when two independent

research groups identified the compounds as lignans, which weren't known to exist in humans. The two lignans, named enterolactone and enterodiol, are actually the products of microbial metabolism of secoisolariciresinol and matairesinol, compounds found in whole grains, fibers, and flax seeds, as well as several fruits and vegetables. Enterodiol may be further oxidized to enterolactone. All four of the lignans may be absorbed from the gut.

Isoflavones, which are abundant in legumes, have also been identified in human biological fluids. Within plant tissues, isoflavones exist as sugar derivatives called glycosides, and concentrations vary widely depending on stressors such as viral, bacterial, fungal, or herbivore attack. These compounds undergo hydrolysis in the human gut, yielding aglycones. Like the lignans, these aglycones meet one of three fates: they may be excreted or absorbed from the gut, or undergo further metabolism. The four most common isoflavones are formononetin, daidzein, genistein, and biochanin A. Biochanin A is metabolized to genistein. If it is not absorbed into the body, it may be further metabolized to p-ethylphenol, a hormonally-inert compound. Formononetin may be metabolized to daidzein, which is further metabolized mostly to equol, a more potent phytoestrogen, and to O-desmethyleriodiol.

Products of both lignan and isoflavone metabolism may be excreted or absorbed. If absorbed, the phytoestrogens undergo conjugation in the liver with glucuronic acid, or to a lesser extent, sulfate, and are excreted in the urine or in the bile. Some intestinal bacteria produce beta-glucuronidases, enzymes that can deconjugate phytoestrogen metabolites when they pass through the intestine, setting the stage for their recirculation through the body.

Nons teroidal plant estrogens were first identified in the early 1930s, with the discovery that soybeans, willows, dates, and pomegranates contain compounds with structural similarity to estrogens. It was unknown whether these compounds could have biological activity in animals until a concurrent discovery was made in phytoestrogens' effects on Australian sheep. Female sheep were plagued by reproductive system lesions and sharp declines in fertility. Animal husbandry experts linked the problem to the sheep's grazing on *Trifolium subterraneum*, a species of clover.

Researchers identified the clover compounds equol and coumestrol (another phytoestrogen) as being responsible for the sheep's reproductive problems. Once the etiology of clover disease had been established, scientists began to question whether these compounds also affected other species. Equol and other phytoestrogens such as enterolactone and enterodiol were discovered in human biological fluids in concentrations as much as 5,000 times greater than endogenous estrogens. The question then became whether these compounds presented a reproductive or other risk to humans or whether their presence might be in some way beneficial. "Unless you ask what is the quantitative risk [of phytoestrogens]," says Michael Boiger, a toxicologist at the FDA's Center for Food Safety and Applied Nutrition, "how can you integrate it with the benefits to get the full picture?"

P.05
64+9+4340567
04 APR - 98 11:38
000207

BENEFITS

Scientists have begun to piece together the full picture of phytoestrogens by looking at populations who consume them the most. Asian populations consume a diet that is very rich in the phytoestrogens genistein and daidzein, which are found in soybeans and soy products. These phytoestrogens occur at levels of 50-300 milligrams per 100 grams in soy beans, and in lower levels in soy products such as miso, soy milk, and tofu.

Asian populations also suffer a significantly lower rate of hormone-dependent cancers compared to westerners. They also have a much lower incidence of other hormonally-associated problems such as osteoporosis and menopausal symptoms. The presence of phytoestrogens in Asian diets and the comparatively low rates of diseases prevalent in western populations—including breast, endometrial, prostate, and colon cancers, as well as coronary heart disease—suggests that phytoestrogens may have protective effects.

Studies of immigrants have bolstered the argument that different disease rates between eastern and western populations may spring more from diet than from other factors such as genetics. Although the genetic link to several cancers has been well established, the genetic propensity to develop cancer does not vary significantly between eastern and western populations. For example, Japanese men develop small latent prostatic carcinomas at the same rate as western men, although their mortality from prostate cancer is much lower. However, Asian immigrants to western countries tend to alter their diets to the typical western diet that includes more protein and fat, less fiber, and fewer soy products. As their diets change, their risks for certain hormonally-related diseases increase.

These effects may be due to certain properties of phytoestrogens. For example, lignans are associated with the fiber portion of seeds and grains. Because fiber increases fecal bulk and decreases intestinal beta-glucuronidase levels, it reduces the circulation of conjugated estrogens in the liver and intestines. By indirectly reducing the amount of bioavailable hormone, fiber may reduce cancer risk.

Other research has focused on the effects of a lack of beneficial dietary factors rather than the presence of detrimental components. Herman Adlercreutz, professor and chairman of the Department of Clinical Chemistry at the University of Helsinki, reviewed phytoestrogens in articles in the October 1995 EHP Supplement and (with colleagues) the March 1995 supplement of the Journal of Nutrition. Adlercreutz presented evidence from many studies which shows that lignan and isoflavonoid excretion rates correlate with dietary and population groups. People who are a macrobiotic diet and vegetarians had significantly higher urinary excretion rates of lignans compared to meat eaters and subjects with breast cancer. Low urinary lignan values were also seen in Japanese men and women, consistent with their low consumption of whole grain products. This group's high consumption of soy products was reflected in their high urinary and plasma concentrations of isoflavonoids. Metabolites of phytoestrogens in biological fluids indicated that people who consumed phytoestrogens in their diets also metabolized and absorbed them. Results showing that variations exist among dietary and population groups with regard to plasma, urinary, and fecal concentrations of

estrogens and estrogen metabolites provide some evidence that phytoestrogens affect sex hormone metabolism. How these differences relate to disease rates is being investigated.

IN VITRO AND ANIMAL STUDIES

Many of the studies that provide evidence for the benefits of phytoestrogens were conducted for the purpose of investigating other endpoints and, therefore, are not accepted as definitive. "These studies add weight to the questions, but they don't answer them," cautions Daniel Sheehan, research biologist, at the National Center for Toxicological Research. Still, in vitro studies have served as a springboard for phytoestrogen research.

In vitro studies using radio-labeled estradiol have helped to demonstrate that phytoestrogens and endogenous estrogens have a common mechanism of action namely through the estrogen receptor. It has been shown that phytoestrogens can elicit both estrogenic and antiestrogenic responses.

According to Newbold, most researchers accept that phytoestrogens such as lignans and isoflavones are weakly estrogenic. For example, the equilibrium dissociation constant for genistein is 100 to 10,000 times greater than for estradiol or DES, which means that genistein's ability to stay bound to an estrogen receptor is less than one-hundredth that of the more potent estrogens. Furthermore, if genistein binds with an estrogen receptor, it elicits less than one-thousandth the response of an endogenous estrogen.

In vitro data have demonstrated that phytoestrogens may inhibit cell cancer growth. For example, using the MCF-7 human breast cancer cell line, which depends on estrogen for proliferation, researchers have shown that the lignan enterolactone inhibits proliferation in the presence of estradiol, a stronger estrogen. Alone, enterolactone stimulates proliferation. In vitro data have also demonstrated biological activity by phytoestrogens that is not associated with estrogenicity namely inhibition of protein tyrosine kinases, DNA topoisomerases, and angiogenesis by genistein. Of particular interest are reports of inhibition of protein tyrosine kinase, an enzyme associated with oncogene products of the retroviral src gene family. Such variety in biological action might explain why genistein is able to inhibit cancer cell growth in both the estrogen-dependent MCF-7 and estrogen-independent MDA-468 breast cancer cell lines. Other effects include stimulation of sex hormone-binding globulin (SHBG) synthesis and inhibition of aromatase, both of which indirectly affect the amount of free steroidal hormones in the body. Also, some evidence exists for inhibition of 17 α -hydroxysteroid oxidoreductase (type I), the enzyme responsible for reversible conversion of 17 α -estradiol to estrone. In addition to the inhibiting cancer cell growth, genistein has also been shown to induce differentiation of cells into mature phenotypes.

The in vitro studies have provided the basis for in vivo studies of phytoestrogens. Most such in vivo studies have been conducted in rats and mice, but some have been conducted on nonhuman primates. These studies highlight the difficulties in extrapolating from in vitro results to whole systems. For example, in a 1994 study in Anticancer Research, oncologist Harnesh R. Naik and colleagues at Wayne

The hypothesis that soy products could stimulate SHBG was proposed by Adlercreutz. It, however, has not been demonstrated to be the case (e.g. the "Cassidy" study).

State University School of Medicine compared in vitro and in vivo data on genistein's ability to affect hormone refractory prostate cancer. Although genistein was cytotoxic in both rat and human prostate cancer cell lines, it failed to inhibit proliferation of implanted prostate cells in rats.

EXPERIMENTAL STUDIES IN HUMANS

Researchers are becoming fascinated by phytoestrogens' potential as cancer preventatives and as nonpharmaceutical interventions for menopausal symptoms and osteoporosis. Current estrogen replacement therapies treat menopausal symptoms and may help to prevent health problems such as breast and endometrial cancers and osteoporosis. Many women are reluctant to take estrogenic drugs because of side effects such as resumption of menstrual bleeding, breast tenderness, and weight gain. The first studies in this area have focused on whether dietary amounts of phytoestrogens are sufficient to alter menstrual cycles. Aedin Cassidy and colleagues at the Dunn Clinical Nutrition Center in Cambridge, England, investigated the effect of a high soy-protein diet on hormonal status and the menstrual cycles of nine women between the ages of 21 and 29. In a study published in the September 1994 issue of the American Journal of Clinical Nutrition, they found that ingesting 60g of soy protein (equivalent to 45mg of isoflavones) daily for 1 month was sufficient to disrupt the menstrual cycle by increasing the length of the follicular phase and delaying the onset of menstruation. Flax seed, a rich source of lignans, has also been demonstrated to induce cycle changes.

Experiments with menopausal women have been less conclusive. Epidemiologist Donna Baird and colleagues at the NIEHS looked at a variety of measurements in an attempt to determine the estrogenicity of dietary soy in postmenopausal women in a study published in the May 1995 issue of the Journal of Clinical Endocrinology and Metabolism. After consuming a soy diet including 165 mg of isoflavones daily for one month, the subjects showed very little estrogenic response: levels of follicle-stimulating hormone, luteinizing hormone, and SHBG did not change significantly. There was a small effect on the maturation of the vaginal epithelium.

Setchell, who was involved with the study, speculated that more effect might have been shown if the experiment had lasted longer, citing an Australian study that ran for three months. This study, which appeared in the April 1995 issue of *Menopause*, was conducted by Alice L. Murkies, a doctor of medicine at the Brighton Medical Clinic in Victoria, Australia. Murkies and colleagues measured hot flashes as a determinant of estrogenic activity in response to phytoestrogen-rich diets. This experiment found significant reductions in the occurrence of hot flashes when the diets of postmenopausal women were supplemented with soy or wheat flours for 12 weeks. Of the test diets, soy seemed to yield the best results, but the authors did not indicate the amounts of actual phytoestrogens the test subjects consumed on a daily basis. Neither soy nor lignans have been examined for protective effects against osteoporosis. However, isoflavone, a phytoestrogen similar to genistein, has been shown to stimulate osteoblasts.

Human studies on hormones and diet may be confounded by a number of largely uncontrollable variables including genetics, individual intestinal flora, transit time

effects of medicines, health, and hormonal status. Many of the diseases being studied for phytoestrogenic effects are also multifactorial. Researchers have indicated that a prospective study is needed to tease out phytoestrogens' effects.

RISKS

Although studies on phytoestrogenic benefits seem promising, researchers have voiced concerns that consumption of large amounts of phytoestrogens may cause adverse health effects, especially with regard to development and fertility. "My position is that, while we are intrigued by the possible benefits, the fact of the matter is that no safety studies, especially with regard to development, have been done," says Sheehan. "Estrogens are clearly a two-edged sword in humans." Newbold also expresses concern that the effects of developmental exposure to phytoestrogens is simply not known. "We are not sure for adults if the 'natural' doses are helpful, or at least not harmful." Also, the possible interactions of phytoestrogens with established medical treatments such as estrogen replacement therapy or cancer therapy are unknown.

Neonatal and in utero exposures to sex steroids regulate the development of sexually differentiated behavior, reproductive physiology, and central nervous system anatomy and neurochemistry. In a letter published in the 24 May 1995 issue of the *New Zealand Medical Journal*, Cliff Irvine, a professor of animal and veterinary science, and colleagues at Lincoln University in New Zealand indicated that soy-based infant formulas in New Zealand contain 3-5 times as much daidzein and genistein as the amount that will disrupt a woman's menstrual cycle. Considering diethylstilbestrol's estrogenic effects on development, Irvine stated that this exposure should be investigated. Setchell says that infants metabolize the phytoestrogens, but how these compounds act in their bodies is unknown. One point of view is that they might negatively affect development, while others believe developmental effects would be negligible and that exposure might actually help ward off hormone-related ill health in the future.

Patricia Whitten, an associate professor of anthropology, and colleagues at Emory University have investigated possible developmental effects of coumestrol. Results of the study, published in the March 1995 supplement to the *Journal of Nutrition*, showed that in neonatal and immature rats, endogenous estrogens were low, and that coumestrol induced estrogenic responses, including premature estrous cycles. In normal adult female rats, coumestrol proved antagonistic to the endogenous estrogens and disrupted the ovarian cycle. Whitten and colleagues have also found that male and female neonatal rats exposed to coumestrol via their mothers' milk had altered numbers of progesterin receptors in the pituitary and hypothalamus, as well as altered sexual behavior and gonadotropin function. These results led researchers to conclude that coumestrol has a negative effect on neuroendocrine development. Although coumestrol has shown fertility and developmental effects, it's difficult to extrapolate these results to humans, whose exposure to coumestrol is dwarfed by their exposures to genistein, daidzein, and lignans.

Very little research has examined genistein's effect on development outcomes. Claude Hughes, now an associate professor of comparative medicine and obstetrics and gynecology at the Bowman Gray School of Medicine at Wake

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Forest University, examined neonatal exposure to genistein while working at Duke University Medical Center. Hughes and colleague Jill R. Levy in Duke's Department of Obstetrics and Gynecology found that exposure to genistein disrupted secretion of luteinizing hormone by the pituitary gland. In a recent paper published in the January 1995 issue of the Proceedings of the Society for Experimental Biology and Medicine, Levy and colleagues revealed that in utero exposure of rats to genistein may decrease markers such as birth weight and anogenital distance, and in female rats may delay the onset of puberty. Though these results do not confirm any risks for human infants, they do not discount them either.

Coral Lamartiniere, a professor of pharmacology and toxicology at the University of Alabama, and other researchers offer the argument that early exposure to genistein might actually be beneficial. In one study, published in the January 1995 issue of the Proceedings of the Society for Experimental Biology and Medicine, Lamartiniere and colleagues injected neonatal rats with genistein. Once the rats reached maturity, they were exposed to dimethylbenz[a]anthracene to induce mammary carcinogenesis. Rats that had been exposed to genistein as neonates had significantly increased latency in developing tumors, as well as reduced incidence of tumors. Lamartiniere said similar chemopreventive results were observed in rats exposed to genistein during puberty only. Despite lengthening their estrous cycles, their follicular development, sex steroid concentrations, and fertility seemed fine. "Common sense would tell us that soy does not pose a problem for fertility," said Setchell, pointing to the reproductive success of Asians. However, he added, that fact could be countered with other similarly logical arguments. One such argument, according to Hughes, is that Asians have been consuming these diets for centuries, and any soy-related fertility problems may have been selectively bred out of the population generations ago. In that case, westerners suddenly switching to a soy-based diet might not have the advantage of that natural selection. Sheehan also adds that, especially with developmental toxicants, there is a long latency period, which makes it difficult to associate an event with a negative outcome. "The fact that there aren't any negative reports can't be taken as an argument that soy diets are safe," he said.

The current knowledge base on phytoestrogens fuels speculation and arguments, but doesn't yield definitive answers. For all the varying opinions about phytoestrogens, however, researchers are united in the call for more definitive research. "What seems eye-opening to me is that we are looking at the development of a field," says Newbold. "It's just now come to the forefront."

Julia Barrett

National Institute of Environmental Health Sciences,
Research Triangle Park, North Carolina.

Last Update: May 22, 1996

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

27 January 1995

~~Martin J. Edwards~~
Food Administration
133 Molesworth Street
Wellington, NEW ZEALAND

Dear Dr. Edwards:

This letter is in response to your request of 20 December 1995 for information regarding toxicity of soybeans.

Soybeans and products made from soybeans have a long history of use as human foods and animal feeds in the United States. We are not aware of particular public health concerns in this country of such magnitude that would warrant regulatory action at this time. However, we are well aware that soybeans, like all other foods, can be toxic under particular circumstances. Any other food product which replaced soybeans in the diet could represent a public health concern under certain conditions.

The toxicity in birds mentioned in the material you sent, adds to a large body of literature on the subject of soybean toxicity. We have recently installed in Internet a large bibliography on poisonous plants, including many records dealing with soybeans and potentially toxic substances in them such as phytoestrogens. It can be accessed by Anonymous FTP `VAES.CFSAN.FDA.GOV`. A small readme file is called `ANON.FILES.PUBLIC.PLANT-TOX.README.TXT`. The large bibliography is `ANON.FILES.PUBLIC.PLANT-TOX.PLANT-TOX.REF`. Another access route is the World Wide Web. If you need further information please contact me by E-mail, `DJW@FDACFSAN.BITNET`.

More specific information about phytoestrogens can be obtained from an Internet file maintained by the National Center for Toxicologic Research (NCTR). It can be accessed through the NCTR Gopher or through World Wide Web.

Some general comments about estrogens we have made in response to a consumer inquiry may be useful for you. Estrogens have essential functions in control of reproduction and growth in humans and animals. An optimum estrogen level is needed at each stage of life to maintain good health. Either deficiency or excess can be associated with health problems. There are a large number of estrogenic compounds. The most important and some of the more potent estrogens are produced in our own bodies.

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A few very active compounds such as diethyl stilbesterol have been manmade. Several with marked therapeutic activity are used to treat certain clinical conditions.

Other compounds are industrial or natural contaminants which have variable estrogenic activity. When concentrated in the environment they may affect the health of wild or domestic animals. But they are present in the human diet at far lower concentrations than those estrogenic compounds produced naturally in our bodies or those which are used therapeutically.

The largest variety of estrogens are produced by plants. The most important dietary sources are legumes especially soybean products. Whole grains contain moderate levels but all types of food plants contain some. On the other hand, foods of animal origin (meat, milk, and eggs) are low in estrogens. But regardless of the type of foods eaten, it is virtually impossible to have a diet free of estrogens.

Sincerely yours,


D. Jesse Wagstaff, D.V.M., Ph.D., D.A.B.V.T.
Contaminants Standards Monitoring
and Programs Branch (HFS-308)
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
200 C Street S. W.
Washington, DC 20204

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**PLANT OESTROGENS AND THEIR RELATION TO
HORMONAL STATUS IN WOMEN**

A dissertation submitted to the University of Cambridge
for the degree of Doctor of Philosophy

by

AEDIN CASSIDY

Darwin College

October 1991

000212

TABLE 1.1 AGE SPECIFIC INCIDENCE RATES OF BREAST CANCER IN SEVERAL COUNTRIES (MUIR et al 1987)

Country	Age (Years)	
	40 - 44	65 - 69
	Incidence rate (per 100,000)	
Ireland	117.5	214.2
United Kingdom	94.0	199.4
Scotland	108.5	202.5
Canada	98.1	268.0
USA - Hawaii		
White	126.6	410.4
Japanese	120.7	178.1
Hawaiian	133.2	365.4
Filipino	62.0	101.6
Chinese	148.7	126.8
Japan		
Hiroshima	59.5	73.7
Nagasaki	53.7	52.3
China		
Hong Kong	44.3	117.4
Shanghai	41.9	60.6
India		
Bombay	31.7	81.7
Madras	43.4	77.8
	25.9	53.4

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The figures circled (which are about 65-69 year old women) are quoted in scientific papers to show that Japanese have low rates of breast cancer for pre-menopausal women. Asian societies do have high rates of rich men's (or women's) disease. Poor countries do not.

Look at Indian rates or the Middle East. Also these figures are taken from a book of statistics with almost 1,000 pages: Every page is filled with statistics. To quote just a few is to misrepresent the truth.

FOOD IN CHINESE CULTURE

ANTHROPOLOGICAL AND HISTORICAL PERSPECTIVES



EDITED BY K. C. CHANG

NEW HAVEN AND LONDON
YALE UNIVERSITY PRESS
1977

000214

Winter Wheat-Millet	21	17	63	5	23	7	2	*	—	—	—	—	—
Winter Wheat-Kaoliang	33	40	59	8	5	16	8	1	3	—	*	—	—
Rice Region Areas:													
Yangtze Rice-wheat	22	98	58	24	1	8	2	2	3	*	1	—	1
Rice-Tau	19	106	54	31	*	5	1	3	4	1	1	—	—
Szechwan Rice	6	165	48	37	2	3	7	1	1	—	1	—	—
Double Cropping Rice	11	102	42	22	*	3	7	6	8	6	2	4	—
Southwestern Rice	11	196	46	44	*	2	4	2	*	1	1	—	—

Actually Usage is low

Percentage of Calories Supplied by Different Important Staple Crops

17,351 persons, 2,727 families, 136 localities, 131 hsien, 21 provinces, China, 1929-1933

Regions and areas	Number of localities	Rice	Wheat	Millet	Kaoliang	Corn	Millet, proso	Potatoes, sweet	Barley	Soybeans	Green beans	Oats	Potatoes, Irish	Field peas	Rice, glutinous	Broad beans	Soybeans black	Other grains	Other legumes
China	136	35.0	14.4	10.4	7.5	7.5	2.8	2.6	2.0	2.0	1.2	1.2	0.9	0.8	0.7	0.6	0.6	0.2	1.3
Wheat Region	67	0.6	23.5	19.9	14.6	11.9	5.8	2.5	1.3	3.2	2.0	2.2	1.7	1.3	—	0.2	1.2	2.3	1.4
Rice Region	69	68.4	5.6	1.2	0.6	3.2	—	2.8	2.7	0.9	0.4	0.1	0.1	0.3	1.5	1.1	*	1.3	1.3
Wheat Region Areas																			
Spring Wheat	13	*	15.3	20.0	4.5	*	21.7	—	1.3	0.4	0.4	10.9	7.3	3.2	—	1.0	0.7	8.6	0.8
Winter Wheat-Millet	21	1.0	26.7	23.2	11.5	17.4	4.1	0.3	0.8	2.2	1.4	0.3	0.8	1.5	—	—	0.9	1.4	1.9
Winter Wheat-Kaoliang	33	0.6	24.7	17.9	20.5	13.0	0.5	4.9	1.7	4.9	3.0	—	*	0.5	—	—	1.6	0.5	1.2
Rice Region Areas:																			
Yangtze Rice-wheat	22	57.8	12.8	1.2	1.6	2.1	—	1.8	7.4	0.7	1.2	—	—	0.2	1.3	1.5	—	1.9	1.1
Rice-Tau	19	75.8	3.1	0.4	0.1	2.1	—	2.0	0.3	0.8	*	*	0.1	0.1	1.8	0.5	0.1	1.7	1.5
Szechwan Rice	6	56.9	5.3	—	0.1	14.0	—	3.1	0.3	1.9	0.3	1.5	0.1	2.2	0.8	1.2	—	0.7	1.2
Double Cropping Rice	11	76.9	0.5	0.1	—	—	—	8.2	1.2	0.6	0.1	—	—	*	0.6	0.1	—	0.1	1.5
Southwestern Rice	11	74.7	1.0	*	—	4.8	—	0.5	0.3	1.4	*	—	0.3	0.1	2.4	2.1	—	1.3	1.1

↑
protein

↑
Sauce

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OU



TO DR LINDA S KAHL
REGULATORY POLICY BRANCH H.F.S 206
FOOD & DRUG ADMINISTRATION
FAX (202) 418 3131

~~Robbins Kaplan Miller & Cress~~
RD 7
WHANGAREI NZ
APRIL 5 1998

Dear Dr Kahl,

G. R. N. # 000001
Soy Isoplaugen

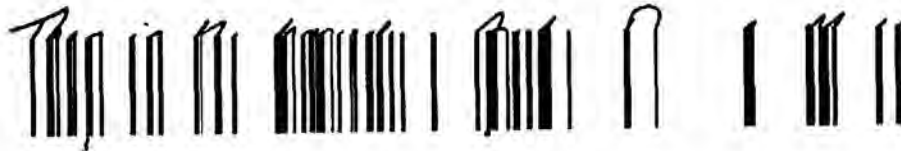
Could you please confirm by return fax whether the procedure on the following page is the one that is still in effect for normal G.R.A.S. application processing?

If not, please advise what the current normal procedure involves.

Sincerely

~~Robbins Kaplan Miller & Cress~~

Copied to Bruce Finzer esq
Robbins Kaplan Miller & Cress (612) 339 4181



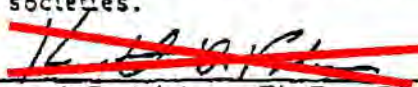
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NOTICE

This report is one of a series concerning the health aspects of using the Generally Recognized as Safe (GRAS) or prior sanctioned food substances as food ingredients, being made by the Federation of American Societies for Experimental Biology (FASEB) under contract no. 223-75-2004 with the Food and Drug Administration (FDA), U.S. Department of Health, Education, and Welfare. The Federation recognizes that the safety of GRAS substances is of national significance, and that its resources are particularly suited to marshalling the opinions of knowledgeable scientists to assist in these evaluations. The Life Sciences Research Office (LSRO), established by FASEB in 1962 to make scientific assessments in the biomedical sciences, is conducting these studies.

Qualified scientists were selected as consultants to review and evaluate the available information on each of the GRAS substances. These scientists, designated the Select Committee on GRAS Substances, were chosen for their experience and judgment with due consideration for balance and breadth in the appropriate professional disciplines. The Select Committee's evaluations are being made independently of FDA or any other group, governmental or nongovernmental. The Select Committee accepts responsibility for the content of each report. Members of the Select Committee who have contributed to this report are named in Section VII.

Tentative reports are made available to the public for review in the Office of the Hearing Clerk, Food and Drug Administration, after announcement in the Federal Register, and opportunity is provided for any interested person to appear before the Select Committee at a public hearing to make oral presentation of data, information, and views on the substances covered by the report. The data, information, and views presented at the hearing are considered by the Select Committee in reaching its final conclusions. Reports are approved by the Select Committee and the Director LSRO, and subsequently reviewed and approved by the LSRO Advisory Committee (which consists of representatives of each constituent society of FASEB) under authority delegated by the Executive Committee of the Federation Board. Upon completion of these review procedures the reports are approved and transmitted to FDA by the Executive Director of FASEB.

While this is a report of the Federation of American Societies for Experimental Biology, it does not necessarily reflect the opinion of all of the individual members of its constituent societies.


Kenneth D. Fisher, Ph.D., Director
Life Sciences Research Office
FASEB

000217

To: Linda Kahl@OPA@FDA.CFSAN
From: "Esther Fitzpatrick" <e.fitzpatrick@extra.co.nz>
Certify: N
Subject: Re: GRAS and soy isoflavones
Date: Tuesday, April 7, 1998 at 4:43:30 am EDT
Attached: None

OU



WARNING: this mail message was sent from a host for which the identity cannot be verified.
Hostname given was 'MajorD.xtra.co.nz' but is actually 'terminator.xtra.co.nz'

----- Original Message Follows -----
Dear Dr Kahl

I have read in Food Chemical News recently that ADM have made application to the FDA to have the soy isoflavones classified as GRAS in foodstuffs. I was quite amazed that Food Chemical News stated that ADM had determined that the soy isoflavones had been shown to be safe to humans and animals. In my opinion this is a grossly inaccurate.

Although recent research has shown that the soy isoflavones may reduce the risk of certain hormone-dependant diseases and may also have be beneficial in the prevention of bone loss, much research into the effects of isoflavones has shown quite conclusively their reproductive toxicity to a range of animals. These included rats, mice, pigs, cheetah, quail and fish. It is well established that the soy-isoflavones can modify the menstrual cycle of human females at moderately low doses. I am involved with current research that shows that similarly low doses to adult male humans reduces testosterone levels; this confirms the earlier work by L-J Lu.

Other researchers (at NCTR!) have shown that isoflavones have a marked impact on thyroid function. To any neutral person with a knowledge of endocrine disruptors it is clear that the soy isoflavones are still very much an unknown quantity in the human diet. It is beyond me how anyone can state with confidence that they are GRAS. I am particularly concerned about the very high doses of isoflavones that infants fed soy-infant formula receive and understand that this very issue is being closely followed by the EPA.

To classify soy isoflavones as GRAS would be premature. To respond to industry claims that soy isoflavones have part of the diet for thousands of years or that millions of babies have been fed soy-formulas without any negative effects (both claims are inaccurate) without first conducting a rigorous investigation of the claims, would be inconsistent with the duty that a regulatory organization, such as the FDA, has to

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the worldwide community.

I am not familiar with the GRAS process but I assume I am able to make a submission as per the usual Federal Register procedure. Could you please advise on the submission deadline and exactly where to direct the submission?

Yours faithfully

Mike Fitzpatrick PhD MNZIC
Environmental Scientist

mfitzpatrick@kma.co.nz or
e.fitzpatrick@xtra.co.nz

000220

To: ISMTP@FDA-OC-TRAINING@FDAOC[<e.fitzpatrick@extra.co.nz>]
From: Linda Kahl@OPA@FDA.CFSAN
Certify: N
Subject: re: Re: GRAS and soy isoflavones
Date: Tuesday, April 7, 1998 at 8:04:45 am EDT
Attached: None

Under sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act (the Act), a food ingredient is not subject to premarket approval if the ingredient is generally recognized, among experts qualified by training and experience to evaluate its safety, as having been adequately shown to be safe under the conditions of its intended use - i.e., to be GRAS. Under these provisions of the act, there is no requirement that a person who determines that a use of a substance is GRAS inform FDA of that determination or obtain FDA's concurrence.

Under the act, a substance may be GRAS through scientific procedures or, for substances used in food before passage of the 1958 Food Additives Amendment, through experience based on common use in food before January 1, 1958. Shortly after the 1958 amendment, FDA assembled a partial list of substances that were known to be commonly used in food prior to 1958. This was the first GRAS list and is now in Part 182 of our regulations. In the 1970's, FDA began a scientific safety review of all substances on this "history of use" GRAS list. If, following completion of its review of a particular substance, FDA affirmed that the use of the substance is GRAS the agency deleted the listing in Part 182 and added a new listing in Part 184 - for "affirmed GRAS" substances. FDA went through rulemaking to establish the procedures that the agency would use to conduct this review. Because not all substances used in food on the basis of the GRAS exemption were on the agency's GRAS list, FDA also established a voluntary process, the GRAS petition process, whereby persons who had made their own GRAS determination could request that FDA affirm that determination and list the affirmed use in Part 184. That procedure involved "notice and comment rulemaking," in which FDA conducts a pre-filing review to see if the petition meets certain format requirements, publishes a filing notice in the Federal Register, and in that filing notice requests comment on the proposed use. If FDA affirms the proposed use as GRAS, the agency publishes a final rule in the Federal Register and includes in that document a discussion of comments received.

In the Federal Register of April 17, 1997, FDA published a proposed rule that would eliminate the voluntary GRAS petition process. In its place, FDA proposed to establish a voluntary notification procedure whereby any person may notify FDA of a GRAS determination. In so doing, FDA stated that a goal of this process was to increase the agency's knowledge of substances that are being added to food based on the GRAS exemption.

000221

Under the proposed notification procedure, FDA would not affirm the notifier's GRAS determination and therefore FDA would not itself "classify"

the use of the substance as GRAS. The procedure does not involve notice-and-comment rulemaking because the endpoint of the proposed notification procedure is not a regulation. Instead, the endpoint would be a letter from FDA to the notifier. This letter could identify a problem with the notifier's GRAS determination; however, a letter that does not identify a problem with the notice would not provide an affirmative statement that FDA agreed with the notifier.

Although that proposed rule has not become final, FDA invited interested parties to participate in the notification procedure during the interim between the proposed and final rules. The subject Archer Daniels Midland (ADM) submission is a notice to FDA that ADM has determined that the use of soy isoflavones as a micronutrient in food is GRAS. FDA is evaluating ADM's notice to determine whether it provides a sufficient basis for ADM's GRAS determination. Because the procedure does not involve rulemaking, there is no formal mechanism for submission of "comments" within the meaning of "notice and comment rulemaking." Nonetheless, FDA welcomes fact-based information on food safety issues. Accordingly, you may submit such information to the Office of Premarket Approval (HFS-200), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 200 C Street SW, Washington, DC 20204 and request that it be considered with respect to GRAS Notice Number GRN 000001.

The April 17, 1997, proposed rule is available electronically on CFSAN's home page at <http://vm.cfsan.fda.gov>, within the section regarding Food Additives and Premarket Approval, Documents for Industry. Please note that the proposal to establish the notification procedure is in fact notice and comment rulemaking; the comment period closed on July 16, 1997. However, GRAS notices sent to the agency under the auspices of the proposed program are NOT notice-and-comment rulemaking.

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C St., SW, Washington DC 20204
Phone: (202)418-3101 Fax: (202)418-3131
Internet: LKAHL@BANGATE.FDA.GOV

000222

OU



~~R. F. Jones~~

R. J. 4

WHITING/ARE1

1998 APR -8 A 8:15 APRIL 8 1998

Dr Linda S Kahl,
Office of Pre Market Approval
Food and Drug Administration

Fax 001 (202) 418-3131

Dear Dr Kahl

Thank you for your facsimile of 7 APRIL,
received here this morning, on general matters
relating to G. R. A. S.

We will take time to evaluate the situation.

But at first flush, I personally wonder how
G. R. A. S. status for soy protein isolate was ever
granted in 1979. The work of Carter and
Madhore in 1957, 1958 and 1986 in North
Carolina quite clearly showed reproductive system
damage in mice by Genistein isolated from
soybean, and by the beans as a dietary
component.

Why on earth allow it into the human diet?
And especially into infant foods

And a quick glance at the reference list
I gave the Director of Pre Market Approvals
raises the whole question of product safety.

Sincerely

~~R. F. Jones~~

000227

OU



~~W. E. R. Jones~~

R. D. 4

WINDHURST

APRIL 10 1998

Dr Linda Kahl
Office of Pre market Approval
F. D. A

FAX (202) 418 3131

Dear Dr Kahl

G.R.N 000001

We have your E-Mail to Yvonne Clapherton.
The mail delivery will be satisfactory, thank
you. We can collect hard copies from her office

I'm putting copies of the following papers
in the mail to you
"Anti Thyroid Isoflavones From Soybean"
and "Abnormal Thyroid Function Tests in Infants"

It is already Good Friday here. Even in the
outer world environment of N.Z., we're regarded as
off-the-universe-bonies, so it might take a
while for the mail to arrive. It's a 4 day
holiday weekend here

Sincerely

~~R. D. Jones~~

000229

OU



DR LINDA S KAHL
Regulation Policy Branch
F. D. A
(202) 418 3131

~~Mr. & Mrs. [unclear]~~
R.D. 4
WINDYBARKI
APRIL 10 1998

Dear Dr Kahl

C. R. N. 000001
I have collected the hardcopies of the A.D.M
submission from Yvonne Clapperton

Many thanks indeed for your prompt response

It's what's not said that will get you I'll
give A.D.M the credit of knowing it's our business
as well as I do

At the sake of brevity, I'm sending you now
the front pages of 3 papers, all of which make it
clear there is a 50 year history of thyroid damage
The one from N.C.J.R. also demonstrates that
the isoflavones are carcinogens.

Sincerely,

~~Dr. [unclear]~~

1998 APR 13 A 9:56

000231

Clinical and Laboratory Pearl

Abnormal Thyroid Function Tests in Infants with Congenital Hypothyroidism: The Influence of Soy-Based Formula

Muhammad A. Jabbar, MD; Jennifer Larrea, RN; and Renee A. Shaw, BS

Department of Pediatrics (M.A.J., J.L.) and the Department of Research (R.A.S.), Hurley Medical Center, Flint, Michigan

Key words: soy formula, thyroxine, thyrotropin, hypothyroidism

Objective: To assess the etiology of hyperthyroxinemia or hyperthyrotropinemia in infants with congenital hypothyroidism who are on replacement therapy with L-thyroxine.

Methods: These infants were treated with recommended doses of L-thyroxine following the diagnosis of congenital hypothyroidism. Because of hyperthyroxinemia (2 patients) and hyperthyrotropinemia (1 patient), medication compliance and dietary practices (formula type, age of introduction, and discontinuation or change of the formula) were assessed. Clinical evaluation was also performed.

Results: Elevated thyroxine level in 2 infants was associated with discontinuation of soy formula 4 weeks previously; reduction of L-thyroxine dose normalized serum levels in both of these infants. In the third infant, who received soy formula from 1 week of age, TSH remained elevated despite incremental L-thyroxine doses of 19 µg/kg/day; discontinuation of soy formula was followed by normalization of the TSH in 3 weeks and helped attain a subsequent decrement of L-thyroxine dose to 8.6 µg/kg/day. Neither the hyperthyroxinemia nor hyperthyrotropinemia in these infants was associated with any adverse behavioral-developmental consequences.

Conclusion: When initiating soy formula feeding in infants with congenital hypothyroidism, the L-thyroxine dose should be increased because of significant reduction in intestinal absorption; conversely, when soy feeding is discontinued, the L-thyroxine dose should be decreased.

INTRODUCTION

Infants with congenital hypothyroidism require adequate thyroxine replacement therapy as a prerequisite for a good outcome. In infants and young children, overtreatment with thyroid preparations may result in enhanced linear growth, poor weight gain, and behavioral changes. On the other hand, undertreatment of congenital hypothyroidism may lead to poor linear growth, excessive weight gain, and impaired developmental function. While the daily dose requirement and adequacy of treatment primarily depend on the severity of the disease and patient compliance, dietary constituents may affect the various indices of thyroid function [1-3].

Among the dietary factors, soy protein is important for two reasons. First, it has been reported to cause goiter and hypothyroidism in clinical and animal studies [4-5]. Second, soy-based formulas are often chosen by parents and pediatricians

for feeding of infants with presumed "milk allergies" [5]. In this report, we describe a sequential relationship between soy formula feeding and abnormalities in thyroid function tests in three infants with congenital hypothyroidism. The clinical course is also described. We conclude that soy formula feeding increases the thyroxine dose requirement, while a change from soy to non-soy formula reduces the thyroxine dose requirement.

METHODS

All infants with congenital hypothyroidism were followed monthly for 3 months and twice monthly thereafter, unless an abnormality was detected. This follow-up included clinical and laboratory studies. Because of elevated thyroxine (T_4) or thyrotropin (TSH) level, we analyzed the L-thyroxine dose and

compliance, type of feeding, and the clinical course of three infants with congenital hypothyroidism.

Born at full-term, none of these infants had a maternal history of thyroid disease before or during pregnancy. The diagnosis of congenital hypothyroidism was suspected from abnormal newborn screening (TSH level >20 µU/L) and was later confirmed by clinical or laboratory tests. Confirmatory T_4 (µg/dL) and TSH (µU/L) levels, respectively, in these infants were: 6.5 and 100 (patient 1), 1.5 and 200 (patient 2), and 6.3 and 77 (patient 3). During the initial evaluation, imaging studies with technetium scans showed hypoplasia in all three infants. These studies excluded thyroid agenesis or dysmorphogenesis. All of the infants were given L-thyroxine (Synthroid, Knoll Pharmaceuticals, Mount Olive, NJ; half-life = 6 to 7 days) as replacement therapy. The dosage (µg/kg/day) was calculated at the time of initiation and follow-up visits. Compliance to the medication and adherence to the specified dose was also determined during these visits. Serum T_4 and TSH levels were measured by the radioimmunoassay laboratory of our institution.

RESULTS

T_4 and TSH Levels

Abnormal serum T_4 and TSH levels in relation to the change of formula are shown in Table 1. Of a total of 22 infants and children who were receiving L-thyroxine replacement therapy, two infants (patients 1 and 2) had elevated total and free T_4 levels. Parental compliance to the recommended medication dose and schedule was adequate. However, dietary history revealed that feeding was switched from soy formula (Nusoy-3) to cow's milk formula 4 weeks prior to laboratory tests, accounting for elevated serum thyroxine levels. Decreasing the L-thyroxine dose (from 9.2 µg/kg/day to 7.4 µg/kg/day in patient 1 and from 9.3 to 7.6 µg/kg/day in patient 2) normalized serum thyroxine levels in 3 weeks. Serum thyroxine levels were within high normal limits in both these infants prior to soy formula discontinuation.

One infant (patient 3, Table 1) had elevated TSH (with normal total T_4) that failed to normalize until 5 months of age. This infant, who was fed soy formula (Isomil®) from 1 week of age, was started on L-thyroxine (8 µg/kg/day) at 2 weeks of

Soy Formula and Congenital Hypothyroidism

age. This dose, started on an empiric basis, was within the recommended guidelines and was similar to the dose given to other infants with congenital hypothyroidism at that time. Thyroxine remained high despite a gradual increase in L-thyroxine dose to 19 µg/kg/day during the next 4 months. At 5 months of age, feeding was changed from soy formula to cow's milk formula, which normalized TSH level. Subsequently, L-thyroxine dose was decreased to 14 µg/kg/day, and TSH level remained normal during the follow-up.

Nineteen of our 22 patients (including infants and older children) who received L-thyroxine replacement therapy during this time had no abnormalities in their T_4 and TSH levels during follow-up. None of these patients received soy formula during infancy.

Feeding History:

These three infants with thyroid function abnormalities were fed soy formula because of parental concern about "milk allergies." Soy formula was started at 1 month, 2 weeks and 1 week of age and was continued until 6 months, 2.5 months, and 5 months of age, respectively. At that time, soy formula was gradually discontinued and cow's milk formula and supplements were introduced. None of the three infants had any obvious behavioral/clinical alterations attributable to high or low thyroxine levels. However, long-term, comprehensive follow-up data is not available.

The timing of soy introduction related to thyroid replacement therapy was also reviewed in these patients. In patient 1, introduction of soy feeding was preceded by initiation of thyroid replacement therapy; in patients 2 and 3, it was vice versa. In patients 1 and 2, the serum T_4 and TSH normalized within 4 weeks of start of therapy, while in patient 3, TSH was not normalized until soy feeding was discontinued 5 months later. This suggests that thyroid function test abnormalities were unrelated to the timing of soy feeding introduction.

DISCUSSION

The effect of soy formula feeding (or its withdrawal) on serum T_4 and TSH level is shown in three congenitally hypothyroid infants who were on replacement therapy. The introduction of soy formula feeding increases the dose requirement,

Table Thyroid Function Abnormalities and Altered L-Thyroxine Dose Requirement During and After Discontinuation of Soy Feeding

Patient number	TFT abnormalities & relation to soy feeding	Age of soy introduction (weeks)	Age of soy discontinuation (months)	Maximum dose while on soy (µg/kg/day)	Decrement of dose after soy discontinuation
1	$T_4 > 20$ µg/dL after soy was discontinued	8	6	9.2	25%
2	$T_4 = 18.2$ µg/dL after soy was discontinued	2	2.5	9.3	18%
3	TSH = 25 µU/L during soy feeding	1	5	19	25%

TFT = thyroid function test.

Address reprint requests to Muhammad A. Jabbar, MD, Department of Pediatrics, Hurley Medical Center, Flint, MI 48906.

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Anti-Thyroid Isoflavones from Soybean

ISOLATION, CHARACTERIZATION, AND MECHANISMS OF ACTION

Rao L. Ditt, Hebron C. Chang and Daniel R. Doerge*

NATIONAL CENTER FOR TOXICOLOGICAL RESEARCH, JEFFERSON, AR 72079, U.S.A.

ABSTRACT. The soybean has been implicated in diet-induced goiter by many studies. The extensive consumption of soy products in infant formulas and in vegetarian diets makes it essential to define the goitrogenic potential. In this report, it was observed that an acidic methanolic extract of soybeans contains compounds that inhibit thyroid peroxidase (TPO)-catalyzed reactions essential to thyroid hormone synthesis. Analysis of the soybean extract using HPLC, UV-VIS spectrophotometry, and LC-MS led to identification of the isoflavones genistein and daidzein as major components by direct comparison with authentic standard reference isoflavones. HPLC fractionation and enzymatic assay of the soybean extract showed that the components responsible for inhibition of TPO-catalyzed reactions coeluted with daidzein and genistein. In the presence of iodide ion, genistein and daidzein blocked TPO-catalyzed tyrosine iodination by acting as alternate substrates, yielding mono-, di-, and triiodoisoflavones. Genistein also inhibited thyroxine synthesis using iodinated casein or human goiter thyroglobulin as substrates for the coupling reaction. Incubation of either isoflavone with TPO in the presence of H_2O_2 caused irreversible inactivation of the enzyme, however, the presence of iodide ion in the incubations completely abolished the inactivation. The IC_{50} values for inhibition of TPO-catalyzed reactions by genistein and daidzein were ca. 1–10 μ M, concentrations that approach the total isoflavone levels (ca. 1 μ M) previously measured in plasma from humans consuming soy products. Because inhibition of thyroid hormone synthesis can induce goiter and thyroid neoplasia in rodents, delineation of anti-thyroid mechanisms for soy isoflavones may be important for extrapolating goitrogenic hazards identified in chronic rodent bioassays to humans consuming soy products. *BIOCHEM PHARMACOL* 54:10:1087–1096, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. thyroid peroxidase; soybean; isoflavonoid; inhibitor; goitrogen; mechanism

The soybean and its products have been considered goitrogenic in humans and animals. Goiter and hypothyroidism were reported in infants receiving soy-containing formula [1–4], and such feedings in early life have also been associated with the development of autoimmune thyroid disorders [5]. Several investigators have reported induction of goiter in iodine-deficient rats maintained on a soybean diet [6–11]. Furthermore, Kimura *et al.* [9] reported the induction of thyroid carcinoma in rats fed an iodine-deficient diet containing 40% defatted soybean. Konijn *et al.* [10] showed that the anti-thyroid activity present in acidic acetone soybean extracts is water soluble, is dialyzable, and is not precipitated by either ammonium sulfate or trichloroacetic acid. The active ingredient was characterized partially by these workers as a small molecular compound of non-peptide origin, since it was not destroyed by either digestion with pancreatin or by boiling for 2 hr.

The function of the thyroid is synthesis of thyroid hormones, and TPO catalyzes iodination of tyrosyl residues on Tg and the subsequent coupling of iodotyrosyl residues required for iodothyronine hormone formation.

Inhibition of TPO-catalyzed reactions results in decreased levels of circulating thyroid hormones, which lead to increased secretion of TSH by the anterior pituitary. The increased levels of TSH provide a growth stimulus to the thyroid, and it has been proposed that a prolonged stimulus can select for clones of follicular cells with the potential for transformation [12]. This mechanism predicts that any compound that inhibits TPO mediated thyroid hormone synthesis is a potential thyroid carcinogen.

The widespread use of soy products in infant food formulas and the significant consumption of soy products by people consuming a vegetarian diet require a closer evaluation and examination of the anti-thyroid activity of the soybean. This is important because of the current promotion of soy-based products as healthy foods possessing putative beneficial estrogenic and anti-carcinogenic properties. For example, genistein, but not daidzein, inhibits tyrosine kinase activity, and this property has been explored for

*Corresponding author. Tel. (501) 543-7943; FAX (501) 543-7770.
Index to FDA Response to NRDC's FOIA Request No. 2013-8042 for GRN-1
Received 24 February 1997; accepted 23 May 1997.

Abbreviations: APCI, atmospheric pressure chemical ionization; C₆P, cytochrome c peroxidase; CID, collision induced dissociation; DIT, 3,5-diiodotyrosine; I-PC₃, isopropylcarbamate; MIT, 3-iodo-L-tyrosine; PEG, polyethylene glycol; rT₄, 3,3',5'-triiodo-L-tyrosine; L₄, thyroxine; T₄, 3,5,3'-triiodo-L-tyrosine; Tg, thyroglobulin; TPO, thyroid peroxidase; TSH, thyroid stimulating hormone; and MFS, 2,4,6-trimethyl-5-methyl-4-pyridylmethyl sulfonate.

000233

Soyabean, Goitre, and Prevention

By Theodore Kay*, Miesko Kimura, Koroake Nishino, and Yoshinori Itokawa
Department of Hygiene, Faculty of Medicine, Kyoto University, Kyoto, 606, Japan

Introduction

Thyroid enlargement in rats and humans, especially children and women, fed with soyabeans has been known for half a century. However, this thyroid enlargement can be reversed to normal by increasing the daily intake of dietary iodine.¹⁻³

Soyabeans have been the main protein source for vegetarian Buddhist monks and nuns in East Asia, particularly in Japan. However, almost no case of endemic goitre has been reported in Japan other than some cases of coast goitre on the northern coast of Japan due to the over-consumption of iodine rich seaweeds there. Iodine-rich seaweed is a part of daily diet in Japan. The daily urinary excretion of iodine in these who regularly consume such seaweed has been between 1 and 3 mg (per person).⁴

Recently, soyabeans have been promoted as a daily staple food in many parts of the developing world. Soyabeans have been proved to be one of the best and the cheapest protein sources and the problems such as the elimination of beany taste and flatus factors have been fully solved.⁵⁻⁷ In West Africa, more than a half of the daily foods there can be prepared from or mixed with soyabeans with improved palatability and without passing through sophisticated industrial processes.⁸ However, because most of these areas do not have iodine-rich foods, such as seaweeds, endemic goitre, due to the increased consumption of soyabeans, has been expected because soyabeans contain goitrogens. So the necessity of iodine supplementation has been emphasized.

In general iodine supplementation has been achieved by popularizing iodized salt, by evenly mixing potassium iodide (in dry regions) or potassium

iodate (in humid regions) into table salt, or injecting intramuscularly iodized oil, 1-2 ml, once in every 3-5 years.⁹ However, the minimum safety iodine requirement or the appropriate amount of iodine requirement for a fixed amount of soyabeans have never been well studied. This study is an attempt to suggest the appropriate method of determining the minimum safety amount of daily iodine intake for humans in those areas where soyabeans have been popularized.

Materials and methods

Experimental animals

Male rats of Wistar strain weighing between 40 and 50 g were used. The rats were divided into groups of five animals, each fed with the experimental diets for 3 months. Distilled water and the experimental rations were fed *ad libitum*. The composition of the experimental ration is shown in Table 1. The soyabean flour was added in place of protein in the basal diet.

Heading measurement

The heading measurements were as follows: thyroid gland weight (mg per 100 g body weight), thyroxine levels of sera (T_4) ($\mu\text{g/dl}$) were determined by utilizing the thyroxine measurement kit purchased from Wako Company of Chemicals, Osaka, Japan. For histological observation, the thyroid was fixed in 10 per cent neutral formaldehyde solution, was mounted in paraffin, stained with haematoxylin-eosin and examined microscopically.

Preparation of pre-cooked soyabean flour

Production of pre-cooked soyabean flour, free of beany taste and flatus factors is as follows:

1. Soak dry soyabeans in plenty of fresh water* overnight (about 12 hours).
2. Place overnight-soaked beans in a pot about half-full and cover them with the same amount of fresh water.
3. Heat to boil and keep simmering for a half hour.
4. Remove the pot from fire and leave to cool; drain the water to separate beans. Rinse the cooked beans once with fresh water.
5. Dry the cooked beans in the oven at 82°C. (In the tropics it should be dried in the sun.)
6. Grind the dry beans into a flour.

* De ionized water was utilized as fresh water

TABLE 1
Composition of low iodine basal diet (1000 g)

	Soyabean containing diets			
	Control A	B	C	D
Sucrose (g)	353	366	366	366
Starch (g)	300	—	—	—
Soyabean flour (g)	—	36	36	263
Citric acid (g)	150	—	—	—
Soyabean oil (g)	24	24	24	24
Cellulose (g)	20	—	—	—
Salt mixture (g) (iodine free)	40	40	40	40
Vitamin mixture (g)	5	5	5	5
Choline chloride (g)	2	2	2	2
Iodine (g) (in potassium iodide)	1.36	0	1.36	272

TABLE 2
Levels of thyroid and thyroxine

Groups	Body weight (g)	Iodine supplement (g/100 g diet)	Thyroid (mg/100 g body weight)	Thyroxine ($\mu\text{g/dl}$) (normal value 2.9-6.1 $\mu\text{g/dl}$)
A (control)	361 ± 45	13.6	10.5 ± 2.2	5.25 ± 1.90
B (soyabean containing diet)	375 ± 38	0.0	10.86 ± 11.2	1.86 ± 0.50
C (soyabean containing diet)	388 ± 49	13.6	11.6 ± 0.7	5.12 ± 1.10
D (soyabean containing diet)	345 ± 48	27.2	11.50 ± 1.10	4.06 ± 1.10

The main composition of this soyabean flour is as follows:

Dry soya- beans	Water	Protein (g/100g)	Sugar	Cellu- lose	Other
100 (g)	12.2	41.4	21.0	13.4	5.5
363 (g)	44.29	150.28	76.23	48.6	20.0

The iodine free salt mixture was prepared according to the Jonsson salt mixture formula excluding potassium iodide. This iodine-free salt mixture formula is described in the following:

NaCl	243.2
K ₂ C ₂ H ₃ O ₄ · H ₂ O	513.0
CaHPO ₄ · 2H ₂ O	800.0
CaCl ₂	168.0
MgCO ₃	92.0
FeC ₂ H ₃ O ₄ · 3H ₂ O	76.0
MnSO ₄	2.8
CuSO ₄ · 5H ₂ O	0.4
CoCl ₂ · 6H ₂ O	0.2
K ₂ Al ₂ (SO ₄) ₃ · 24H ₂ O	0.2
ZnCl ₂	0.1
NaI	0.002

Results

The effect of iodine supplement in diets based on thyroid weight, goitre was prevented easily



Fig. 1. Histology of the thyroid of a rat fed with control diet for 3 months. Morphologically thyroid gland is normal. Each follicle contains a low cuboidal and follicles contain colloid 14 & 20 x 10. The same applies to the other four rats.

when iodine was added to the diet. All animals on the iodine-supplemented diet showed normal thyroxine levels in sera. The results of this experiment are summarized in Table 2. However, histological examinations indicated that the development of goitre in the rats fed with soyabeans was not completely prevented by such an addition of iodine (see Figs 1-4) although

The first author, Theodore Kay, is very grateful to Dr and Mrs (Dr) Shozo Tateuchi, Dr Shin-ichiro Shino, their colleagues and friends of Kyoto Municipal Hospital, Dr Junji Konoishi and his colleagues of the Radiation and Nuclear Medicine Department, Professor Mitsuo Inada of the Second Department of Internal Medicine (Endocrinology), Mr Hiroshi Morino of the Hygiene Department, and all other members of the Hygiene and Public Health Departments of Kyoto University Faculty of Medicine who have provided invaluable assistance and encouragement.

* Present address: Department of Chemistry, National Taiwan College of Education, Chang-Hua, Taiwan 300, R.O.C.

000234

To: Linda Kahl@OPA@FDA.CFSAN
From: "Ian & Yvonne Clapperton" <safetywize@clear.net.nz>
Certify: N
Subject: RE: RE: Electronic copy of ADM GRAS notice
Date: Saturday, April 11, 1998 at 7:55:27 pm EDT
Attached: attach1

OU



everything worked fine thanks - Dick James is now in receipt of the copy
and very busy!

From: ~~William T. [redacted]~~, R.D. Whangarei, Fax 66-4-4340564
April 10th, 1998.

To: Dr. L. Kahl { Premarket Approval Office, Center for Food Safety and Applied
Nutrition, Food and Drug Administration,
200 C Street, S.W. HFS 206, Washington, D.C. 20204

OU



Re: GRAs Notice # GRN 000001.

Dear Dr. Kahl,

Thank you for your information. In further considerations of GRN 000001 occur, I recommend that you obtain a copy of "Phytoestrogens in the Human Diet" a 170 page report and review of literature commissioned by the U.K. Ministry of Agriculture, Fisheries, and Food. This work which considered only potentially beneficial effects of phytoestrogen administration was completed in Nov. 1997. I am sure that the British Embassy officials would willingly provide you with a copy of this authoritative work.

The conclusions were (p. 7, # 7) "Though some epidemiological studies suggest that consumption of foods may have beneficial effects, almost no evidence exists to link these effects directly to phytoestrogens." Among the scientific literature considered by the reviewers were many published papers by Prof. H. Adlercreutz.

That brief was only to consider literature concerned with positive effects of phytoestrogen administration but the introduction warns (p. 11) that, "clearly in any risk of phytoestrogens in the human diet it is essential to balance beneficial effects against potential adverse effects."

Currently the U.K. MAFF and Department of Health have established a "Joint Food Safety and Standards Group". Their research programme is yielding important information, including the above report. Recent personal communication from them (March 27 1998) states, "we hope that the results of these projects together with those of other workers in the field will help resolve the questions regarding both potential beneficial and potential adverse effects of phytoestrogens"

Yours sincerely,

000236



Institute for Environment
and Health

IEH assessment on

PHYTOESTROGENS IN THE HUMAN DIET

FINAL REPORT TO THE
MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

November 1997

000237

The Institute for Environment and Health (IEH) was established by the Medical Research Council at the University of Leicester in 1993. The Institute is partly funded by the various UK government departments and agencies by way of specific research and consultancy contracts.

This literature review^{*} has been prepared by IEH for the Ministry of Agriculture, Fisheries and Food. The principal focus of this document is on the potential beneficial effects of phytoestrogens on adults. Potential detrimental effects on adults and the influence on other life stages were specifically excluded from consideration. It also contains an assessment of the factors influencing the phytoestrogen content of food and the relative potencies of the various phytoestrogens. The assessment incorporates the output of a workshop held in Leicester in March 1997 which was chaired by Professor Lewis Smith, IEH. The Institute gratefully acknowledges the contribution of all those who attended the workshop and provided material for inclusion in the assessment but assumes no endorsement from these scientists for the conclusions and recommendations contained herein.

The Ministry of Agriculture, Fisheries and Food has provided funding for this project but has not conducted the research or written this report. The views expressed here do not necessarily represent those of any government department or agency.

Prepared by:

Dr Charles Humfrey and Mr Philip Holmes, IEH

^{*} Literature search to November 96; supplemented by additional papers to June 97

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FINAL REPORT TO MAFF

List of Participants

PHYTOESTROGENS IN THE HUMAN DIET

WORKSHOP HELD AT LEICESTER, UK ON 21 MARCH 1997

Members

Professor M Ashwell, Ashwell Associates, Ashwell St, Ashwell, Herts, SG7 5PZ

Dr S Bingham, Dunn Clinical Nutrition Centre, Hills Road, Cambridge CB2 2DH

Mr N Bundred, Department of Surgery, University Hospital of South Manchester, Nell Lane, West Didsbury, Manchester M20 8LR

Dr A Collins, Rowett Research Institute, Green Burn Road, Bucksburn, Aberdeen AB21 9SB

Dr D Ferry, Department of Clinical Oncology, University of Birmingham, Queen Elisabeth Hospital, Birmingham B15 9TH

Professor A Gescher, MRC Toxicology Unit, Hodgkin Building, University of Leicester

Dr M Joffe, Department of Epidemiology and Public Health, Imperial College of Medicine at St. Mary's, Norfolk Place, London W2 1PG

Dr H Makin, Department of Clinical Biochemistry, St. Bartholomew's and The Royal London School of Medicine and Dentistry, Queen Mary and Westfield College, Turner St, London E1 2AD

Professor C Rice-Evans, Division of Biochemistry and Molecular Biology, UMDS Guy's Hospital, London SE1 9RT

Professor K Setchell, Clinical Mass Spectrometry, Department of Paediatrics, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, Ohio 45229, USA

Dr A Wilkinson, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA

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FINAL REPORT TO MAFF

Representatives

Dr D Aldridge, Department of Health, Room 651c Skipton House, 80 London Road, Elephant and Castle, London SE1

Dr R Burt Food Contaminants Division, MAFF, Room 231, Ergon House, c/o Nobel House, 17 Smith Square, London SW1P 3JR

Dr L. Smith, Department of the Environment, Transport and the Regions, Room, A3141 Romney House, 43 Marsham Street, London SW1P 3PY

Dr C Tahourdin, Food Contaminants Division, MAFF, Room 209, Ergon House, c/o Nobel House, 17 Smith Square, London SW1P 3JR

Dr A Zampelas, Chief Scientist's Group (Food and Veterinary Science Division), MAFF, Room 666, St Christopher House, Southwark Street, London SE1 0UD

IEH Secretariat

Professor L Smith (Chairman)

Dr C Humfrey

Mr P Holmes

Dr P Harrison

000240

FINAL REPORT TO MAFF

References

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FINAL REPORT TO MAFF

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OU



Dr Linda S Kahl
Office of Pre Market Approval
Food & Drug Administration

FAX (202) 418 3131

~~Dr. T. Jones~~
A.D.M.
WANNAREI
N.Z.
APRIL 11 1998

Dear Dr Kahl,

I may have missed a trick - I don't believe I have actually objected to the fast track C.R.A.S experimental proposed procedure.

The A.D.M. case illustrates my point - a radical doctoring of the food supply requires careful consideration, and open and full discussion. Submissions must be invited and the process should be transparent to all. That is what democracy is all about.

The current attempt to contaminate the food chain with potential carcinogens is profane.

G R N 000001

I am sure you will eventually receive voluminous comment on the A.D.M submission. Apart from the total omission to reveal any of the voluminous reports of thyroid damage and potential carcinogenesis, the submission does not mention three epidemiologies showing high levels of autoimmune diseases and hormonal damage in children who were fed soy formulas as infants.

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Worse, it misrepresents existing literature in a way that is almost fraudulent. At this time three examples will suffice.

① On page 14, they allege that "Eastern" (whatever that means) diets contain 10 times higher levels. Yet based on Japanese Ministry of Health figures, per head consumption is only 8g a day. This in fact is much

lower than the quantities shown to disrupt hormones of adult women (Cassidy et al: 45 mg, Petrankis: 38 mg)

② Reference 49 is represented as evidence of non-toxicity. I follow with the front page of that Lancet paper. Judge for yourself.

③ At page 32 you quote an organization called the New Zealand Nutrition Foundation. This organization is the mouthpiece for the food industry, of which at least three infant formula/food manufacturers are prominent members. To my knowledge, its opinion has never been peer reviewed or published and its authorship is not revealed.

Perhaps you should demand a copy of it.

I think you will hear more about that document in the future.

The best calculation for the quantities of soy solids we can get is around 12-15 gr/day in the New Zealand diet, and the advent of soy as a local ingredient here was about 1985. One could more easily attribute the fact that New Zealand is around top in the world in asthma and diabetes to the advent of soy, than to credit it for any advantages in "Eastern" diets. The lowest rate of breast and prostate cancers is in the near East and the Arab states, where soy is unknown, and rice is the common factor. And, of course, low fat and high fiber. We have one paper here which insists prostate cancer rates are the same in Japan. The mortality is attributed to other causes.

~~Sincerely,
[Signature]~~

000244

VIS-a-VIS Ref 49

Early

Exposure of Infants to phyto-oestrogens from soy-based Infant formula

Kenneth D R Satchell, Linda Zimmer-Nechemias, Jinnan Cai, James E Heubi

Summary

Background The isoflavones genistein, daidzein, and their glycosides, found in high concentrations in soybeans and soy-protein foods, may have beneficial effects in the prevention or treatment of many hormone-dependent diseases. Because these bioactive phyto-oestrogens possess a wide range of hormonal and non-hormonal activities, it has been suggested that adverse effects may occur in infants fed soy-based formulas.

Methods To evaluate the extent of infant exposure to phyto-oestrogens from soy formula, the isoflavone composition of 25 randomly selected samples from five major brands of commercially available soy-based infant formulas were analysed, and the plasma concentrations of genistein and daidzein, and the intestinally derived metabolite, equol, were compared in 4-month-old infants fed exclusively soy-based infant formula ($n=7$), cow-milk formula ($n=7$), or human breast-milk ($n=7$).

Findings All of the soy formulas contained mainly glycosides of genistein and daidzein, and the total isoflavone content was similar among the five formulas analysed and was related to the proportion of soy isolate used in their manufacture. From the concentrations of isoflavones in these formulas (means 32–47 $\mu\text{g/mL}$), the typical daily volume of milk consumed, and average body-weight, a 4-month-old infant fed soy formula would be exposed to 28–47 μg per day, or about 4.5–8.0 mg/kg body-weight per day, of total isoflavones. Mean (SD) plasma concentrations of genistein and daidzein in the seven infants fed soy-based formulas were 684 (443) ng/mL and 295 (80) ng/mL , respectively, which was significantly greater ($P<0.05$) than in the infants fed either cow-milk formulas (3.2 (0.7) and 2.1 (0.3) ng/mL), or human breast-milk (2.6 (0.7) and 1.4 (0.1) ng/mL), and an order of magnitude higher per bodyweight than typical plasma concentrations of adults consuming soy foods.

Interpretation The daily exposure of infants to isoflavones in soy infant-formulas is 8–11 fold higher on a bodyweight basis than the dose that has hormonal effects in adults consuming soy foods. Circulating concentrations of isoflavones in the seven infants fed soy-based formula were 13 000–20 000 times higher than plasma oestradiol concentrations in early life, and may be sufficient to exert biological effects, whereas the contribution of isoflavones from breast-milk and cow-milk is negligible.

Introduction

More than a decade after attention was first drawn to the levels of phyto-oestrogens in soy infant-formulas,¹ concerns are being expressed about the possibility of hormonal effect from exposure of infants to phyto-oestrogens from soy-based infant formulas.² These concerns have prompted at least one government agency to issue statements and recommendations about the use of soy-based infant formulas in early life.³

The phyto-oestrogens in all soy-protein foods belong to the isoflavone class.⁴ With few exceptions all soy-protein products and soybeans are rich in isoflavones.^{5,6} Variation in amount of isoflavones in different soy foods is accounted for mainly by the differences in industrial processing of the soybean, and the type and extent of incorporation of the soy protein into the food matrix.⁷ Isoflavones when ingested are metabolised extensively in the intestinal tract, absorbed, transported to the liver, and undergo enterohepatic recycling.^{8,9} Intestinal bacterial glucosidases cleave the sugar moieties and release the biologically active isoflavones, daidzein and genistein, and in the adult these can be further biotransformed by bacteria to the specific metabolites, equol,¹⁰ desmethylequol,¹¹ and p-couplediphenol.¹² All of these phyto-oestrogens are then eliminated, mainly by the kidney, and therefore share the physiological features and behaviour of endogenous oestrogens.¹³

In addition to acting as oestrogen mimics,¹⁴ isoflavones have important non-hormonal activities. Genistein, for example, is a potent inhibitor of tyrosine kinases¹⁵ and interferes with cell signal-transduction pathways.¹⁶ The ingestion of high concentration of phyto-oestrogens has adversely affected reproduction in several animal species,^{17,18} and in premenopausal women daily ingestion of soy protein lengthens the menstrual cycle and suppresses the usual mid-cycle surge in pituitary gonadotropins,¹⁹ effects that epidemiological evidence suggests are beneficial in decreasing risk of breast cancer. The hypocholesterolaemic action of soy protein is well established and anticancer actions of soy isoflavones have been shown in in-vitro studies and in several classic animal models of chemically-induced breast cancer.²⁰

Although urinary analyses have indicated that isoflavones are absorbed by the infant fed soy-based infant formula, data on the composition of phyto-oestrogens in infant formulas are scant and the level of exposure of the infant fed soy-based formula to phyto-

oestrogens is uncertain. We now describe a

000245



Pergamon

Food and Chemical Toxicology 34 (1996) 457-461



VISA
P. 14

Quantification of Genistein and Genistin in Soybeans and Soybean Products

M. FUKUTAKE^{*†}, M. TAKAHASHI^{*}, K. ISHIDA^{*}, H. KAWAMURA[†],
T. SUGIMURA^{*} and K. WAKABAYASHI^{*‡}

^{*}Biochemistry Division, National Cancer Center Research Institute, 1-1 Tsukiji, 5-chome, Chuo-ku, Tokyo 104 and [†]Tsumura & Co., 3586 Yoshiwara, Ami-cho, Inashiki-gun, Ibaraki 300-11, Japan

(Accepted 12 January 1996)

Abstract—It has been suggested that the isoflavone, genistein, may have some role as a chemopreventive agent against cancer in humans. Levels of genistein and its β -glucoside conjugate, genistin, ingested in soybeans and related bean products by the Japanese were quantified by HPLC, to estimate daily intake of these compounds. Amounts of genistein and genistin in soybeans, soy nuts and soy powder were in the range of 4.6 to 18.2 and 200.6 to 968.1 $\mu\text{g/g}$ food, respectively. The values for soy milk and tofu (bean curd) were 1.9 to 13.9 and 94.8 to 137.7 $\mu\text{g/g}$ food, respectively. Levels of isoflavones in fermented soybean products, miso (bean paste) and natto (fermented soybeans), were 38.5 to 229.1 $\mu\text{g/g}$ food for genistein and 71.7 to 492.8 $\mu\text{g/g}$ food for genistin. Thus, the level of genistein in the fermented soybean products was higher than in soy beans and soybean products such as soy milk and tofu. From these observations, it is suggested that the β -glycosyl bond of genistin is cleaved to produce genistein by microbes during fermentation to yield miso and natto. Soy sauce was also found to contain both isoflavones, but at levels lower than in miso and natto. On the basis of these data for average annual consumption of soybeans and related products, daily intake of genistein and genistin by the Japanese is calculated to be 1.5–4.1 and 6.3–8.3 mg/person, respectively. These levels are much higher than those for Americans or Western Europeans, whose mortality rates for breast, colon and prostate cancers are greater than the Japanese. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

Currently, the search for chemopreventive agents acting against human cancer development has become an important high-priority subject. Ideally, such agents should be administrable safely over long periods. From this point of view, food components taken in daily life are good candidates for this purpose. It is known that food components, including α - and β -carotenes (Malone, 1991; Murakoshi *et al.*, 1992; Santamaria and Bianchi, 1989), vitamins (Halter, 1989; Lippman *et al.*, 1989; Smith *et al.*, 1975), omega-3 fatty acids (Karmali, 1989; Narisawa *et al.*, 1991; Takahashi *et al.*, 1993 and 1994) and tea polyphenols (Narisawa and Fukaura, 1993; Wang *et al.*, 1992 and 1994; Yamane *et al.*, 1995; Yoshizawa *et al.*, 1987), can suppress tumorigenesis in rodents. However, it has not as yet been fully elucidated whether these compounds actually serve as chemopreventive agents in man.

One isoflavone compound, genistein, which is present in soybeans has been found to inhibit development of aberrant crypt foci, considered to be preneoplastic lesions in the colon of rats (Pereira *et al.*, 1994), as well as the growth of human breast

and prostate cancer cell lines (Barnes *et al.*, 1994; Peterson and Barnes, 1991 and 1993). The plasma concentrations of isoflavone derivatives, including genistein, in Japanese men are known to be seven to 110 times higher than those in Finnish men (Adlercreutz *et al.*, 1993), suggesting that the large intake of soybean products may be related to the low mortality from prostate cancer in Japan (Yatani *et al.*, 1982). Epidemiological data are also available showing a positive association between high intake of soybean products and a low risk of cancer in other organs, such as the breast and colon (Adlercreutz *et al.*, 1991; Messina *et al.*, 1994; Nomura *et al.*, 1978). From these observations, genistein might play some role as a chemopreventive agent in man. By contrast, it has been reported that several dietary factors, for example fat and meat, are involved in increasing the risk of cancer in the prostate, breast and colon in man (Armstrong and Doll, 1975; Carroll and Khor, 1975).

Japanese people frequently consume soybeans and soybean products in daily life. Although the amounts of genistein and its β -glucoside conjugate, genistin, in soybean food in the US were recently documented (Coward *et al.*, 1993), detailed quantification data for these compounds in Japanese foodstuffs are lacking. In the present study, the levels of genistein and genistin in soybeans and soybean products ingested by the Japanese, were analysed and daily intake

[‡]Author for correspondence.

To: Dr. Linda Kahl, Office of Premarket Approval, Center for
Food Safety and Applied Nutrition, Food and Drug Administration
200 C Street, S.W. (H.F.S. - 206) Washington D.C.
20204.

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At: Facsimile 001-202-418-3131

1998 APR 16 A 9:13

Re: GRAS Notice on Phytoestrogens, # GRN 000001

From: ~~Delose A. Jones~~ R.D.4 whiggore.

Date, April 16th 1998.

Dear Dr. Kahl,

Thank you for arranging for the transcript of the
"GRAS Notification for Isoflavones Derived from Soybeans"
by the Archer Daniel Midland Company, to be sent to New
Zealand. Since that report, which apparently makes a
considered evaluation of the benefits of phytoestrogen consumption,
contains many mistakes of fact including the misrepresentation
of a number of published documents, I shall submit a detailed
critique in the future should that become necessary.

However, please consider the following:—

- ① "The essential justification for the use of an additive in
food processing - or of a pesticide that leaves a toxic
residue in foods - is some direct or indirect benefit to
the consumer." P. 586, "Toxicants occurring naturally in foods"
Nat. Acad. Sciences, Washington D.C. in chapter entitled, "Toxicology
of Natural Food Chemicals: A perspective by J.H. Conner."

Please note in this respect:—

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- ① (a) "Final Report to HAF" P. 7, # 7, states "Though some
epidemiological studies suggest that consumption of foods
containing phytoestrogens may have beneficial effects, almost

2.

No evidence exists to link these effects directly to phytoestrogens"

①(b) " — and —

Dietary Genistein Exerts Estrogenic Effects upon the uterus, Mammary Gland and the Hypothalamic/Pituitary Axis in Rats" Santol et al, American Soc. for Nutritional Sciences 1996, p 203, " This raises some concern with regard to mammary tumorigenesis, which initially requires estrogen, because these studies have demonstrated that dietary genistein has estrogenic effects in the mammary gland of ovariectomized rodents. Further research is required... " " Genistein is receiving much attention as a potential chemopreventative/therapeutic agent in the treatment and/or prevention of various cancers. The results of these studies demonstrate the need for additional experiments on the biological effects of dietary genistein, particularly in tumor models, before any dietary recommendations can be made or supported "

— and —

①(c) "Isoflavone Content of Breast Milk and Soy Formulas: Benefits and Risks", David H. Sheehan, Nat. Center for Toxic Res. USFDA, Clinical Chemistry, p 43, No 5, 1997 000248

" Estrogens are two-edged swords in human health, both risks and benefits can be demonstrated in the same person... As the editorials point out, the beneficial effects of soy-based formulas or of milk from mothers consuming phytoestrogens is speculative. The same is true for potential risks... while metabolism and disposition data are important in both animals and humans, another crucial need is to define appropriate animal models and to explore phytoestrogen effects in these models — and —

①(d) "Estrogenic Soybean Isoflavones and Chronic Disease Risks" [unclear] et al, Environ Health Persp 1995 p 11

3.

" Potential risks ~~or~~ benefits of phytoestrogens depend not only on dose and potency but also on duration ~~and~~ pattern of exposure. Further, risk assessment must be considered relative to the developmental phase or life stage of the individual at the time of exposure."

- and -

① (e) "Anti-Thyroid Isoflavones from Soybean, isolation, characterization and mechanisms of action" Devi. et al,

Elsevier Science Inc. 1997 p 1086.

" The widespread use of soy products in infant food formulas and the significant consumption of soy products by people consuming a vegetarian diet require closer evaluation and examination of the anti-thyroid activity of the soybean. This is important because of the current promotion of soy-based products in health foods possessing putative beneficial estrogenic and anticarcinogenic ~~effects~~ properties."

② The submission of A.O.M. defines isoflavones as "micro-nutrients". They are not. The definition of a nutrient (Webster's dictionary) is "furnishing nourishment". Isoflavones do not furnish nourishment. Rather, since 1931 when isoflavones were first isolated from soybeans (Waltz), they have always traditionally been described as toxicants.

Therefore, since there are no demonstrated benefits for isoflavone administration and they are not nutrients, the A.O.M. submission is out of order. An appropriate submission by them could be for consideration as a medicine, but not as a food or food additive.

Please do keep me informed.

Yours sincerely,

000249

OU



Dr Linda Kahl
Office of Pre-Market Approvals,
Food & Drug Administration

FAX 001(202)418 3131

~~R. F. Tarnet~~
R.D. 4
WILMANIAREI
N. 2

April 17 1998
64 9 4340567

Dear Dr Kahl,

GRN # 000001

Mary Hodge, at your F.O.I.A. office, most graciously included the Review by Adlercreutz with papers she sent by mail.

It is weird that A.D.M. uses this as a centerpiece for their "determination". I am forwarding back the final "Conclusions" page, which is essentially the same as the British Ministry of Agriculture Fish and Food reached: there are no proven benefits to these chemicals. And quite likely, adverse effects.

Surely the F.D.A. cannot rely on this to proceed with a monumental change to the food chain?

A further point of procedure: since there is no history of isoflavones as a "nutrient," how can A.D.M. ask for them to be designated as such?

Sincerely

~~R. F. Tarnet~~

1998 APR 17 A 8:41

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BEST ORIGINAL COPY

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Adlercreutz • Mazur

Bone metabolism was studied in rats and it was shown that a low dose (1 mg/day) but not a high dose (2 or 10 mg/day) of genistein was equivalent to conjugated equine oestrogens (5 µg/day) in maintaining bone mass in ovariectomized rats (347). Another study in this field showed that 10 g of soy protein containing 10 mg of genistein twice daily reduced hypo-oestrogenic symptoms ($P < 0.004$ vs. placebo) in post- and perimenopausal women and had a borderline significant effect on general symptoms ($P < 0.07$) and sleep quality ($P < 0.1$). Serum alkaline phosphatase decreased ($P < 0.03$) suggesting an effect on the bones. Coumestrol in an experimental system both inhibits bone resorption and stimulates bone mineralization (348). Furthermore, genistein inhibited avian osteoclastic activity and reduced bone loss in ovariectomized rats (349). Soybean protein administered to rats prevented bone loss, but it was not established whether this protective effect on bone was due to the protein itself or to the presence of isoflavones in soybean protein (350). These studies suggest a possible beneficial effect of this isoflavonoid in prevention of osteoporosis.

In conclusion, there is a great interest among women to try to treat their menopausal symptoms by natural food products instead of using synthetic drugs containing oestradiol or oestrol, or equine oestrogens extracted from horse urine. In particular, subjects with treated breast cancer present a problem that has to be solved. In addition, to alleviate the symptoms, the food should have a protective effect with regard to coronary heart disease and osteoporosis. Climacteric symptoms are relatively rare in China and Japan (343, 351) and the incidence of hip fractures is also lower than in the USA and Europe (352). However, Japanese women have lower femoral neck bone mass but they still have substantially lower incidence of hip fractures. This seems to be due to shorter femoral necks and to a lesser degree, to a smaller femoral angle (353). Osteoporosis is now becoming a serious problem in the Japanese society and it starts already in the early postmenopausal period (354). This may be due to a shift from traditional diet to a more westernized one resulting in a reduction of isoflavonoid-rich food. One important observation seems to be the fact that the effects on menopausal symptoms, osteoporosis and plasma lipids occur with moderate amounts of isoflavonoids achievable by changing the normal diet and that high amounts may not have any effect.

Possible Cancer-stimulating Effects

There is no evidence in the literature suggesting that phyto-oestrogens, present in such amounts in human food that they could have biological effects, stimulate already existing cancer, and there is also no evidence that such phyto-oestrogens could initiate cancer. The high plasma levels in Japanese subjects having low breast, prostate and colon cancer risk would also suggest that soy consumption is not associated with any risk. However, we have to remember that the

Japanese have traditionally consumed a very low-fat diet and their endogenous oestrogen levels tend to be lower than those in Western populations (see above). The combination of high phyto-oestrogen intake with a Western diet may not be beneficial. On the other hand, it seems that phyto-oestrogens tend to act as anti-oestrogens when oestrogen levels are high and as oestrogens when the levels are low.

That xeno-oestrogens may cause breast cancer is still an open question (355, 356) and Safe in his review (356) summarizes that 'results would suggest that the linkage between dietary or environmental oestrogenic compounds and breast cancer has not been made'.

Conclusions

Despite an already abundant literature at this early stage of dietary phyto-oestrogen research it seems that much work is needed before any recommendation as to phyto-oestrogen consumption can be made. However, the experimental and epidemiological evidence obtained strongly supports the view that these compounds do not have any negative effects and that they may form a group of substances with great potentialities in preventive medicine. However, at present no definite recommendations can be made as to the dietary amounts needed for prevention of disease.

The research carried out in our laboratory since 1979 was initially supported by the Medical Research Council of the Academy of Finland and the Sigrid Jusélius Foundation, Helsinki and later by the National Institutes of Health grants 1 R01 CA56289-01 and 2 R01 CA56289-04, and by a grant from the Nordic Industrial Foundation, by EC contract FAIR-CT95-0894, and by grants from the King Gustav VII and Queen Victoria's Foundation, Sweden and the Finnish Cancer Foundations.

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OU



D^r Linda Kahl
Office of Premarket Approval
W.F.D.A.

~~R. F. JAMES~~
R.D. +
LUHMANAREI NZ
18 APRIL 1998
64.9.4340567

FAX 001(202) 418 3131

Dear D^r Kahl
G.R.N # 000001

I have only just examined the A.D.M.
"determination" regarding "Saponins" (references
35(a) 35(b) 35(c))

It is very curious that A.D.M. do not cite
the most significant review that exists, nor do they
disclose the numerous accounts of deleterious
effects associated with consumption of saponins,
especially the soya saponins.

You should obtain a copy. The reference is
"THE CHEMISTRY & BIOLOGICAL SIGNIFICANCE OF
SAPONINS IN FOOD AND FEEDING STUFFS": PRICE, JOHNSON,
FENWICK; IN "CRITICAL REVIEWS IN FOOD SCIENCE &
NUTRITION" 26, 1, 1987 pp 27-129

The failure of A.D.M. to mention this monumental
work smacks of a deliberate intention to deceive

I understand that Professor J. E. Lienes of
the University of Minnesota Biochemistry Department
has chaired two conferences on the anti-nutritional
factors in soya, and has edited the two Conference
books.

Why not consult him?

000252



DEPARTMENT OF HEALTH & HUMAN SERVICES

Phone: (501) 543-7000
(FTS) (501) 543-7000

Public Health Service
Food and Drug Administration

National Center
For Toxicological Research
3900 NCTR Road
Jefferson AR 72079-9502

OU



Daniel M. Sheehan, Ph.D.
Director, Estrogen Base Program
Division of Genetic and Reproductive Toxicology
and
Dan Doerge, Ph.D.
Division of Chemistry

April 22, 1998

Linda Kahl
Division of Product Policy
Mail Code HFS-205
FDA
200 "C" St, SW
Washington, DC 20204

Dear Dr. Kahl,

We are writing in reference to the application for GRAS status for isoflavones, such as genistein, by ADM. We oppose GRAS status because there is abundant evidence that some of the isoflavones, including genistein and equol, are toxicants. This is true for a number of species, including humans. Additionally, the adverse effects in humans occur in several tissues and, apparently, by several mechanisms.

Genistein is clearly estrogenic; it possesses the chemical structural features necessary for estrogenic activity (Miksicek, 1998; Sheehan and Medlock, 1995; Tong, et al, 1997) and induces estrogenic responses in developing and adult animals and in adult humans. In rodents, equol is estrogenic and acts as an estrogenic endocrine disruptor during development (Medlock, et al, 1995a,b). Faber and Hughes (1993) showed alterations in LH regulation following developmental treatment with genistein. Thus, consumption of isoflavones during pregnancy in humans could be a risk factor for abnormal brain and reproductive tract development. In adults, genistein could be a risk factor for a number of estrogen-associated diseases.

Additionally, isoflavones are inhibitors of the thyroid peroxidase which makes T3 and T4. Inhibition can be expected to generate thyroid abnormalities, including goiter and autoimmune thyroiditis. There exists a significant body of animal data that demonstrates goitrogenic and even carcinogenic effects of soy products (cf., Kimura et al., 1976). Moreover, there are significant reports of goitrogenic effects from soy consumption in human infants (cf., Van Wyk et al., 1959; Hydovitz, 1960; Shepard et al., 1960; Pinchera et al., 1965; Chorazy et al., 1995) and adults

(McCarrison, 1933; Ishizuki, et al., 1991). Recently, we have identified genistein and daidzein as the goitrogenic isoflavonoid components of soy and defined the mechanisms for inhibition of thyroid peroxidase (TPO)-catalyzed thyroid hormone synthesis in vitro (Divi et al., 1997; Divi et al., 1996). The observed suicide inactivation of TPO by isoflavones, through covalent binding to TPO, raises the possibility of neoantigen formation and because anti-TPO is the principal autoantibody present in autoimmune thyroid disease, this hypothetical mechanism is consistent with the reports of Fort et al. (1986, 1990) of a doubling of risk for autoimmune thyroiditis in children who had received soy formulas as infants compared to infants receiving other forms of milk.

The thyroid findings in infants receiving soy formula are a result of serum levels of isoflavones that are about five times higher than in women receiving soy supplements who show menstrual cycle disturbances, including an increased estradiol level in the follicular phase (Setchell, et al, 1997). Assuming a dose-dependent risk, it is unreasonable to assert that the infant findings are irrelevant to adults who may consume smaller amounts of isoflavones. Additionally, while there is an unambiguous biological effect on menstrual cycle length (Cassidy, et al, 1994), it is unclear whether the soy effects are beneficial or adverse. Furthermore, we need to be concerned about transplacental passage of isoflavones as the DES case has shown us that estrogens can pass the placenta. No such studies have been conducted with genistein in humans or primates. As all estrogens which have been studied carefully in human populations are two-edged swords in humans (Sheehan and Medlock, 1995; Sheehan, 1997), with both beneficial and adverse effects resulting from the administration of the same estrogen, it is likely that the same characteristic is shared by the isoflavones. The animal data is also consistent with adverse effects in humans.

Finally, initial data from a robust (7,000 men) long-term (30+ years) prospective epidemiological study in Hawaii showed that Alzheimer disease prevalence in the Hawaii men was similar to European-ancestry Americans and to Japanese (White, et al, 1996a). In contrast, vascular dementia prevalence is similar in Hawaii and Japan and both are higher than in European-ancestry Americans. This suggests that common ancestry or environmental factors in Japan and Hawaii are responsible for the higher prevalence of vascular dementia in these locations. Subsequently, this same group showed a significant dose-dependent risk (up to 2.4 fold) for development of vascular dementia and brain atrophy from consumption of tofu, a soy product rich in isoflavones (White, et al, 1996b). This finding is consistent with the environmental causation suggested from the earlier analysis, and provides evidence that soy (tofu) phytoestrogens causes vascular dementia. Given that estrogens are important for maintenance of brain function in women; that the male brain contains aromatase, the enzyme that converts testosterone to estradiol; and that isoflavones inhibit this enzymatic activity (Irvine, 1998), there is a mechanistic basis for the human findings. Given the great difficulty in discerning the relationship between exposures and long latency adverse effects in the human population (Sheehan, 1998), and the potential mechanistic explanation for the epidemiological findings, this is an important study. It is one of the more robust, well-designed prospective epidemiological studies generally available. We rarely have such power in human studies, as well as a potential mechanism, and thus the results should be interpreted in this context.

Does the Asian experience provide us with reassurance that isoflavones are safe? A review of several examples lead to the conclusion "Given the parallels with herbal medicines with respect to attitudes, monitoring deficiencies, and the general difficulty of detecting toxicities with long latencies, I am unconvinced that the long history of apparent safe use of soy products can provide confidence that they are indeed without risk" (Sheehan, 1998).

Taken together, the findings presented here are self-consistent and demonstrate that genistein and other isoflavones have adverse effects in a variety of species, including humans. Animal studies are the front line in evaluating toxicity, as they predict, with good accuracy, adverse effects in humans. For the isoflavones, we additionally have evidence of two types of adverse effects in humans, despite the very few studies that have addressed this subject. While isoflavones may have beneficial effects at some ages or circumstances, this cannot be assumed to be true at all ages. Isoflavones are like other estrogens in that they are two-edged swords, conferring both benefits and risk (Sheehan and Medlock, 1995; Sheehan, 1997). As the benefits are not under consideration, the addition of isoflavones to foods needs to be considered just as would the addition of any estrogen or goitrogen to foods, which are bad ideas.

Finally, NCTR is currently conducting a long-term multigeneration study of genistein administered in feed to rats. The dose range-finding studies were just completed. As preliminary data, which is still confidential, may be relevant to your decision, I suggest you contact Dr. Barry Delclos at the address on the letterhead, call him at 870-543-7372, or email him at <bdelclos@nctr.fda.gov>.

Sincerely,

~~Daniel M. Sheehan~~
Daniel M. Sheehan

~~Daniel R. Doerge (by JMS)~~
Daniel R. Doerge

Enclosures

cc: Dr. Bernard Schwetz, Director, NCTR
Dr. Barry Delclos

TABLE 4

Urinary excretion of isoflavones during the period of dietary intervention with soy protein¹

Subject	Daidzein	Genistein	Equol	Total isoflavone excretion	Percent of isoflavones excreted ²
	$\mu\text{mol/d}$	$\mu\text{mol/d}$	$\mu\text{mol/d}$	$\mu\text{mol/d}$	%
1	0.79 \pm 0.79 (0.24–2.76) ¹	0.74 \pm 0.74 (0.12–2.96)	12.81 \pm 4.55 (6.61–23.14)	13.33 \pm 4.71 (7.45–22.7)	7.6
2	0.79 \pm 0.39 (0.28–1.18)	0.3 \pm 0.15 (0.11–0.37)	6.61 \pm 3.30 (2.89–12.0)	8.2 \pm 3.14 (3.92–12.54)	4.7
3	15.35 \pm 3.93 (11.8–24.0)	8.51 \pm 2.96 (5.55–13.33)	0.01 \pm 0.01 (0.001–0.41)	22.74 \pm 4.70 (17.64–29.41)	12.9
4	2.76 \pm 1.97 (1.18–7.09)	0.30 \pm 0.15 (0.07–0.70)	0.02 \pm 0.02 (0.01–0.04)	3.14 \pm 1.96 (1.57–7.05)	1.8
5	10.63 \pm 8.27 (2.76–26.77)	1.48 \pm 1.18 (0.30–3.33)	1.24 \pm 0.82 (0.12–2.48)	12.16 \pm 7.06 (3.14–24.31)	6.9
6	13.0 \pm 5.12 (7.48–24.41)	2.59 \pm 1.48 (0.33–4.44)	ND	17.25 \pm 1.96 (16.07–18.82)	9.8

¹ Urinary isoflavone excretion during the control period ranged from 5.6 to 67.3 nmol/d. ND, not detected.² Expressed as % of total daily dietary isoflavone intake (44.93 \pm 0.21 mg/d).³ \bar{x} \pm SD, range in parentheses.

spectively). Figure 1 illustrates mean plasma concentrations of LH and FSH for the 4 d either side of ovulation, where day 0 is assigned the day of ovulation. As a group there was a threefold reduction in peak plasma LH concentration and twofold reduction in FSH during the period that soy protein was ingested.

TABLE 5

Plasma hormone concentrations during the control period and during intervention with soy protein¹

	Control diet	Soy diet
Progesterone		
Luteal phase		
(nmol/L)	21.4 \pm 6.0	18.4 \pm 4.8
(ng/mL)	6.72 \pm 1.89	5.79 \pm 1.50
Estradiol		
Follicular phase		
(pmol/L)	246.2 \pm 38.4	362.5 \pm 82.0 ²
(pg/mL)	66.96 \pm 10.44	98.60 \pm 22.31 ²
Midcycle		
(pmol/L)	520.6 \pm 153.5	572.0 \pm 180.20
(pg/mL)	141.61 \pm 41.75	155.72 \pm 49.1
Luteal phase		
(pmol/L)	416.5 \pm 110.6	415.0 \pm 170.3
(pg/mL)	113.30 \pm 37.08	112.93 \pm 46.33
SHBG (nmol/L)		
Follicular phase	58.57 \pm 13.61	57.54 \pm 9.39
Luteal phase	58.33 \pm 1.25	55.32 \pm 9.96
Entire menstrual cycle	58.48 \pm 14.6	56.98 \pm 9.3
Testosterone		
Entire menstrual cycle		
(nmol/L)	1.25 \pm 0.38	1.46 \pm 0.52
(ng/mL)	0.36 \pm 0.11	0.42 \pm 0.15
Cholesterol (mmol/L)		
Entire menstrual cycle	4.27 \pm 1.08	3.86 \pm 1.01 ¹

¹ \bar{x} \pm SD. SHBG, sex-hormone-binding globulin.² ¹ Significantly different from control diet: ² P < 0.02, ¹ P < 0.05.

Mean plasma progesterone concentrations during the luteal phase of the menstrual cycle were within the normal range of the assay (8.0–89.2 nmol/L; 2.5–28.0 ng/mL) and were not significantly different between the control and soy-diet periods (Table 5). Compared with the control period, the maximum plasma progesterone concentration during the soy-diet period occurred later in the cycle in all six subjects (Fig 2).

There was no change in the concentration of SHBG during the two dietary periods and no significant difference in SHBG concentration between the follicular phase and luteal phase of the menstrual cycle for either diet period. In addition, the soy diet had no effect on SHBG concentration (Table 5). Although there were individual variations in plasma testosterone concentrations during the two diet periods, mean values for all subjects compared over a complete menstrual cycle for the two diet periods did not differ significantly (Table 5).

TABLE 6

Midcycle plasma concentrations of luteinizing hormone and follicle-stimulating hormone on the days surrounding ovulation (days -1 to +1)

Subject	Luteinizing hormone		Follicle-stimulating hormone	
	Control diet	Soy diet	Control diet	Soy diet
	U/L			
1	9.4	2.9	10.8	4.8
2	4.3	5.4	4.3	2.9
3	26.0	9.3	21.0	17.3
4	21.0	10.8	17.4	7.3
5	22.5	7.0	15.3	8.5
6	44.0	7.4	18.8	6.0
\bar{x} \pm SD	21.2 \pm 12.7	7.1 \pm 2.6 ¹	14.6 \pm 5.6	7.8 \pm 4.6 ²

¹ ² Significantly different from control diet: ¹ P < 0.05 (r = 2.69), ² P < 0.02 (r = 4.01)

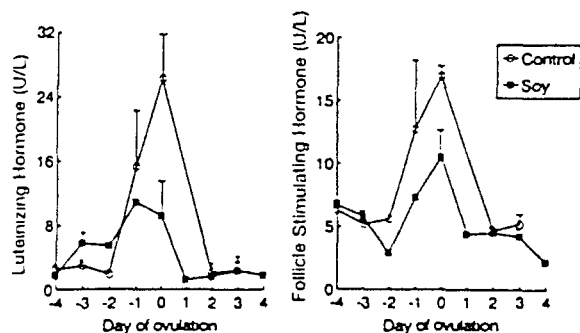


FIG 1. Mean (\pm SD) group luteinizing hormone and follicle-stimulating hormone concentrations in the control- (○) and soy-diet (■) periods.

Plasma estradiol concentrations in the follicular phase, at mid-cycle (the 3 d surrounding ovulation), and in the luteal phase are compared in Table 5. Concentrations of estradiol were significantly higher in the follicular phase of the menstrual cycle ($P < 0.02$) after exposure to isoflavones. However, there were no significant differences in plasma estradiol concentrations mid-cycle or during the luteal phase between the two diet periods (Table 5).

Plasma cholesterol concentrations (Table 5) were 4.3 ± 1.1 mmol/L ($\bar{x} \pm$ SD) in the control-diet period and decreased 9.6% during the period of soy intake (3.9 ± 1.0 mmol/L). This change was statistically significant ($P < 0.05$).

Transit time

Each subject was given 2100 radiopaque markers over the course of the dietary-intervention study, and marker recovery ranged from 99% to 100%, confirming that all of the subjects had provided complete fecal collections during the study. Soy had no significant effect on gut transit time and transit time did not significantly change during the menstrual cycle. Mean transit time during the follicular phase was 64.2 ± 24.2 h during the soy-diet period and 62.2 ± 34.4 h during the luteal phase of the soy-diet period.

Discussion

In view of the concerns over the contamination of meat products by synthetic estrogens such as diethylstilbestrol (DES), it is surprising that there has been relatively little attention given to the potential effects of ingestion of plant (dietary) estrogens by humans (18). In the last 50 y, several important examples of the way in which nonsteroidal dietary estrogens can influence reproductive physiology in animals have been described (10–12). Many plants consumed by humans contain dietary estrogens and although the concentrations are generally low, several plants contain high concentrations of isoflavones (19, 20), and if ingested in large quantities may evoke significant biological effects. Soy protein, for example, is a relatively rich source of isoflavones, which have partial estrogen agonist-antagonist characteristics (7). Because soy protein and other legumes are consumed in significant quantities by humans, the acute and chronic effects of exposure to these dietary estrogens may be biologically important. Although soy-protein products form a major component of the diet of the Japanese and Chinese, where the incidence of breast

cancer and other common Western diseases is low, they are less frequently ingested by Western populations. It has therefore been proposed that the presence of dietary estrogens may contribute to differences in incidence of diseases such as breast cancer between Eastern and Western populations (8, 21–23). Evidence substantiating this contention includes the recent epidemiological data indicating that a high soy intake is associated with a decreased risk for breast cancer in premenopausal women from China and Singapore (24) and the earlier studies showing that soy protein will reduce the number of tumors, in a dose-dependent manner, in animal models of chemically induced mammary carcinoma (9). There also appear to be differences in hormonal status and menstrual cycle length in Japanese women (25) compared with Western women (26–29) and it is possible that these differences could be accounted for by a high dietary estrogen intake.

To our knowledge this is the first report that demonstrates physiological effects of dietary estrogen intake in humans. Six premenopausal women were studied over a 9-mo period, in which two menstrual cycles were spent on controlled diets. Menstrual cycle length appeared to be unaffected by the move to the metabolic suite because there was no significant change in cycle length before the study began, and during the control-diet period (Table 3).

The amount of textured vegetable (soy) protein (60 g/d) used in the study exposed each individual to a daily intake of 45 mg isoflavones. This is a relatively modest amount of dietary estrogen compared with the amounts generally found in soy-protein foods typically consumed in the Far East (9, 30, 31). Separate studies we carried out indicate that all soy-derived foods contain isoflavones (30), and on the basis of typical intake we estimate that the Japanese consume 150–200 mg isoflavones/d (9, 30, 31). The effectiveness of 45 mg soy isoflavones/d raises the issue of whether a greater effect would occur with higher doses, but the complexity of the study precluded examination of dose-response effects. The order of presentation of the control and test diets was not randomized because of the small number of subjects studied and because of the uncertainty of the prolonged effect of con-

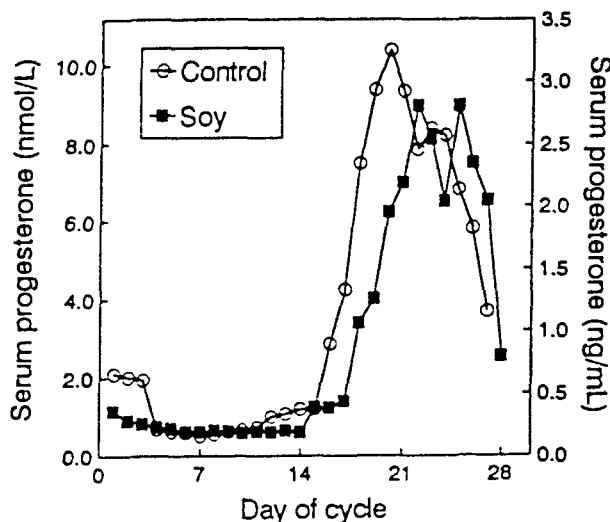


FIG 2. Mean (\pm SD) progesterone concentration during the control- (○) and soy-diet (■) periods.

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Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women¹⁻³

Aedin Cassidy, Sheila Bingham, and Kenneth DR Setchell

ABSTRACT The influence of a diet containing soy protein on the hormonal status and regulation of the menstrual cycle was examined in six premenopausal women with regular ovulatory cycles. Soy protein (60 g containing 45 mg isoflavones) given daily for 1 mo significantly ($P < 0.01$) increased follicular phase length and/or delayed menstruation. Midcycle surges of luteinizing hormone and follicle-stimulating hormone were significantly suppressed during dietary intervention with soy protein. Plasma estradiol concentrations increased in the follicular phase and cholesterol concentrations decreased 9.6%. Similar responses occur with tamoxifen, an antiestrogen undergoing clinical trial as a prophylactic agent in women at high risk for breast cancer. These effects are presumed to be due to nonsteroidal estrogens of the isoflavone class, which behave as partial estrogen agonists/antagonists. The responses to soy protein are potentially beneficial with respect to risk factors for breast cancer and may in part explain the low incidence of breast cancer and its correlation with a high soy intake in Japanese and Chinese women. *Am J Clin Nutr* 1994;60:333-40.

KEY WORDS Soy, hormonal status, menstrual cycle, tamoxifen, breast cancer, isoflavones

Introduction

Breast cancer is the second most common cancer in Western countries but its incidence is significantly less in Third World and Asian populations. Age-specific rates for breast cancer in England and Wales are 199.4 per 100 000 in women 60-65 y of age compared with 52.3 per 100 000 for women of similar age in Japan (1). Epidemiological data from migrant studies suggest that in most cases the susceptibility to breast cancer is the result of environmental rather than genetic differences between these populations and that diet is a major contributing factor (2). The largest and most carefully controlled prospective study of diet and breast cancer, however, failed to provide conclusive evidence to support an association between relative risk and a diet high in fat (3). Furthermore, although elevated free estrogen concentrations have been associated with increased risk (4), there have been few adequately controlled studies of the influence of fat on hormonal status.

Apart from fat, there are many other differences between the typical diets of Far Eastern and Western populations (5). In the Far East a significant quantity of soybean protein is consumed in many different forms, including beans, miso, tofu, and soy milk.

Soy is a rich source of nonsteroidal estrogens of the isoflavone class (6). These compounds, which are structurally similar to estrogens, bind to the estrogen receptor and behave as partial estrogen antagonists (7). For this reason we previously suggested that a diet of soy protein may be beneficial in the protection against and/or treatment of breast cancer (8). This hypothesis is strengthened by recent studies, which have shown that a diet of soy protein leads to a significant dose-dependent reduction in mammary tumor growth in two animal models of chemically induced mammary carcinoma (9). In these studies, tumor formation was negatively correlated with total dietary isoflavone concentration, and in particular with the dietary intake of genistein and the urinary isoflavone excretion (9).

Because little is known about the biological and physiological effects of dietary estrogens in humans and in view of well-documented examples of the biological potency of isoflavone ingestion by animals (10-12), we investigated in a controlled dietary-intervention study the influence of soy-protein intake on hormonal status of premenopausal women. We hypothesized that a constant diet of soy protein containing isoflavones would lead to significant modifications to the hormonal status of the menstrual cycle and that these changes would be beneficial with regard to risk factors for breast cancer.

Subjects, materials, and methods

Study protocol

Six healthy nonvegetarian women (21-29 y of age) were enrolled in the study. The protocol was approved by the Dunn Nutrition Unit Ethical Committee. All women had normal menstrual cycles and had taken no medication for ≥ 6 mo before starting

¹ From the Dunn Clinical Nutrition Centre, Cambridge, UK, and the Division of Clinical Mass Spectrometry, Department of Pediatrics, Children's Hospital Medical Center, Cincinnati.

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³ Address reprint requests to KDR Setchell, Division of Clinical Mass Spectrometry, Department of Pediatrics, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229, or A Cassidy, Dunn Nutrition Center, 100 Tennis Court Road, Cambridge, UK, CB2 1QL.

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to record menses data. For 4 mo before the study began, and for several months after completion, each subject monitored basal body temperature at the same time each morning to determine cycle length, regularity, and ovulatory status. The women lived in the metabolic suite at the Dunn Clinical Nutrition Centre for a total of 4–6 mo. During two of these months, diets and activities were closely monitored and controlled. The time between the control diet and soy diet was separated by a 1–4-mo period because the soy diet had to commence on the first day of a menstrual cycle. Basal metabolic rate was estimated from body weight (13), and the energy intake necessary to maintain a constant body weight throughout the study was calculated, assuming a ratio of total energy intake to basal metabolic rate of 1.4.

The first complete menstrual cycle served as a control period during which time each subject consumed a constant daily diet of nonsoy-containing foods provided by the metabolic kitchen. During the second month, and starting on the first day of menses (which was defined as the first day of menstrual bleeding), appropriate modifications were made to the basal diet to maintain amounts of macronutrients and nonstarch polysaccharide with the addition of 60 g/d (dry wt) soy protein/d (Proteveg; Direct Foods Ltd, Manchester, UK) to meals. All meals were prepared in advance, accurately weighed, and deep frozen until required. All frozen and canned foods were of the same batch to minimize interbatch variability, and bread that contained no soy flour was specially prepared for the study. The same batch of soy protein was used throughout the study period.

Collection of biological samples

Every 3 d during each diet period, a fasting blood sample was collected between 0730 and 0830 (10 mL) and a 24-h urine sample was obtained. Throughout the two diet periods, all fecal samples were collected to measure intestinal transit time. Urine volumes were recorded and aliquots taken and stored at -20°C . Completeness of the 24-h urine collection was assessed by using a previously validated PABA check method (14). Early morning urine samples were collected on the other 2 d and were used to define the day of ovulation with the First Response ovulation prediction test kits (Carter-Wallace Ltd, Folkestone, Kent, UK).

Blood samples were collected into heparinized tubes and immediately centrifuged at $3000 \times g$ for 10 min at room temperature. Plasma was separated and stored at -20°C before analysis. Transit time and completeness of fecal collections were assessed continuously throughout the study by using a radiopaque-marker technique (15). The subjects consumed 10 radiopaque plastic pellets with each meal, three times each day. Stools were collected from the time the first marker was taken until all markers were recovered. Each stool was weighed and x rayed to determine the recovery of markers.

Analytical methods

Duplicate diets were collected at the beginning and end of each diet period for each of the 3 d of the rotation diet. Six diets were therefore analyzed per subject for each diet period. Samples were freeze-dried and analyzed for nitrogen content by using the Kieldahl technique (Tecator Kjeltac System 1002, Bristol, UK). Plant estrogen concentrations were determined in the diets by using previously described HPLC-mass spectrometry (MS) techniques (16, 16).

Urinary isoflavone concentrations were determined in urine by using previously published gas chromatography (GC)-MS techniques (8, 17, 18). The following plasma hormone concentrations were determined by using commercially available radioimmunoassays: sex-hormone-binding globulin (SHBG) (Pharmacia, Milton Keynes, UK), progesterone, testosterone, estradiol, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), all obtained from Diagnostic Products Ltd (Abingdon, Oxon, UK). The plasma cholesterol concentration was measured by using the Cobas-Bio automatic centrifugal analyzer (Roche Diagnostics, Welwyn Garden City, UK).

Two sets of internal quality-control samples were used: trilevel immunoassay control serum (Lyphocheck; Biorad Laboratories, Hemel Hempstead, UK) and trilevel control serum (Diagnostics Product Ltd). Interbatch variability was avoided by assaying all samples from each subject in a single batch and samples were assayed in duplicate.

The cross-reactivity of the dietary estrogens daidzein and genistein in the estradiol radioimmunoassay was determined from solutions of the pure precursors of daidzein and genistein in the concentration range 40 pmol/L–4 mmol/L (10 pg/mL–1 mg/mL). Over this range there was negligible cross-reactivity.

Statistical analysis

Statistical analysis of the data was carried out on an Apple Macintosh LC computer by using the *Systat* 5.1 program (Systat Inc, Evanston, IL). All results are expressed as mean \pm SD, and differences were assessed by using paired *t* tests. Pearson's correlation coefficients were used to assess associations between variables. The CV (%) was used to assess the intra- and interassay variability.

Results

The demographics of the six women participating in the study are given in Table 1. Body weight was monitored during the two diet periods and did not change significantly during the study. Mean body weight was 61.59 ± 5.28 kg at the beginning of the study and 61.34 ± 5.45 kg at the end of the study. Nutrient intakes during the control- and soy-diet periods are compared in Table 2. There were no significant differences in calculated nutrient intake or dietary nitrogen content between the two diet periods. The total dietary isoflavone content of the control diets

TABLE 1
Demographics of the six premenopausal women studied

Subject	Age	Energy intake	Height	Weight before study	Weight after study
	y	MJ/d	m	kg	kg
1	25	8.5	1.68	62.58 \pm 0.11 ¹	61.34 \pm 0.23
2	24	9.0	1.75	63.57 \pm 0.11	64.54 \pm 0.25
3	21	10.0	1.64	69.30 \pm 0.26	69.54 \pm 0.27
4	21	8.0	1.60	53.03 \pm 0.24	51.60 \pm 0.29
5	26	8.0	1.68	56.84 \pm 0.39	56.03 \pm 0.21
6	29	8.5	1.70	64.23 \pm 0.19	64.66 \pm 0.17

¹ $\bar{x} \pm$ SD

TABLE 2
Nutrient intake during the two diet periods¹

Nutrient	Control diet	Soy diet
Calculated		
Energy (MJ)	8.5 ± 0.1	8.5 ± 0.3
Protein (g)	97.2 ± 0.6	98.7 ± 0.8
Total fat (g)	61.4 ± 0.7	60.0 ± 1.7
Carbohydrate (g)	258.2 ± 1.9	260.3 ± 1.0
Nonstarch polysaccharide (g)	16.0 ± 0.6	16.3 ± 0.7
Starch (g)	108.3 ± 2.2	106.7 ± 2.7
Analyzed		
Nitrogen (μg/g)	2.09 ± 0.03	2.10 ± 0.06
Daidzein (mg)	0.76 ± 0.03	25.08 ± 0.31 ²
Genistein (mg)	0.49 ± 0.03	19.85 ± 0.43 ²

¹ $\bar{x} \pm SD$ ² Significantly different from control diet, $P < 0.0001$.

measured from triplicate samples was significantly lower (1.26 ± 0.02 mg/d) than that of the soy diet (44.93 ± 0.21 mg/d).

Menstrual cycle lengths before, during, and for the 3 mo after the study are summarized in Table 3. Subjects 1, 2, 3, and 4 had menstrual cycles of relatively constant length before commencing the dietary study (CV ranged from 0% to 2.2%). In subjects 5 and 6 the menstrual cycle length over the 4-mo period before the control period was more variable but these women had previously used oral contraceptives.

There was no significant difference in the average menstrual cycle length between the control period (27 ± 2 d) and the 4-mo period before the study (28 ± 3 d). However, during the period the soy diet, menstruation was delayed by 1–5 d in five of the six subjects (Table 3). Subject 5 had a shorter cycle on the soy diet, but nevertheless the length of the follicular phase increased. The average length of the follicular phase length was 15 ± 0.9 d during the control-diet period and after the introduction of soy, the mean follicular phase length increased (17.5 ± 2.3 d). The average change in follicular phase length was 2.5 ± 1.6 d, which was statistically significant ($P < 0.01$).

Despite the increase in follicular phase length and total cycle length, no change in the length of the luteal phase was observed during soy consumption (Table 3). There was a trend toward a shorter menstrual cycle in the first month after dietary intervention with soy in four of the six subjects, but this was not statistically significant. Within 3 mo, menstrual cycle length had returned to prestudy values in all six subjects (Table 3).

Table 4 summarizes the urinary excretion of the isoflavones daidzein, genistein, and equol for the six subjects during the period of soy intake. Total urinary isoflavone excretion (5.6 – 67.3 nmol/d; 1.4 – 17.1 μg/d) was low during the control period but after 60 g soy/d over a complete menstrual cycle, a 1000-fold increase in total urinary isoflavone excretion occurred for all subjects, with values ranging from 1400 to 29 400 nmol/d (from 0.35 to 7.49 mg/d) (Table 4). From the estimated dietary intake of 44.9 mg total isoflavones/d, urinary excretion accounted for 1.8–12.9% of the total intake. Only subjects 1 and 2 excreted high amounts of equol during the soy-diet period. Subject 5 excreted considerably lower amounts of equol, and only traces of equol were detected in the other three subjects. The highest urinary excretion of daidzein was observed in the four subjects excreting low or negligible concentrations of its specific metabolite equol, and daidzein excretion was quantitatively more important than was genistein in these four subjects (Table 4). Conversely, the two subjects excreting substantial amounts of equol excreted low concentrations of the precursors daidzein and genistein.

Plasma hormones

The plasma concentrations of progesterone, estradiol, testosterone, SHBG, and cholesterol at times before and during soy-protein intake are summarized in Table 5. Table 6 shows the mean plasma LH and FSH concentrations at the time of ovulation, determined from the samples obtained on the day of ovulation and 1 d either side of ovulation as defined by the First Response assay kit for predicting ovulation from the early morning and 24-h urine samples.

The midcycle peaks of LH and FSH were significantly suppressed by soy-protein ingestion ($P < 0.05$ and $P < 0.01$, re-

TABLE 3
Length of menstrual cycle and follicular phase over a 4-mo period before the study, during the study periods, and 3 mo after the soy diet

Subject	Menstrual cycle length							
	Before study ¹				After soy diet ²		Follicular phase length	
	$\bar{x} \pm SD$	CV	Control diet	Soy diet	$\bar{x} \pm SD$	CV	Control diet	Soy diet
	d	%	d	d	d	%	d	d
1	28.25 ± 0.50	1.8	29	32	24.33 ± 2.31	9.5	16	21
2	26.50 ± 0.58	2.2	25	30	29.00 ± 2.65	9.1	16	19
3	27.75 ± 0.50	1.8	27	29	26.33 ± 1.53	5.8	15	15
4	28.00 ± 0.00	0.0	28	29	28.67 ± 1.16	4.1	14	16
5	35.00 ± 4.83	13.8	31	28	33.67 ± 4.62	13.7	15	18
6	25.00 ± 1.41	5.6	25	26	24.67 ± 1.16	4.7	14	16
$\bar{x} \pm SD$	28.42 ± 3.45	—	27.50 ± 2.35	29.00 ± 2.00	27.78 ± 3.48	—	15.00 ± 0.90	17.50 ± 2.30 ³

¹ $n = 4$ mo² $n = 3$ mo.³ Significantly different from control diet, $P < 0.01$ ($t = 3.73$).

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sumption of soy on menstrual cycle length. In several women, after the soy diet, three menstrual cycles elapsed before the cycle length returned to its original value (Table 3). Subjects lived in the metabolic suite for an average period of 4–6 mo, and for at least one complete menstrual cycle before initiation of the study.

With the amount of soy protein used in this study there occurred a significant increase in follicular phase length and a delay in menstruation. The midcycle surges of the gonadotrophins LH and FSH were significantly suppressed during the soy-diet period. In these studies, no special attempt was made to ascertain the exact day of ovulation and because peak urinary LH and plasma LH concentrations can be out of phase by a day, it could be argued that the differences between the soy and control cycles could be the result of differences in sampling times. In more recent studies when sampling was carried out daily for 1 wk before ovulation was established by urinary LH concentrations, a 1-d delay was found between the peak plasma and the urinary LH concentrations; however, when an identical soy protein devoid of isoflavone was used, no significant change in gonadotrophins was observed. These observations suggest that the agonist-antagonistic action of dietary estrogens in soy may be responsible for the hormonal modification to the cycle. Equol, a specific metabolite of the ingested soy isoflavones daidzein and genistein (17), had the greatest influence; the two subjects with the highest urinary equol excretion showed the largest increase in follicular phase length. Interestingly, in our recent studies Arcon F (Archer Daniels Midland, Decatur IL)—a soy-protein product devoid of isoflavones—was found to have no effect on either plasma gonadotrophins or follicular phase and menstrual cycle lengths (32), whereas miso—a fermented soy protein that has high concentrations of unconjugated isoflavones (30)—had a greater effect than did textured vegetable protein, providing further evidence that isoflavones may be the biologically active components of soy.

The major influence on menstrual cycle length is variation in the length of the follicular phase (33). In the present study follicular phase length was significantly increased by an average of 2.5 d when soy protein was consumed daily, whereas no significant change in luteal phase length was observed. One possible explanation for the relationship between menstrual cycle length and breast cancer risk may be that shorter menstrual cycles would lead to a greater life-time exposure to estrogen. Furthermore, because the mitotic rate for breast tissue is almost fourfold greater during the luteal phase than during the follicular phase (27, 34), women with shorter cycles and consequently at high risk for breast cancer, will therefore spend proportionally more of their lifetime in the luteal phase of the cycle. A significant increase in menstrual cycle length, particularly follicular phase length, as was observed in our study, would therefore be potentially beneficial in lowering risk for breast cancer. The influence of soy protein containing dietary estrogens on the menstrual cycle may explain the reduced risk for breast cancer in premenopausal women consuming soy-protein products (24). Interestingly, Olsson et al (26) made a retrospective assessment of cycle length and found a significantly shorter cycle length for breast cancer patients compared with control subjects (26.4 vs 28.6 d). Menstrual cycle length is also significantly longer in Asian women than in Western women. Menstrual cycle length of women from Western populations ranges from 26 to 29 d (26–29),

whereas the average cycle length for Japanese women is longer (27). It is conceivable that the differences in hormonal status and characteristics of the menstrual cycle of Japanese and Chinese women (35) may be in part due to the ingestion of substantial amounts of nonsteroidal estrogens present in soy protein. The results of the present study provide evidence that soy-protein-containing dietary estrogens when ingested daily will lead to significant biological effects in premenopausal Western women, which are potentially beneficial with regard to breast cancer risk.

Some of the biological effects of a soy-protein diet containing isoflavones are similar to those induced by the potent synthetic antiestrogen tamoxifen (36). Tamoxifen, when used therapeutically in breast cancer patients, leads to decreases in circulating concentrations of LH and FSH (37–39) and a reduction in total serum cholesterol and LDL-cholesterol concentrations; however, increases in SHBG are consistently observed in both pre- and postmenopausal women after prolonged antiestrogen therapy (40–46). The magnitude of the change in LH and FSH concentrations when soy protein is included in the diet is markedly greater than that observed in studies of tamoxifen. Jordan et al (40) showed that 10 mg tamoxifen/d suppressed LH concentrations by 39% in premenopausal women and 17% in postmenopausal women with breast cancer. With a dose of 40 mg/d, Willis et al (37) showed a 41% decrease in plasma LH and a 29% decrease in plasma FSH concentrations after 6 wk of therapy. These data compare with a 300% reduction of LH and 200% reduction of FSH during a 1-mo period in which soy protein is consumed.

The hypocholesterolemic effect of soy protein may relate to its nonstarch polysaccharide content (47, 48), but isoflavones may also play a role in cholesterol homeostasis (20), because we recently showed that soy-milk formulas containing isoflavones will influence cholesterol fractional synthesis rates in newborn infants (49). In the present study a mean reduction in serum cholesterol of 9.6% is impressive given the relatively short duration of soy-protein intake and the fact that these women had normal serum cholesterol concentrations. No change in plasma SHBG concentration was observed during soy intake but it is possible that the study period was too short to observe an effect. A similar lack of effect was also observed in a separate study of postmenopausal women consuming soy protein (≈ 50 g/d) for 1 mo (DD Baird, KDR Setchell, unpublished data, 1988).

Recent and controversial trials of tamoxifen as a prophylactic agent are underway in women at high risk for breast cancer, but with no evidence of disease (50–52). The earlier demonstration that soy-protein-containing isoflavones have anticancer actions in animal models of breast cancer (9) raises the question of whether dietary intervention with soy protein should be considered as an alternative approach to drug therapy for breast cancer prevention. Significantly higher concentrations of estradiol (measured by immunoassay) were found in the plasma during the follicular phase when the women were given soy protein. Similar increases have been found in women given 20 mg tamoxifen/d (52). Whereas a high estrogen concentration in the follicular phase may be considered undesirable from the point of view of breast cancer, this finding did not deter the recent clinical trials of tamoxifen.

In summary, our observations clearly indicate how dietary modifications can lead to significant changes in the regulation of

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the menstrual cycle. Such changes may be beneficial with regard to risk factors for breast cancer and suggest that a diet rich in dietary estrogens may be protective against breast cancer. ■

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Persistent Hypothyroidism in an Infant Receiving a Soy Formula: Case Report and Review of the Literature

Soy-induced goiter was a well-known phenomenon before 1966, the back date used in many computerized literature databases.¹⁻⁴ In the mid-1960s, iodine-supplemented infant soy formulas prepared from isolated soy protein were introduced by commercial manufacturers.⁵ Since then, there have not been any documented cases of soy formula-associated hypothyroidism.

We present the case of a patient with congenital hypothyroidism who remained persistently hypothyroid while on a soy formula diet despite large doses of L-thyroxine (T₄). This case made us reaware of the historical data on the effects of soy on thyroid function. It also realerted us to the thoughtful use of formula preparations and close dietary monitoring of hypothyroid infants.

CASE SUMMARY

The patient was a full-term male infant born from an uncomplicated pregnancy and delivery. Birth weight was 3.4 kg (75%); length, 54 cm (90%); and head circumference, 35 cm (50%). He received a soy formula diet at his parents' request because of a family history of intolerance of cow's milk in the patient's older siblings. Poor feeding, hypotonicity, and continuous sleeping was noted during his first week. An abnormal newborn screen (done on day 2 of life) was reported on day 8 of life. Serum confirmation of primary hypothyroidism was obtained on day 9 of life (Table).

A physical examination showed a weight of 3.46 kg (75%), a length of 52 cm (75%), and a head circumference of 36 cm (75%). He had a mild degree of generalized mottling. His skin was warm to the touch. There was some wrinkling over his forehead. His anterior fontanelle was 3 × 3 cm, and his posterior fontanelle was 1 × 1 cm. No lingual protuberance was noted on examination of the pharynx. His thyroid was not palpable. A grade II/VI systolic ejection murmur was heard at the left sternal border.

He had a small umbilical hernia. His genitalia were those of a healthy male infant, and he seemed to have good muscle tone.

Bone age was compatible with 34 weeks' gestation. A technetium thyroid scan showed evidence of an ectopic thyroid (lingual) gland. Treatment with L-T₄, 0.05 mg (14.5 µg/kg per day), was initiated on day 11 of life.

One month later, the infant's thyrotropin (TSH) level remained markedly elevated (Table). Compli-

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Reprint requests to (N.J.H.) Department of Pediatrics, Division of Endocrinology, University of Michigan, Room D3252, Medical Professional Building, Ann Arbor, MI 48109-0718.
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TABLE. Serial Measurements in Our Infant With Congenital Hypothyroidism

Age (d)	T ₄ (μg/dL)	TSH (μU/mL)	Free T ₄ (ng/dL)	TBG (%)	T ₃ (ng/mL)	Reverse T ₃ (ng/mL)	Free T ₃ (pg/mL)	Weight (kg)	L-T ₄ Dose (μg/kg/d)
Formula									
2	8.4†	201†						3.4	
9	8.6	342		26	132			3.4	14.5
39	4.8	98							
44	6.9	180	0.7	29					
49	7.8	196	0.9		(124+)‡	(40+)‡	(282+)‡	5.1	12.2
Nonsoy formula									
57	9.3	58	0.83					5.8	10.9
64		21	1.39		200			6.2	12.1
71		11.4	0.8		157			7	8.9
120		0.98	2.1					9	8.3
180		<0.2	1.65					10.8	6.9
270		1.63	1.65		255			12.1	5.6
368		3.62	1.45					13.3	5.7
Infant Norms ²⁰	6.1–14.9	0.5–4.8	0.9–2.6	NA§	85–250	10–50	NA		

* Abbreviations: T₃, triiodothyronine; NA, not available for infants; TBG, thyroid-binding globulin.

† Newborn screen (filter paper), state of Michigan.

‡ (+), Obtained later on stored serum.

§ 17–26 for children ages 1 to 6 years.

|| 260–480 for adults.

ance and nongeneric L-T₄ dosing was confirmed (Boots/Synthroid). There were no reported episodes of emesis or spitting up after feeding. He was defecating five to seven times per day. He was more alert, hungrier, and less tired after the initiation of L-T₄ therapy. However, his thyroid tests continued to show a lack of TSH suppression. A new prescription and different pharmacy were recommended to rule out the possibility of an inactive preparation.

At 49 days of age, TSH remained high. He weighed 5.1 kg (75%), with a length of 57.1 cm (75%) and a head circumference of 40 cm (75%). His posterior fontanelle was closed. His L-T₄ dose was adjusted for his weight, and he was switched to a cow's milk formula. After 1 week on the cow's milk formula, he showed significant suppression of TSH (Table). He was noted to have a decreased stool frequency of two to three times per day. His thyroid function continued to normalize over time. Subsequent growth and development were normal without a need for increase in his L-T₄ dose (expressed as micrograms per kg/d). His skeletal age at 12 months was equivalent to 10 months (average of hands, feet, and knees).

DISCUSSION

In the early part of the 20th century, the goitrogenic effect of a soybean diet was well recognized in animals.^{6–9} Animal soy diets supplemented with iodine in amounts double the normal daily requirements protected against the development of goiters.^{9–11} The goiters in soy-fed rats exhibited high uptake of iodine ¹³¹I, similar to the effect of iodine deficiency.¹¹

Van Middleworth^{12,13} elucidated the mechanism for the development of goiters in animals fed soy diets. Soy diets are associated with a large fecal mass and an accelerated rate of transport. The high-uptake goiters were caused by an increased iodine requirement after T₄ depletion from increased fecal wastage.

Beck¹⁴ confirmed Van Middleworth's^{12,13} findings and also speculated about the presence of a goitro-

genic factor in soy. Several investigators attempted to characterize this possible goitrogen. Konijn et al¹⁵ purified a goitrogenic substance from soybeans described as a glycopeptide. This glycopeptide blocked iodine uptake by the thyroid and decreased its organification but had little effect on the formation of triiodothyronine and T₄. There have been no further reports characterizing goitrogenic factors in soy.

Relatively little is known about the effects of a soy diet on thyroid function in humans. Before 1960, there were several notable cases of goiters occurring in infants fed soy formulas, which resolved after their diets were changed to a cow's milk formulas.^{1–3,16} Similar to the findings in animals, all the infants demonstrated an increased uptake of ¹³¹I by the thyroid while taking a soy diet. These infants showed normal ¹³¹I uptake when receiving cow's milk diets. Stool frequency and bulk were also noted to decrease after this change.

There may be several different mechanisms by which soybeans induce goiters. Commercial manufacturers have reported that different lots of soy vary widely in their goitrogenic properties. In all cases it was possible to overcome the goitrogenic activity of the soy ration by fortifying it with additional iodine.^{1,11} Since commercial manufacturers began supplementing soy formulas with iodine in 1959,⁵ no further cases of soy formula-induced goiters have been reported.

However, in 1965, an athyreotic cretin fed a soy-flour formula supplemented with iodine was described to be refractory to thyroid hormone.¹⁷ ¹³¹I-labeled T₄ was measured in the feces, along with the ¹³¹I content in the urine and serum at the time the patient was receiving the soy formula and later a cow's milk formula. Higher fecal ¹³¹I excretion and lower levels of radioactivity in the urine and serum were found during soy feeding but not in cow's milk feedings. These findings provided evidence that soy feeding interferes with exogenous T₄ absorption primarily by fecal wastage in humans. This is similar to

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the earlier observations of soy feeding on endogenous T_4 in animals.^{12,13}

In the mid-1960s, commercial manufacturers replaced the high-fiber soy-flour preparation formulas with a formula prepared from isolated soy protein.⁵ The isolated soy protein formula is more like a cow's milk formula in color and odor. Most of the fiber is removed during the protein isolation process, and the stools of infants receiving the isolated soy protein formula were found to be similar to those of infants fed a cow's milk formula. This is the preparation of the soy formula widely used today.

Our patient was found to have congenital hypothyroidism from his neonatal screen, confirmed by serum values (Table). He continued to have persistent hypothyroidism despite receiving a dose of $L-T_4$ that is usually adequate for infants with congenital hypothyroidism. His soy formula contained 10.2 μg of iodide/4200 J) similar to several other commercial soy formulas (15 to 20 μg /4200 J) and several cow's milk infant formulas (6 to 9 μg /4200 J). After the change from a soy formula to a cow's milk formula, our patient's thyroid functions normalized while he received the same or a smaller dose of $L-T_4$ in micrograms per kg/d. The patient's stool frequency decreased from five to seven stools per day while receiving the soy formula to two to three stools per day while receiving the cow's milk formula. Although T_4 excretion was not measured in this infant's stools, his clinical course suggests that fecal bulk and accelerated transit time may have played a role in the absorption of exogenous thyroid hormone.

There is a recent report of an infant with congenital hypothyroidism who, in the course of thyroxine replacement, developed a cow's milk protein intolerance. There was a need to increase his thyroxine replacement dose due to rising TSH levels.¹⁸ After the patient was switched to a hydrolyzed milk diet, his thyroid function improved. A rechallenge with the cow's milk formula caused subsequent decreases in the patient's serum T_4 and increases in his TSH.

Our patient was given a soy formula from birth because of a family history of allergies to milk. Allergies to soy formulas and protein intolerances do occur.¹⁹ Although there are no reports of intolerance of a formula inducing hypothyroidism, it is theoretically possible that the increased stools associated with intolerance of a formula may cause a persistence of hypothyroidism in a congenital hypothyroid infant receiving replacement thyroid hormone.

In summary, soy diets are known to induce high-uptake goiters from an increased iodine requirement after T_4 depletion through fecal wastage. Other mechanisms may be involved in the development of goiters, and evidence exists that soy contains a goitrogenic factor. The addition of iodine supplementation to commercial soy formulas in the 1960s has eliminated the development of hypothyroidism caused by iodine deficiency in soy-fed infants.

However, it is still necessary to reemphasize the effects of soy diets on thyroid function, particularly in hypothyroid infants receiving thyroid hormone. This case presentation should realert physicians that dietary monitoring and close follow-up are necessary in hypothyroid infants.

PAULA ANN CHORAZY, MD, PhD

SETH HIMELHOCH, MD

NANCY J. HOPWOOD, MD

Department of Pediatrics

Division of Pediatric Endocrinology

University of Michigan Medical Center

Ann Arbor, MI

NANCY G. GREGER, MD

DANIEL C. POSTELLON, MD

Department of Pediatrics

Division of Pediatric Endocrinology

Children's Hospital of Michigan

Detroit, MI

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Anti-Thyroid Isoflavones from Soybean ISOLATION, CHARACTERIZATION, AND MECHANISMS OF ACTION

Rao L. Divi, Hebron C. Chang and Daniel R. Doerge*

NATIONAL CENTER FOR TOXICOLOGICAL RESEARCH, JEFFERSON, AR 72079, U.S.A.

ABSTRACT. The soybean has been implicated in diet-induced goiter by many studies. The extensive consumption of soy products in infant formulas and in vegetarian diets makes it essential to define the goitrogenic potential. In this report, it was observed that an acidic methanolic extract of soybeans contains compounds that inhibit thyroid peroxidase- (TPO) catalyzed reactions essential to thyroid hormone synthesis. Analysis of the soybean extract using HPLC, UV-VIS spectrophotometry, and LC-MS led to identification of the isoflavones genistein and daidzein as major components by direct comparison with authentic standard reference isoflavones. HPLC fractionation and enzymatic assay of the soybean extract showed that the components responsible for inhibition of TPO-catalyzed reactions coeluted with daidzein and genistein. In the presence of iodide ion, genistein and daidzein blocked TPO-catalyzed tyrosine iodination by acting as alternate substrates, yielding mono-, di-, and triiodoisoflavones. Genistein also inhibited thyroxine synthesis using iodinated casein or human goiter thyroglobulin as substrates for the coupling reaction. Incubation of either isoflavone with TPO in the presence of H_2O_2 caused irreversible inactivation of the enzyme; however, the presence of iodide ion in the incubations completely abolished the inactivation. The IC_{50} values for inhibition of TPO-catalyzed reactions by genistein and daidzein were ca. 1–10 μM , concentrations that approach the total isoflavone levels (ca. 1 μM) previously measured in plasma from humans consuming soy products. Because inhibition of thyroid hormone synthesis can induce goiter and thyroid neoplasia in rodents, delineation of anti-thyroid mechanisms for soy isoflavones may be important for extrapolating goitrogenic hazards identified in chronic rodent bioassays to humans consuming soy products. *BIOCHEM PHARMACOL* 54:10:1087–1096, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. thyroid peroxidase; soybean; isoflavonoid; inhibitor; goitrogen; mechanism

The soybean and its products have been considered goitrogenic in humans and animals. Goiter and hypothyroidism were reported in infants receiving soy-containing formula [1–4], and such feedings in early life have also been associated with the development of autoimmune thyroid disorders [5]. Several investigators have reported induction of goiter in iodine-deficient rats maintained on a soybean diet [6–11]. Furthermore, Kimura *et al.* [9] reported the induction of thyroid carcinoma in rats fed an iodine-deficient diet containing 40% defatted soybean. Konijn *et al.* [10] showed that the anti-thyroid activity present in acidic acetone soybean extracts is water soluble, is dialyzable, and is not precipitated by either ammonium sulfate or trichloroacetic acid. The active ingredient was characterized partially by these workers as a small molecular compound of non-peptide origin, since it was not destroyed by either digestion with pancreatin or by boiling for 2 hr.

The function of the thyroid is synthesis of thyroid hormones, and TPO[†] catalyzes iodination of tyrosyl residues on Tg and the subsequent coupling of iodotyrosyl residues required for iodothyronine hormone formation.

Inhibition of TPO-catalyzed reactions results in decreased levels of circulating thyroid hormones, which lead to increased secretion of TSH by the anterior pituitary. The increased levels of TSH provide a growth stimulus to the thyroid, and it has been proposed that a prolonged stimulus can select for clones of follicular cells with the potential for transformation [12]. This mechanism predicts that any compound that inhibits TPO-mediated thyroid hormone synthesis is a potential thyroid carcinogen.

The widespread use of soy products in infant food formulas and the significant consumption of soy products by people consuming a vegetarian diet require a closer evaluation and examination of the anti-thyroid activity of the soybean. This is important because of the current promotion of soy-based products as health foods possessing putative beneficial estrogenic and anti-carcinogenic properties. For example, genistein, but not daidzein, inhibits tyrosine kinase activity, and this property has been explored for

* Corresponding author. Tel. (501) 543-7943; FAX (501) 543-7720; E-mail: DDOERGE@NCTR.FDA.GOV

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† Abbreviations: APCI, atmospheric pressure chemical ionization; CcP, cytochrome c peroxidase; CID, collision-induced dissociation; DIT, 3,5-diiodotyrosine; LPO, lactoperoxidase; MIT, 3-iodotyrosine; PEG, polyethylene glycol; rT₄, 3,3',5'-triiodothyronine; T₄, thyroxine; T₃, 3,5,3'-triiodothyronine; Tg, thyroglobulin; TPO, thyroid peroxidase; TSH, thyroid stimulating hormone; and MES, 2-[N-morpholine]ethanesulfonic acid.

potential anti-cancer potential [13]. Information in the scientific literature regarding the chemical nature of the active anti-thyroid component(s) from soybean, as well as the mechanism of action, is far from complete. In the present study, we report the chromatographic separation of the active anti-thyroid compounds of soybean, the elucidation of chemical structures, and the mechanisms for inhibition of TPO-catalyzed reactions.

MATERIALS AND METHODS

Reagents

Genistein, genistin, glucose, and glucose oxidase were obtained from the Sigma Chemical Co. (St. Louis, MO) and used as obtained. Daidzein was a gift from Dr. K. D. R. Setchell. TPO used in the present study was purified from porcine thyroid glands and quantified spectrophotometrically as previously described [14]. Human goiter Tg was a gift from Dr. Alvin Taurog, University of Texas Southwestern Medical School. The isoflavones were dissolved in either ethanol or DMSO that had been purified by distillation. A constant concentration of ethanol or DMSO (5%), which did not affect enzyme activity, was maintained in incubation mixtures.

Preparation of Soybean Extracts

Whole soybeans, advertised as not treated with herbicides or fungicides, were obtained from a local health food store and ground to a fine powder. The powdered sample (5 g) was extracted by stirring with 250 mL of acidic methanol (12 N HCl:CH₃OH, 10:90, v/v) with heating at reflux for 4 hr. The mixture was centrifuged at 20,000g for 20 min, the supernatant evaporated *in vacuo*, and the residue dissolved in 10 mL of 95% ethanol.

Liquid Chromatography

A fraction of the soybean extract was diluted 100-fold with methanol, and a 25- μ L aliquot was injected into a reversed-phase HPLC column (NovaPak C18, Waters Associates, Milford, MA) using a GPM quaternary gradient pump (Dionex, Sunnyvale, CA). The column was eluted using a solvent system consisting of 30% solvent A and linearly increasing to 100% A in 20 min at a flow rate of 1.5 mL/min [A = acetonitrile; B = acetic acid:acetonitrile:water (0.5:5.0:94.5 by vol.)]. The peaks were monitored using a Spectra Focus forward-scanning optical detector (SpectraPhysics, San Jose, CA) at 260 nm and also by obtaining UV spectra between 200 and 350 nm. The same HPLC system was used to separate iodinated isoflavones. Elution was carried out with a mobile phase gradient starting with 50% A and increasing linearly to 100% A in 20 min at a flow rate of 1.5 mL/min. Peaks were detected using 275 nm absorbance.

For determination of TPO-inhibitory activity, individual identifiable peaks were collected after emerging from the

detector, and when peaks were not present, fractions of 1 min were collected across the entire elution profile. The collected fractions were dried *in vacuo* and dissolved in 100–200 μ L of ethanol. A 50- μ L aliquot was then added to the tyrosine iodination assay to determine inhibition of TPO activity (see below).

HPLC was used to monitor TPO-catalyzed iodination of tyrosine to MIT and DIT because the UV absorbance of the isoflavones interfered with the usual spectrophotometric assay [14]. The same NovaPak C18 HPLC cartridge was used with solvent system consisting of A = acetonitrile; B = 0.2% trifluoroacetic acid in 5% acetonitrile/water using a gradient of 10% A to 40% A in 20 min with a flow rate of 1.5 mL/min and UV detection at 230 nm. Retention times for tyrosine, MIT, and DIT were: 2.5, 3.6, and 4.8 min, respectively.

Mass Spectrometry

MS experiments were performed using a VG Platform single quadrupole instrument (Micromass, Altrincham, U.K.) equipped with an APCI interface. The total LC column effluent described above was delivered into the atmospheric pressure ion source (150°) through a heated nebulizer probe (500°) using nitrogen as the probe and bath gas (275 L/hr). Positive ions were acquired in full scan mode (m/z 100–600, 2.1 sec cycle time) in series with a UV detector set at 250 nm. Background-subtracted mass spectra were obtained by averaging spectra across the respective chromatographic peak and subtracting the average background immediately before and after this peak. At a low sampling cone-skimmer voltage (15 V), mass spectra for isoflavones and derivatives consisted predominately of the respective protonated molecule. At a higher voltage (50 V), in-source CID reactions produced numerous fragment ions. For on-line characterization of soy extracts, two separate scan functions were used to simultaneously obtain spectra at 15 and 50 V. The mass spectrometer was calibrated using a solution of PEGs [PEG 200 (25 μ g/mL), 300 (50 μ g/mL), 600 (75 μ g/mL), and 1000 (250 μ g/mL) obtained from Sigma Chemical Co.] in 50% acetonitrile in aqueous ammonium acetate (5 mM) over the range m/z 85–1200.

Inhibition of TPO-Catalyzed Reactions

Different amounts of soy extract, isolated HPLC fractions, or authentic isoflavones (2–80 μ M) were added to reaction mixtures to determine the concentration dependence of TPO inhibition. The solution was incubated for 4 min and the reaction stopped by injection onto the HPLC column. Tyrosine (100 μ M) and iodide (100 μ M) or guaiacol (2.5 mM) were incubated with TPO (10 nM) plus inhibitor, and the reaction was initiated by the addition of H₂O₂ (100 μ M) at 22 \pm 0.1°. HPLC was used to follow the concentration of MIT/DIT and isoflavone present in incubations containing TPO, the H₂O₂-generating system consisting of

glucose (1.25 mM) plus glucose oxidase (10 nM), and genistein or daidzein.

Iodination of bovine casein (Sigma Chemical Co.) was carried out using Iodobeads (Pierce Chemical Co., Rockford, IL) using 10 beads in 5 mL MES buffer, pH 7.0, that contained 250 μ M iodide to generate I_2^- for 1 min. Then a 10-mL aliquot of casein solution (1.25 mg/mL) in the same buffer was added. After incubation for 10–15 min at room temperature, the solution was dialyzed overnight. The degree of iodination was estimated spectrophotometrically using the change in absorbance at 290 nm as a measure of DIT formation ($\Delta A = 0.92/\text{atom I}$, see Ref. 15). The content of MIT and DIT in iodinated casein was confirmed for one sample using HPLC analysis after proteolytic digestion of iodocasein; reasonable agreement with the spectrophotometric determination was seen (not shown).

Measurement of coupling, an *in vitro* assay of thyroid hormone synthesis, was carried out in the presence of TPO (20 nM) using chemically iodinated casein (1.25 mg/mL containing approximately 50–60 atom I/mol) as the source of iodotyrosines [16], various concentrations of isoflavone, and H_2O_2 (100 μ M) for 1 hr in 0.05 M MES buffer, pH 6.5, at $37 \pm 0.1^\circ$. Bovine mucosal alkaline phosphatase (10 units, Sigma Chemical Co.) was added to hydrolyze phosphate groups, including phosphotyrosines. This treatment increased yields of T_4 by approximately 25%. The reaction mixture was digested under a nitrogen atmosphere using pronase (250 μ g/mL final concentration) for 1 hr and then with leucine-aminopeptidase (50 μ g/mL) for 3 hr [17]. Thyroid hormones in the reaction mixture were extracted three times with ethyl acetate, and the extract was dried *in vacuo* and dissolved in a 100- μ L aliquot of starting HPLC solvent. Extraction efficiency for a standard addition of T_4 was determined to be 92–95%. Thyroid hormones were measured by HPLC using a Hamilton PRP-1 reversed-phase column with a solvent system of A = acetonitrile, B = trifluoroacetic acid:acetonitrile:water (0.2:5.0:94.8; by vol.) starting with 10% A in B and increasing linearly to 50% A in 20 min at a flow rate of 1.0 mL/min. The peaks were detected and iodothyronines quantified using 230 nm absorbance.

Measurement of TPO-catalyzed coupling was also carried out using human goiter Tg essentially as described by Taurog *et al.* [18] except that the proteolysis and HPLC analysis described above were used for quantifying iodothyronines. Samples were analyzed in triplicate for at least four different concentrations of isoflavone bracketing the IC_{50} .

Inactivation of TPO by Isoflavones

TPO was inactivated by isoflavones by incubating enzyme (1.0 μ M) with 50 μ M daidzein or 50 μ M genistein and 200 μ M H_2O_2 at $25 \pm 0.1^\circ$ in 0.1 M MES buffer (pH 7.0). After 4 min, aliquots were withdrawn and diluted 1000 to 2000-fold, and the remaining tyrosine iodination activity was measured. The activity was not restored by treatment of

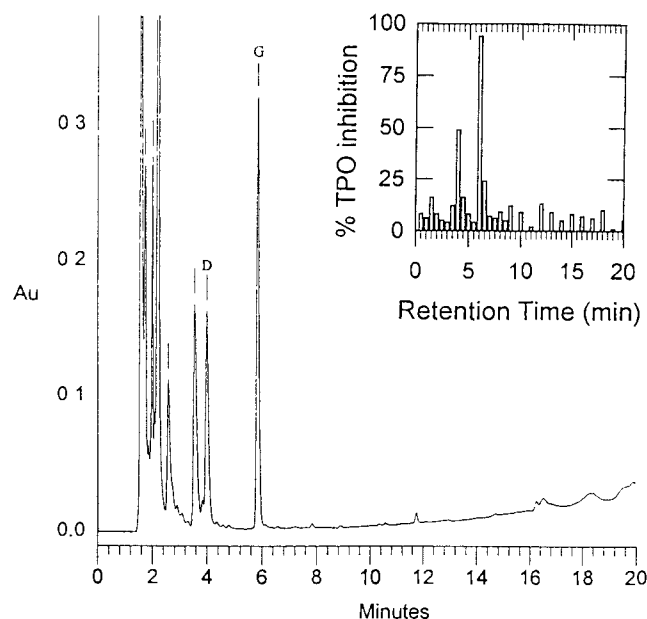


FIG. 1. HPLC fractionation of a soybean extract and inhibition of TPO-catalyzed tyrosine iodination. The soybean extract was fractionated using HPLC and UV detection (260 nm) as described in Materials and Methods, and the individually collected fractions were tested for inhibition of TPO activity (inset). The maximal TPO activity was 9 nmol MIT formed/min.

inactivated enzyme by centrifugal gel filtration or extensive dialysis as previously described [14]. In separate experiments to study its protective effect on inactivation, iodide (0.15 to 5.0 mM) was also included in the reaction mixture.

RESULTS

Characterization of Compounds Inhibiting TPO in Crude Soybean Extract

The presence of anti-thyroid components in soybean was investigated using a heated acidic methanol hydrolysis and extraction procedure. This procedure was selected to liberate the respective aglycones because glucoside conjugates are the predominant form in whole soybean [19, 20]. It was determined that this procedure completely converted genistein and daidzein conjugates to the respective aglycones (data not shown). When the extract was fractionated using HPLC, two distinct peaks of UV absorbance (retention times 4.0 and 5.8 min, see Fig. 1) were found to contain most of the inhibitory activity (Fig. 1 inset). Peaks labeled D and G showed UV absorption maxima at 251 and 259 nm, respectively (data not shown). These chromatographic and spectral properties were identical to those observed from authentic standards of daidzein and genistein, and coinjection of standards with the extract showed no evidence for inhomogeneity. The genistein and daidzein content based on HPLC analysis of the acidic methanol extract was determined to be 1.98 and 0.73 mg/g soybean, respectively, using external standard calibration.

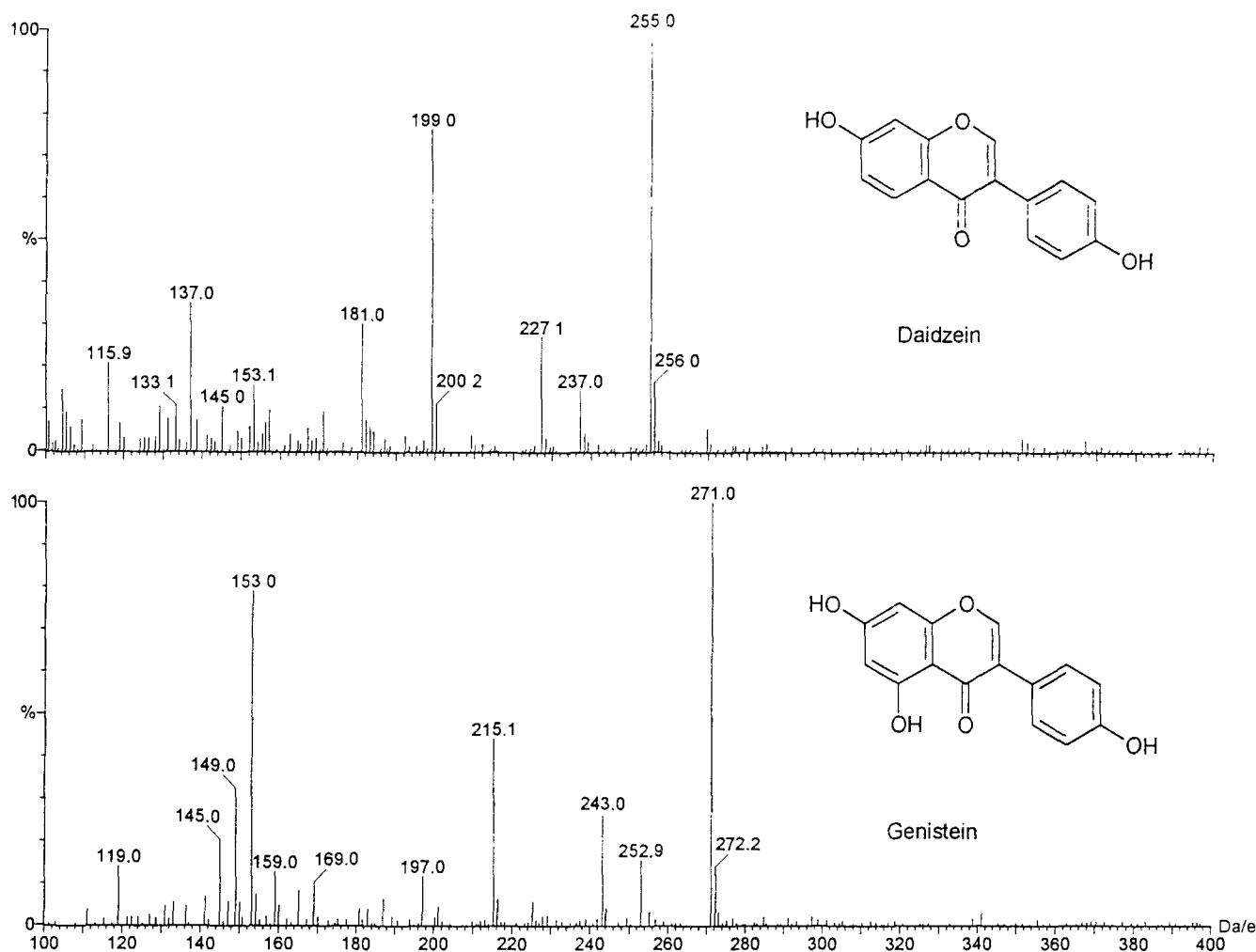


FIG. 2. On-line LC-APCI/MS analysis of genistein and daidzein from a soybean extract. Mass spectra were obtained following on-line LC separation of genistein ($M + H^+ = 271$ Da, bottom panel) and daidzein ($M + H^+ = 255$ Da, top panel) in the soybean extract as described in Materials and Methods. The spectra were obtained using a sampling cone-skimmer potential of 50 V.

These values are consistent with the total isoflavone content in soybean reported previously [9, 20].

The soybean extract was analyzed further by on-line APCI/MS under conditions that produced mass spectra containing protonated molecules ($M + H^+$) and fragment ions (see Fig. 2). Mass spectra from peaks D and G contained protonated molecules corresponding to the masses predicted for daidzein and genistein (mol wt. = 254 and 270, respectively). Diagnostic fragment ions were also observed. Not only were the observed protonated molecules and fragment ions identical to those produced from authentic standards (not shown), but they were also very similar to the CID spectra previously reported for genistein and daidzein using electrospray ionization with tandem mass spectrometry [21, 22]. Since the chromatographic, spectroscopic, and TPO inhibitory properties were found to be identical with those exhibited by authentic isoflavones, subsequent mechanistic studies were carried out with pure isoflavones.

Inhibition of TPO-Catalyzed Iodination and Coupling by Isoflavones

Genistein and daidzein were found to inhibit TPO-catalyzed iodination of tyrosine. The IC_{50} values for these reactions were estimated from concentration-inhibition curves (not shown) to be 3.2 and 7.6 μ M, respectively. These values were similar to those reported previously for related flavonoids [14]. The glycoside genistin was approximately 10-fold less potent than the aglycone with an IC_{50} value of 38 μ M, and HPLC-UV analysis showed the commercial product to be devoid of the aglycone (<0.1%). A 25- μ L aliquot of the crude extract (equivalent to 500 μ g of soybean powder) produced 50% inhibition of TPO-catalyzed tyrosine iodination activity (data not shown). It was possible to compare the inhibition of TPO activity by the crude extract with that predicted from the measured isoflavone content. The extract aliquot contained 0.99 μ g genistein and 0.37 μ g daidzein, and these amounts are predicted to produce approximately 67% inhibition of

TABLE 1. Inhibition of TPO-catalyzed coupling in iodinated casein by genistein

Genistein (μM)	T_4 Residues (% control, average, $N = 2$)
0	100
1	93.8
2	57.0
4	32.7
8	23.8
20	26.3

TPO (20 nM) was incubated with chemically iodinated casein (1.25 mg/mL), alkaline phosphatase, various concentrations of genistein, and H_2O_2 (100 μM) for 1 hr at $37 \pm 0.1^\circ$. Duplicate 1-mL reaction mixtures were digested with proteases, and the thyroid hormones were extracted and then quantified using HPLC as described in Materials and Methods. The content of iodothyronines present in iodocasein was below the detection limit (0.026 $\mu\text{g}/\text{mL}$ casein or 16 ng), and the amount of T_4 formed in the control incubations was 1.02 $\mu\text{g}/\text{mg}$ casein (0.85 newly formed residues per molecule).

TPO-catalyzed iodination in the 1.0-mL incubation. This compares closely with the 50% inhibition of TPO-catalyzed tyrosine iodination observed from addition of the soybean extract. These results also provide evidence that no major additional anti-thyroid compound is present in the soybean extract.

Genistein was also tested for the ability to inhibit iodothyronine formation by using iodinated casein, a model substrate that permits measurement of TPO-catalyzed coupling [16]. In this reaction, casein-bound iodothyrosyl residues are oxidatively converted by TPO in the presence of H_2O_2 to protein-bound T_4 and rT_3 . The formation of prominent amounts of rT_3 , but not T_3 , is not observed *in vivo* or in model systems that use human goiter Tg as a coupling substrate [18, 23]. However, in all experiments, rT_3 formation mirrored T_4 formation, and inhibition by anti-thyroid chemicals was similar for both products. Bovine milk casein is a complex multimeric aggregate (>300 kDa) consisting of subunits with an average monomer molecular mass of 23.3 kDa [24] and contains 3.3 tyrosyl residues/mol [25]. Before treatment with TPO, the amount of total iodothyronines detected by HPLC in hydrolyzed iodocasein was below the detection limit ($\text{T}_4 = 0.026 \mu\text{g}/\text{mg}$ casein or 16 ng on-column). In the absence of an inhibitor, TPO catalyzed the formation of approximately 1

μg T_4/mg casein (approximately 0.85 residues of T_4 , 0.35 residues of rT_3 , and ca. 0.17 residues of T_3 per mol of aggregated iodocasein assuming a molecular mass of 312 kDa, see Ref. 24) under the conditions described. Because the small amounts of T_3 observed were constant and unaffected by inhibitor concentration, we concluded that this resulted from artifactual deiodination of T_4 during sample preparation. Table 1 shows the concentration-dependent inhibition of T_4 synthesis in iodinated casein by genistein for duplicate experiments. Inhibition of rT_3 formation showed similar results (not shown). Using the combined data set for the two separate experiments, the IC_{50} value for genistein was approximately 3 μM .

Additional experiments were performed using human goiter Tg to measure the formation of thyroid hormones and its inhibition by isoflavones in a simultaneous iodination/coupling procedure [18]. When using Tg as the substrate, T_4 was the predominant product (1.06 ± 0.03 newly formed residue/molecule Tg), and only trace amounts of T_3 and rT_3 were observed. These studies confirmed the inhibition of coupling alone by genistein described above for iodinated casein. Genistein, daidzein, and genistin inhibited the formation of T_4 in a concentration-dependent manner (see Table 2), and the IC_{50} values for genistein, daidzein, and genistin were approximately 2.0, 8.8, and 40.6 μM , respectively. Genistein and daidzein also inhibited TPO-catalyzed oxidation of guaiacol. The IC_{50} values for these reactions were 0.7 and 12.4 μM , respectively.

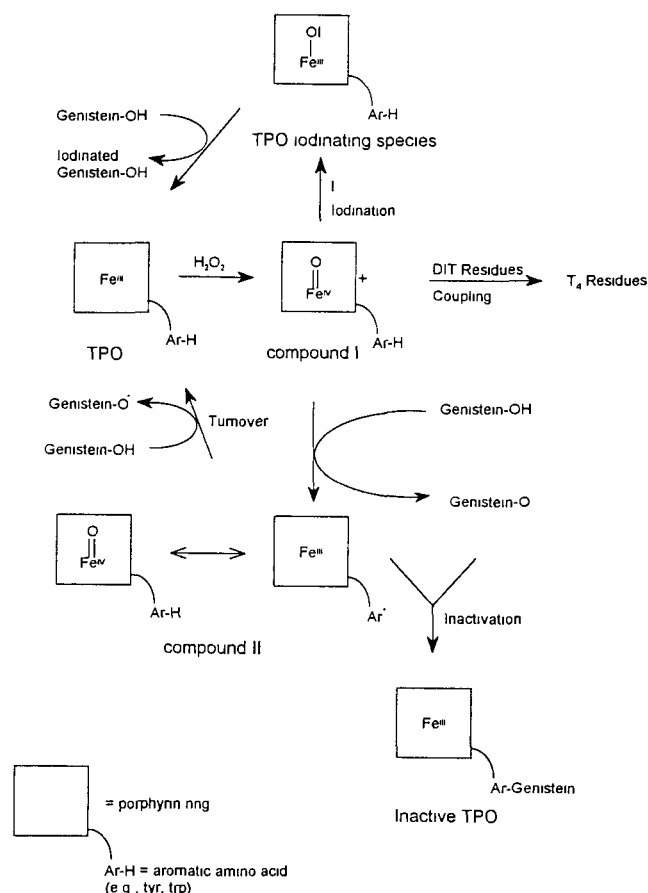
Characterization of Iodinated Isoflavones

Genistein and daidzein were potent inhibitors of TPO-catalyzed tyrosine iodination as described above. Genistein inhibition of TPO-catalyzed tyrosine iodination produced kinetics consistent with alternate substrate inhibition previously described for biochanin A [14]. In this mechanism (see Scheme 1), two-electron oxidation of iodide by TPO compound I produces an iodinating intermediate that is the equivalent of enzyme-bound hypoiodite [26]. Iodination of tyrosine by the enzymatic iodinating intermediate is blocked by isoflavones because of preferential iodination of

TABLE 2. Isoflavone inhibition of TPO-catalyzed coupling in human goiter Tg

Genistein (μM)	T_4 Residues (% control)	Daidzein (μM)	T_4 Residues (% control)	Genistin (μM)	T_4 Residues (% control)
0	100	0	100	0	100
1	52.4 ± 7.6	2.5	76.9 ± 6.5	10	98.3 ± 12.0
2.5	44.0 ± 8.0	5	58.5 ± 2.0	25	70.8 ± 10.8
5	19.8 ± 5.1	10	30.5 ± 4.8	50	46.9 ± 9.8
10	12.2 ± 2.4	20	12.2 ± 4.7	100	11.9 ± 0.7

TPO (24 nM) was incubated with KI (100 μM), 1.5 mM glucose, 5 mU/mL glucose oxidase, 0.76 μM Tg, and various concentrations of isoflavones for 1 hr at $37 \pm 0.1^\circ$. Triplicate 1-mL reaction mixtures were digested with proteases, and the thyroid hormones were extracted and then quantified using HPLC as described in Materials and Methods (means \pm SD). The content of iodothyronines present in the Tg was below the detection limit (0.02 newly formed residues or 16 ng), and the amount of T_4 formed in the control incubations was 1.06 ± 0.03 newly formed residue/molecule Tg.



SCHEME 1. Proposed mechanisms for genistein inhibition of TPO-catalyzed reactions.

the flavonoid, presumably due to its greater reactivity with the electrophilic enzyme species.

Figure 3 shows the conversion of genistein to three products under such conditions. Analysis of reaction products using on-line LC-APCI/MS gave mass spectra consistent with derivatives containing either one, two, or three iodine atoms. The positive ion mass spectra are characterized by abundant protonated molecules (MH^+) as observed for the parent isoflavone (see Fig. 4). In addition, the iodination products show fragment ions corresponding to losses of successive iodine atoms ($-I^- + H^+$, $\Delta m = 126$ Da) including the parent isoflavone. For example, the triiodo-genistein spectrum consists of MH^+ (m/z 649) and fragment ions corresponding to the protonated diiodo (m/z 523) and monoiodo derivative (m/z 397) as well as genistein (m/z 271). Similar results were obtained with daidzein where mass spectra contained MH^+ of m/z 381, 507, and 633, consistent with formation of mono-, di-, and triiodo-daidzein derivatives by TPO-mediated iodination (not shown). These results are similar to those obtained from LC-APCI/MS analysis of TPO-catalyzed iodination of biochanin A to mono- and diiodinated derivatives. In that study, 1H -NMR was also used to determine that the resorcinol moiety was the site for mono- and diiodination [14]. By analogy, we propose that genistein is mono- and

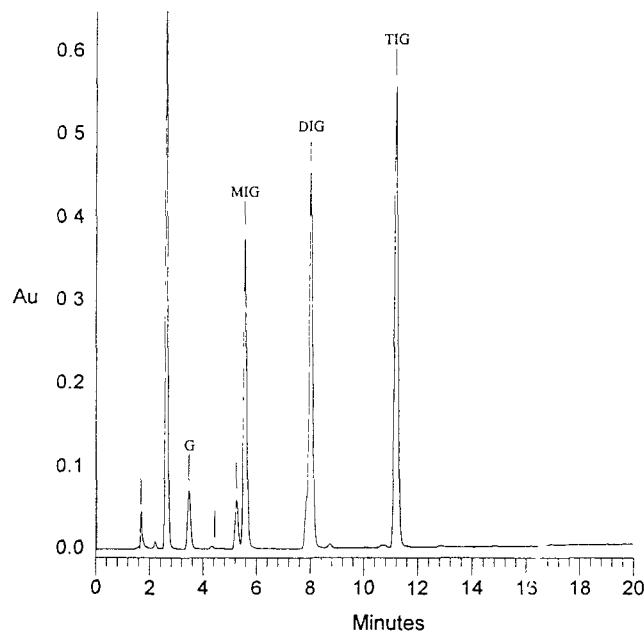


FIG. 3. TPO-catalyzed iodination of genistein. Genistein (25 μM) was incubated with TPO (50 nM) and iodide (100 μM) as described in Materials and Methods. The reaction was initiated by the addition of H_2O_2 (250 μM) and after 5 min the reaction mixture was analyzed using HPLC with UV 275 nm detection. Abbreviations: G, genistein; MIG, monoiodo-genistein; DIG, diiodo-genistein; and TIG, triiodo-genistein.

diiodinated in the resorcinol ring (C6,8), and the third site for iodination is presumably the ortho position on the phenol ring (C3').

Inactivation of TPO by Isoflavones

In the presence of H_2O_2 , genistein and daidzein inactivated TPO by 60–70% (see Table 3). However, the glucoside genistin produced no loss of enzymatic activity under these conditions (data not shown). The inactivation was time dependent, increased with increasing concentrations of isoflavone or H_2O_2 , and was enhanced when the incubation was conducted under anaerobic conditions. The inactivation was concomitant with a 60–80% loss of Soret absorbance and a red shift of the absorbance maximum from 412 nm (native TPO) to 415 nm (genistein-inactivated TPO) or 419 nm (daidzein-inactivated TPO) (Fig. 5). The presence of at least 150 μM iodide in the incubation mixtures completely protected against genistein- or daidzein-mediated TPO inactivation (see Table 3).

DISCUSSION

The results presented here demonstrate that the aglycones genistein and daidzein are the compounds contained in a hydrolyzed extract of soybean that inhibit TPO-catalyzed reactions. The hydrolytic procedure was required to convert the predominately conjugated isoflavones present in soybean to aglycones as previously described [19, 20]. The

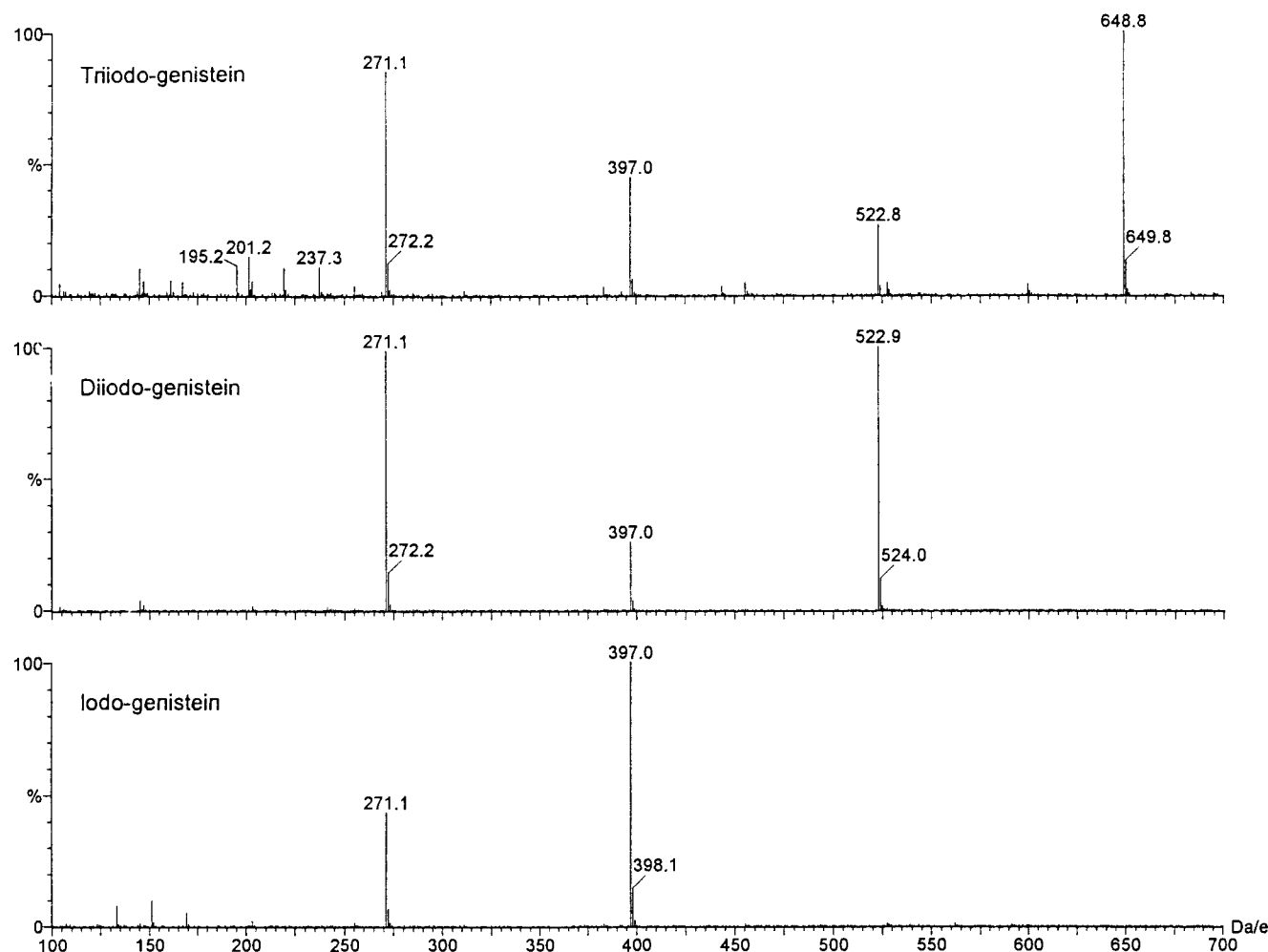


FIG. 4. Mass spectra of iodinated isoflavones. Incubations of TPO with genistein were conducted as described in Fig. 4 except LC-APCI/MS with a sampling cone-skimmer potential of 15 V was used to generate mass spectra of the genistein-derived products.

extract was fractionated by a reversed-phase HPLC separation (see Fig. 1), and the peaks containing inhibitory activity had chromatographic and spectroscopic properties (UV, APCI/MS) identical to authentic genistein and

daidzein (see Fig. 2). All of the TPO inhibitory activity present in the extract was accounted for by the measured amounts of genistein and daidzein. Determining total isoflavones as the respective aglycone after hydrolysis can only give the maximum possible anti-thyroid potential of soy products because glucoside conjugates, which are the predominant forms in soybean, are weakly inhibitory. The mixture of glucoside conjugates and aglycones present in soybean have been shown to be bioavailable through identification of glucuronide and sulfate conjugates of isoflavones in plasma from humans consuming soy products [22]. This suggests that the conjugates are hydrolyzed during absorption from the gut as the aglycone. However, this does not give information about uptake into the thyroid, another critical factor for assessing goitrogenic potential that must be determined in future studies *in vivo*.

Genistein also inhibited TPO-catalyzed phenolic oxidations including guaiacol oxidation and coupling of diiodotyrosyl residues in casein and thyroglobulin to form iodothyronines (see Tables 1 and 2). These reactions proceed by phenoxyl radical intermediates, and the pres-

TABLE 3. H_2O_2 -dependent inactivation of TPO by isoflavones

Reaction conditions	Activity (% of control)
TPO	99 ± 3
TPO + H_2O_2	96 ± 7
TPO + daidzein	97 ± 6
TPO + genistein	95 ± 5
TPO + daidzein + H_2O_2	38 ± 9
TPO + genistein + H_2O_2	29 ± 7
TPO + I^-	97 ± 5
TPO + daidzein + I^- + H_2O_2	94 ± 9
TPO + genistein + I^- + H_2O_2	95 ± 9

TPO (10 μ M) was incubated with 50 μ M daidzein or 50 μ M genistein, 150 μ M iodide ion, and 200 μ M H_2O_2 in the combinations indicated at $25 \pm 0.1^\circ$ in 0.1 M MES buffer (pH 7.0). After 4 min, aliquots were withdrawn and diluted 1000 to 2000-fold, and the tyrosine iodination activity was measured. The values are means \pm SD (N = 6). Under these conditions, maximal TPO activity was 9 nmol MIT formed/min.

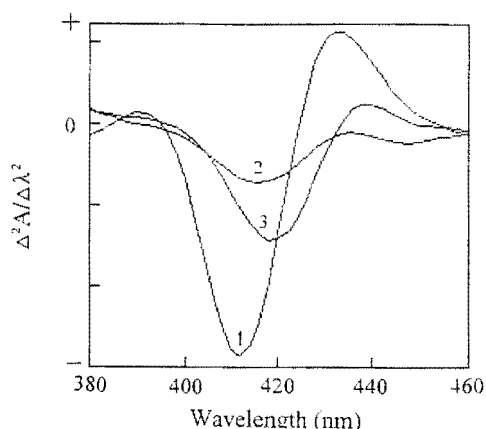


FIG. 5. Soret spectra for TPO and isoflavone-inactivated TPO. TPO (1 μ M) was incubated with genistein or daidzein (50 μ M) and H_2O_2 (200 μ M) as described in Materials and Methods. After a 4-min incubation, second-derivative visible spectra were recorded. The spectra are shown for native TPO (1), genistein-inactivated TPO (2), and daidzein-inactivated TPO (3).

ence of phenolic groups in the isoflavones would be predicted to react with oxidized enzyme species, Tg radical intermediates, or both, to block coupling [14, 18, 27]. The IC_{50} for inhibition of coupling in a simultaneous iodination/coupling assay using Tg was also similar to that observed in a coupling-only assay system using previously iodinated casein as the substrate. Because of the ability of the thyroid both to produce Tg and to concentrate iodide, it is likely that coupling *in vivo* occurs in a manner more comparable to the simultaneous assay. Furthermore, the IC_{50} values obtained for isoflavones were similar for inhibition of iodination and coupling. This suggests a primary interaction between isoflavone and TPO that affects coupling and iodination activity in a similar manner. Furthermore, the levels of total isoflavones observed in human plasma following consumption of soy foods (ca. 1 μ M, see Ref. 22) approach the concentrations required for inhibition of TPO-catalyzed reactions.

In the absence of iodide, genistein and daidzein caused time-dependent, irreversible inactivation of TPO concomitant to distinct changes in the visible spectrum of the heme prosthetic group (see Fig. 5). These observations are similar to those made for inactivation of TPO by other flavonoids (naringenin, quercetin, morin, and kaempferol; see Ref. 14) although the spectra of inactivated TPO have different long wavelength absorption maxima (428 nm for naringenin or kaempferol; 419 nm for daidzein; 415 nm for genistein). These data are consistent with the suicide inactivation mechanism for resorcinol and related flavonoids previously proposed for TPO, LPO, and CcP [14, 28]. Despite the dramatic changes in the heme visible spectrum, the results to date are consistent with enzyme inactivation through covalent binding to the polypeptide chain and not to the heme prosthetic group (see Scheme 1 and Ref. 29).

The inhibition of TPO-catalyzed iodination and coupling *in vitro* is consistent with the numerous reports of anti-thyroid effects in humans and animals from consumption of soy products, especially in cases of iodine deficiency. Many issues regarding the bioavailability of soy isoflavone conjugates and uptake into the thyroid remain unanswered. However, the demonstrated effects of the aglycones presented here, and the well-documented goitrogenic effects of soybeans in humans and animals, do provide a logical starting point from which possible anti-thyroid mechanisms can be examined. The different mechanisms reported for inhibition *in vitro* of the enzymatic reactions in thyroid hormone biosynthesis by isoflavones are useful for predicting potential anti-thyroid effects in animals and humans under several different dosing circumstances:

(A) In the normal case of iodine-sufficient individuals receiving intermittent or low doses of soy isoflavones, alternate substrate iodination would consume the inhibitory compounds after which Tg iodination and coupling reactions would resume unaffected. Since the normal thyroid contains significant amounts of iodide, its high substrate activity should prevent inactivation of TPO.

(B) In the case of iodine deficiency, low or intermittent doses of isoflavones could further deplete iodide levels by covalent incorporation of iodide into iodinated products. Also, enzymatic oxidation of the isoflavone would increase as the intrathyroidal iodide level decreased. Under these conditions, it is possible that inactivation of TPO could occur. This would produce a more long-lasting inhibition of hormone synthesis because enzymatic activity could be replaced only through *de novo* protein synthesis. However, either the alternate substrate inhibition or enzyme inactivation outcome is consistent with the anti-thyroid effects from soy observed in rodents maintained on an iodide-free diet [7, 9] and with the ability of added iodide to reverse the goitrogenic effect of a soybean diet in rats [7]. There are also reports of goiter and hypothyroidism in human infants receiving soy-based formulas [1–4] and evidence for elimination of such effects upon addition of iodide to the diet [3]. For this reason, it appears that iodide supplementation of formulas during manufacturing was implemented [1].

(C) In proposed rodent carcinogenicity bioassays, high doses of isoflavone will be administered chronically in a normal iodide-containing diet. Under these conditions, complete blockade of iodination and coupling is possible even with normal dietary iodide because alternate substrate inhibition would dominate. Since the body burden of isoflavone is replenished continually through feeding, the inhibition of thyroid hormone synthesis would persist throughout the lifetime of the animal. This hypothesis is consistent with observations of the hypothyroid condition that occurs in humans consuming principal foodstuffs (e.g. millet) that contain large amounts of anti-thyroid flavonoids [30, 31]. It is possible that these anti-thyroid effects could persist even if normal levels of iodide were present in the diet through universal iodination programs.

Under either condition B or C, the inhibition of thyroid hormone synthesis would increase TSH levels and could eventually induce thyroid follicular hyperplasia and tumors [12, 32]. Tumor formation through this nongenotoxic, hormonal process results from the growth stimulus provided by TSH, which can provide the selective environment for a transformed phenotype [12]. The observation of metastatic thyroid tumors in iodine-deficient rats, but not in iodide-supplemented rats, receiving a defatted soybean diet [21] is consistent with this proposal. The role of TSH in mediating thyroid tumors is well-documented in rats, but the importance in humans is unclear [32].

Finally, other possible toxicological consequences from ingestion of soy isoflavones come from the demonstrated estrogen receptor binding activity of genistein and daidzein [33, 34]. Although anti-carcinogenic properties from soybean isoflavones have been suggested [13, 35], the dose-response relationships that may separate toxic and beneficial responses are not clear. A possible consequence of TPO-mediated isoflavone iodination is modification of such estrogenic receptor binding activity or changing the pharmacokinetics for elimination. For example, McCague *et al.* [36] reported increased estrogen receptor binding affinity as well as a decrease in metabolism for 4-iodotamoxifen relative to the parent compound. Further experimentation will be required to assess the importance of TPO-mediated iodination in the biological activity and excretion of isoflavones.

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Inhibition of Thyroid Peroxidase by Dietary Flavonoids

Rao L. Divi[†] and Daniel R. Doerge*

National Center for Toxicological Research, Jefferson, Arkansas 72079

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Flavonoids are widely distributed in plant-derived foods and possess a variety of biological activities including antithyroid effects in experimental animals and humans. A structure-activity study of 13 commonly consumed flavonoids was conducted to evaluate inhibition of thyroid peroxidase (TPO), the enzyme that catalyzes thyroid hormone biosynthesis. Most flavonoids tested were potent inhibitors of TPO, with IC_{50} values ranging from 0.6 to 41 μ M. Inhibition by the more potent compounds, fisetin, kaempferol, naringenin, and quercetin, which contain a resorcinol moiety, was consistent with mechanism-based inactivation of TPO as previously observed for resorcinol and derivatives. Other flavonoids inhibited TPO by different mechanisms, such as myricetin and naringin, showed noncompetitive inhibition of tyrosine iodination with respect to iodine ion and linear mixed-type inhibition with respect to hydrogen peroxide. In contrast, biochanin A was found to be an alternate substrate for iodination. The major product, 6,8-diiodo-biochanin A, was characterized by electrospray mass spectrometry and ¹H-NMR. These inhibitory mechanisms for flavonoids are consistent with the antithyroid effects observed in experimental animals and, further, predict differences in hazards for antithyroid effects in humans consuming dietary flavonoids. In vivo, suicide substrate inhibition, which could be reversed only by de novo protein synthesis, would be long-lasting. However, the effects of reversible binding inhibitors and alternate substrates would be temporary due to attenuation by metabolism and excretion. The central role of hormonal regulation in growth and proliferation of thyroid tissue suggests that chronic consumption of flavonoids, especially suicide substrates, could play a role in the etiology of thyroid cancer.

Introduction

Flavonoids (see Figure 1) are a class of naturally occurring, low molecular weight benzo- γ -pyrone derivatives ubiquitously distributed in the plant kingdom. Common human and animal foods contain from traces to several grams of flavonoids per kilogram fresh weight (1). Flavonoids display diverse biological and pharmacological properties, e.g., anti-inflammatory, antiallergic, antiviral, pro- or antimutagenic, pro- or anticarcinogenic, antibacterial, and antioxidant effects (2). Flavonoids inhibit many enzymes including thyroid peroxidase (TPO)¹ (3) and 5'-deiodinase (4), the key enzymes of thyroid hormone synthesis, aldose reductase (5), ATPases (6), neutrophil NADPH oxidase (7), and phosphodiesterases (8). Consumption of flavonoids by experimental animals reduces both iodide ion uptake and iodide ion incorporation into thyroid hormones (9, 10). In vitro, several flavonoids reduce iodide ion uptake as well as incorporation in porcine thyroid slices and inhibited TPO-dependent iodination (3, 11). These data are consistent

with the antithyroid effects of flavonoids observed in humans (goiter) and experimental animals (12, 13); however, the mechanism by which flavonoids block thyroid hormone synthesis is not known. The present study evaluates the effects of 13 commonly consumed flavonoids on TPO to elucidate these mechanisms.

Materials and Methods

Baicalin, biochanin A, catechin, fisetin, flavanone, flavone, and guaiacol were purchased from Aldrich Chemical Co. (Milwaukee, WI); kaempferol, morin, myricetin, naringenin, naringin, quercetin, rutin, horseradish peroxidase, chloroperoxidase, and lactoperoxidase were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine myeloperoxidase (MPO) was obtained from ExOxEmis Co. (San Antonio, TX), and yeast cytochrome c peroxidase (CcP) was a generous gift from Dr. James Erman, N. Illinois State University. The concentration of H₂O₂, obtained from Sigma, was determined by iodometric titration (14), and dilutions were made daily. All chemicals were used without further purification. Stock solutions of the flavonoids were prepared by dissolution in ethanol just before use.

Porcine TPO was purified according to the method of Van Zyl and Van Der Walt (17) with certain modifications. Porcine thyroid glands obtained from a local slaughterhouse were freed from extraneous matter and sliced into small pieces. The slices were homogenized in 50 mM Tris-HCl and 0.5 mM KI buffer (pH 8.0) containing catalase (500 units/L), and microsomes were prepared by the method of Hosoya and Morrison (16). The microsomes were incubated with 0.25% sodium deoxycholate and 5 mM CHAPS in Tris-HCl-KI buffer at 4 °C for overnight. The detergent-solubilized suspension was centrifuged at 100000g for 1 h, and the supernatant was fractionated between 25% and 55% ammonium sulfate saturation. The precipitate was dis-

* Corresponding author, at Tel (501) 543-7943, FAX (501) 543-7720, E-mail DDOERGE@FDANT.NCTR.FDA.GOV.

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¹ Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CPO, chloroperoxidase; CcP, cytochrome c peroxidase; ES/MS, electrospray ionization mass spectrometry; HRP, horseradish peroxidase; IC₅₀, concentration producing 50% inhibition of activity; LPO, bovine lactoperoxidase; MIT, 3-iodotyrosine; MPO, bovine myeloperoxidase; TPO, porcine thyroid peroxidase.

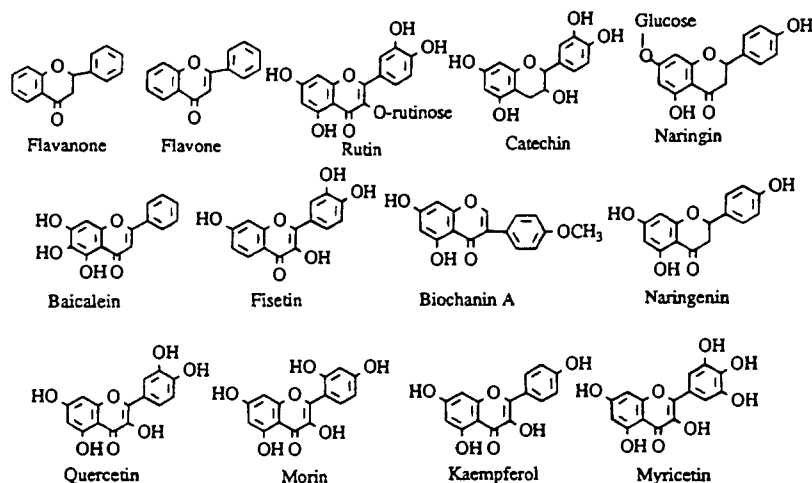


Figure 1. Flavonoid structures.

solved in Tris-HCl-KI buffer containing catalase and dialyzed against the same buffer. The dialyzed enzyme preparation was subjected to trypsin (0.02%) digestion for 1 h at room temperature. The digestion was stopped with soybean trypsin inhibitor (0.02%), the mixture was centrifuged at 100000g for 1 h, and the supernatant was fractionated again with ammonium sulfate as described above. The precipitate from ammonium sulfate fractionation was dissolved in and dialyzed against 0.02 M phosphate buffer (pH 6.8) containing 0.1 mM KI and catalase (500 units/L). The enzyme solution was concentrated by ultrafiltration (MWCO = 30 kDa) and subjected to gel filtration on a Bio-Gel A 1.5 m column (2.0 × 40 cm). The column was equilibrated and eluted with 0.02 M phosphate buffer (pH 6.8). The fractions containing guaiacol oxidation activity were pooled and rechromatographed on a hydroxylapatite column (1.8 × 20 cm) equilibrated with 0.02 M phosphate buffer (pH 6.8) containing 0.1 mM KI. The enzyme was eluted with a linear gradient of 0.02–0.25 M phosphate buffer (pH 6.8) containing 0.1 mM KI. The inclusion of catalase in the buffer during homogenization and initial purification, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), a zwitterionic surfactant, for solubilization greatly increased the yield (ca. 6 mg of purified enzyme/kg thyroid slices) of the purified enzyme. The R_z value of the enzyme preparations used in the current studies ranged from 0.32 to 0.42. The concentration of the enzyme was measured spectrophotometrically by using the extinction coefficient $1.12 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ at 412 nm (17). Guaiacol oxidation activity was determined at pH 7.0 as described previously (18).

Inhibition Assays. TPO (2.0 nM) was incubated with tyrosine (150 μM), iodide ion (150 μM), and hydrogen peroxide (50 μM) with different fixed concentrations of flavonoid, and iodination to 3-iodotyrosine (MIT) was monitored spectrophotometrically at 289 nm. MIT was quantified using HPLC for those flavonoids with absorbance maxima that interfered with spectrophotometric monitoring. It was determined that MIT formation rates were identical within experimental error whether determined by the UV or HPLC methods, although the UV method was more precise. HPLC analysis was performed immediately using a Radial Compression Module equipped with a NovaPak C18 cartridge (5 × 100 mm, 4- μm particle size, Waters Associates, Milford, MA) using UV detection at 289 nm essentially as described earlier (18).

Inactivation Experiments. TPO or lactoperoxidase (LPO, 1.0 μM) was preincubated with the flavonoid (100–150 μM) for 1 min prior to initiation of the reaction by the addition of hydrogen peroxide (200 μM). After 4 min of incubation, aliquots were removed and diluted 1000- to 2000-fold, and the residual enzyme activity was measured using the tyrosine iodination assay as described previously (19). It was determined that, following dilution, the concentration of flavonoid remaining had a minimal effect on the enzyme activity assay. A minimum of triplicate analyses was performed.

Time Course of Inactivation of TPO. Those flavonoids that inactivated TPO by more than 50% in the above experiment were studied further. TPO (1.0–5.0 μM) was incubated with kaempferol, 8–40 μM ; morin, 10–50 μM ; naringenin, 6–30 μM ; or quercetin, 10–50 μM at $25 \pm 0.1^\circ\text{C}$ in 0.1M phosphate buffer (pH 7.4), and hydrogen peroxide (200 μM) was added to initiate the reaction. Aliquots were withdrawn at various time points and diluted 1000- to 2000-fold, and the remaining enzyme activity was determined. The half-times for inactivation were estimated graphically from plots of activity remaining vs time (cf. Figure 3 inset and ref 20). The inactivation of TPO by the solvent ethanol (final concentration <2%, v/v) and H_2O_2 was minimal during the incubation period.

Hydrogen Peroxide Concentration Dependence of Inactivation. TPO (1.0 μM) was incubated with excess naringenin (150 μM), and enzyme activity was titrated by addition of varying but limiting concentrations of hydrogen peroxide. After 4 min of incubation, aliquots were withdrawn and diluted, and residual activity was measured. Soret spectral changes were monitored by second derivative UV-vis spectrophotometry to maximize sensitivity using a Hewlett-Packard 8452A diode array spectrophotometer as previously described (19).

Steady-State Kinetics of Inhibition of TPO-Catalyzed Tyrosine Iodination by Myricetin and Naringenin. The inhibition of TPO (1.25–2.5 nM) by myricetin (0–2.0 μM) or naringenin (0–6.0 μM) was studied in the presence of varying concentrations of either iodide ion (0–100 μM) or H_2O_2 (0–160 μM) at fixed concentrations of H_2O_2 (25 μM) and iodide ion (150 μM), respectively. MIT formation was monitored spectrophotometrically at 289 nm.

Iodination of Biochanin A. TPO (2.5 nM) was incubated with varying concentrations of biochanin A (0–50 μM) at a fixed concentration of iodide ion (250 μM) in 0.1 M phosphate buffer (pH 7.4) at $25 \pm 0.1^\circ\text{C}$, and the reaction was started by addition of hydrogen peroxide (50 μM). The increase in absorbance at 290 nm was monitored for 1–2 min spectrophotometrically.

Competitive iodination kinetics were determined in the presence of an enzymatic hydrogen peroxide-generating system. Biochanin A, TPO (2.5 nM), iodide ion (150 μM), tyrosine (150 μM), glucose (1.25 mM), glucose oxidase (10 nM), and biochanin A (0–20 μM) were incubated in 0.1 M phosphate buffer (pH 7.4) at $25 \pm 0.1^\circ\text{C}$. The reaction was followed by monitoring MIT formation.

For determination of reaction products, biochanin A (50 μM) was incubated with TPO (2.5 nM), iodide ion (0.4 mM), and H_2O_2 (50 μM) at $25 \pm 0.1^\circ\text{C}$ in 0.1 M phosphate buffer (pH 7.4). After 3 min of incubation, biochanin A and its products were extracted with 2 volumes of ethyl acetate. This was repeated twice before concentrating the combined aliquots of the ethyl acetate extract by Speed Vac (Savant Instruments Inc., Farmingdale, NY). The concentrate was reconstituted with 400 μL of HPLC mobile phase. HPLC analysis of 20–40 μL aliquots was performed

Table 1. Effect of Flavonoids on TPO Activity^a

compound	IC ₅₀ (μM) ^b	% inactivation ^c
flavanone	>1500	2.1 ± 0.13
flavone	>2000	6.8 ± 0.34
rutin	40.6 ± 3.88	36.5 ± 4.26
catechin	36.4 ± 3.86	42.9 ± 6.32
naringin	12.6 ± 1.56	2.3 ± 1.52
baicalein	8.3 ± 0.93	8.3 ± 1.76
fisetin	6.3 ± 0.63	31.1 ± 3.97
biochanin A	6.2 ± 0.84	14.6 ± 2.01
naringenin	2.7 ± 0.99	72.4 ± 5.89
quercetin	2.4 ± 0.64	63.9 ± 12.59
morin	2.1 ± 0.82	52.2 ± 6.43
kaempferol	1.2 ± 0.48	76.9 ± 10.67
myricetin	0.6 ± 0.18	8.9 ± 1.02

^a The assay system containing TPO (2.0 nM), tyrosine (0.15 mM), iodide ion (0.15 mM), and the appropriate concentration of test compound was preincubated in phosphate buffer (pH 7.4) for 2 min. The conditions for HPLC analysis are described in Materials and Methods. ^b IC₅₀ values (mean ± SD) are based on 2–5 experiments in which the compounds were tested at 8–12 concentrations in duplicate. ^c Inactivation experiments were carried out in the presence of TPO (1.0 μM), test compound (150 μM), and H₂O₂ (200 μM) at 25 °C in 0.1 M phosphate buffer (pH 7.4). After 2–3 min of incubation, aliquots of the reaction mixture were withdrawn and diluted 1000 to 2000-fold, and tyrosine iodination activity was measured spectrophotometrically.

using a reversed phase column (3 cm × 2 mm, 3 μm particle size, Perkin Elmer, Norwalk, CT) using 60% acetonitrile in H₂O with a flow rate of 1.0 mL/min. The detection was carried out at 230 nm, and UV spectra (220–350 nm) were obtained using a Spectra Focus rapid scanning detector (Spectra Physics, San Jose, CA). Individual product peaks were collected for further analysis (see below).

The products of the biochanin A iodination were investigated by using ES/MS with a VG Platform mass spectrometer (Fisons Instruments, Altrincham, U.K.). The mass spectrometer was operated at the following conditions: capillary voltage 2.63 kV, HV lens voltage 0.57 kV, cone voltage 45–75 V, and source temperature 60 °C; and scanning was carried out at an *m/z* range of 100–600 at a rate of 1.49 s/scan for both positive and negative ion acquisitions. A 10 μL sample containing ca. 150 ng of the products (separated off-line by LC as described above) was introduced by flow injection analysis using a syringe pump delivering 50% acetonitrile in water at 10 μL/min.

The major product of biochanin A described above was further characterized by ¹H-NMR (Bruker AM1 spectrometer operated at 500.13 MHz) in CDCl₃.

Results

All of the flavonoids tested except flavanone and flavone inhibited tyrosine iodination by TPO, but with markedly different potencies (see Table 1). IC₅₀ values ranged from 0.6 to 40.6 μM with the following order of potency: myricetin > kaempferol > morin > quercetin > naringenin > biochanin A > fisetin > baicalein > naringin > catechin > rutin. Subsequent studies were undertaken to determine the mechanisms for the observed inhibitory effects.

Time-Dependent Inactivation of TPO by Flavonoids. Incubation of TPO with kaempferol, morin, naringenin, or quercetin in the presence of H₂O₂ showed time-dependent loss of enzyme activity in an apparent first-order process. No loss of activity was observed in the absence of H₂O₂, and the rate of inactivation was dependent on the concentration of the flavonoid. Inactivation was associated with spectral changes in the Soret absorbance band (Figure 2). Native TPO had an absorption maximum at 413 nm, whereas naringenin- or kaempferol-inactivated TPO had an absorption maxi-

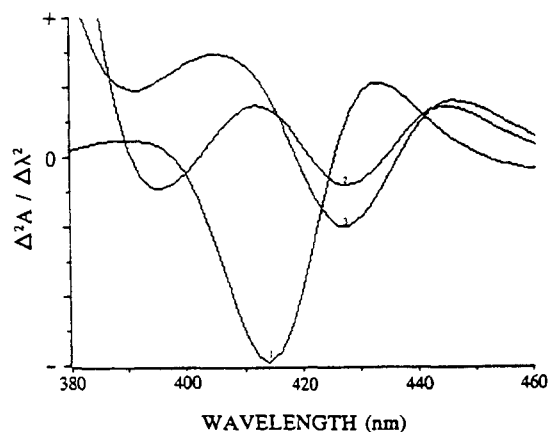


Figure 2. Second-derivative Soret spectra of native TPO and flavonoid-inactivated TPO. TPO (1.0 μM) was incubated with or without kaempferol (150 μM)/naringenin (150 μM) and H₂O₂ (200 μM). After 4 min of incubation, spectra were obtained between 380 and 460 nm. Spectra 1, 2, and 3 are native TPO, kaempferol-inactivated TPO, and naringenin-inactivated TPO, respectively.

mum at 428 nm (a red shift of 15 nm) and about 60–70% decrease in the Soret absorbance. Similar changes were observed during inactivation of the enzyme in the presence of catechin, fisetin, morin, or quercetin. In contrast, analogous incubations with flavanone, myricetin, or naringin resulted in no spectral changes. Control experiments in which the flavonoid was omitted from the incubation mixture resulted in no change in absorbance. It is likely that the 390/393 nm peaks observed were due to the presence of oxidized flavonoids. The levels formed were low in these cases because of the enzyme inactivation that occurred; however, with phenol and pyrogallol, which are good substrates, prominent absorptions in this spectral region are observed (data not shown). As predicted for mechanism-based inhibitors (20), a linear relationship was observed between the concentration of kaempferol, morin, naringenin, or quercetin versus the product of inactivation half-times and the flavonoid concentration. Values for *k_i* and *K_i* ranged from 1.4 to 6.7 and 6.2 to 12.9, respectively. A typical plot obtained with naringenin is shown in Figure 3. The saturation kinetics observed are consistent with formation of a reversible complex between enzyme and the flavonoid prior to inactivation. The apparent enzyme-inhibitor dissociation constants (*K_i*, Table 2) for the flavonoids are approximately one-half to one-third the apparent *K_m* value for iodide ion (22.4 μM, tyrosine iodination) determined under similar conditions for TPO. The apparent maximal inactivation rate constants (*k_i*) for these flavonoids are ca. 700- to 3000-fold lower than the turnover number for the normal enzyme reaction with iodide ion (tyrosine iodination, ~4800 min⁻¹).

Figure 4 shows the titration of TPO activity by varying limiting concentrations of H₂O₂ in the presence of naringenin under aerobic or minimal oxygen conditions. Inactivation was increased by hypoxia at all H₂O₂ concentrations tested. Extrapolation of the linear portion of the curves yielded approximate partition ratio values of 8 and 42, respectively, in hypoxic and aerobic conditions. Addition of other TPO substrates (e.g., pyrogallol, iodide ion) reduced the inactivation of TPO by kaempferol (see Table 3). The presence of pyrogallol, a potent substrate for TPO, in the reaction mixture at ≥150 μM completely protected the enzyme from inactivation by kaempferol. The presence of iodide ion (5.0 mM) in the

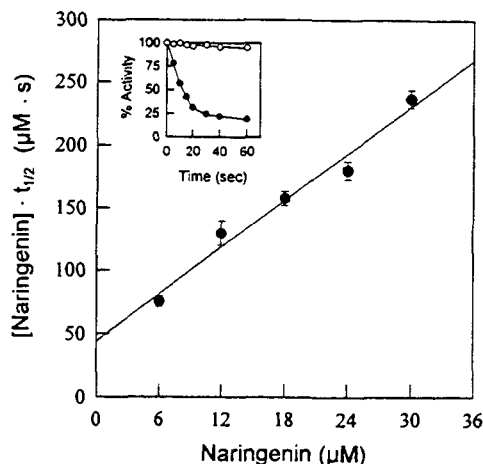


Figure 3. Kinetics of inactivation of TPO by naringenin. Inset: TPO was incubated with (●) naringenin (6 μ M) or without (○) naringenin and hydrogen peroxide (200 μ M) at 25 ± 0.1 °C in phosphate buffer (pH 7.4), and the activity remaining at various time points was determined ($n = 6$). Half-times for inactivation of TPO (1.0–5.0 μ M) by naringenin (6.0–30.0 μ M) were determined graphically from the time course of inactivation.

Table 2. Kinetic Constants for the Inactivation of TPO by Flavonoids^a

compound	K_i (μ M)	k_i (min ⁻¹)
kaempferol	6.23	2.64
morin	12.87	1.36
naringenin	7.06	6.68
quercetin	7.09	1.69

^a Reaction mixtures consisting of TPO (1.0–5.0 μ M), kaempferol (8.0–40.0 μ M)/morin (10.0–50.0 μ M)/naringenin (6.0–30.0 μ M)/quercetin (12.0–60.0 μ M), and H_2O_2 (0.2 mM) were incubated at 25 ± 0.1 °C in 0.1 M phosphate buffer (pH 7.4), and aliquots were withdrawn at various time points and residual activity of TPO was measured. The ratio of TPO/flavonoid was maintained at 0.125, 0.1, 0.167, and 0.083, respectively, for kaempferol, morin, naringenin, and quercetin. Half-time for inactivation was determined from the time course plots as previously described (19, 20).

reaction mixture partially protected the enzyme against inactivation.

Dialysis of inactivated TPO was performed in order to distinguish between covalent or reversible binding of naringenin or kaempferol. Dialysis of enzyme alone resulted in 2–8% loss of enzyme activity with little or no change in the spectral properties. Incubation of TPO with the flavonoids in the absence of H_2O_2 caused no significant changes in either activity or spectra compared to control incubation. However, in the presence of H_2O_2 , kaempferol or naringenin produced ~70% inactivation of TPO and ~65% decrease in the Soret absorbance that remained after dialysis.

Inactivation of Different Peroxidases by Naringenin/Quercetin. Table 4 lists the activities of different enzymes remaining after incubation of these enzymes with naringenin or quercetin in the presence of H_2O_2 . TPO, LPO, and CcP were found to be more susceptible to inactivation by quercetin compared to other enzymes, although significant inactivation of MPO and HRP was observed.

Effect of Myricetin and Naringin on the Kinetics of TPO-Catalyzed Tyrosine Iodination. Myricetin and naringin, the glycoside of naringenin, did not inactivate TPO, but low concentrations did inhibit tyrosine iodination. The iodide ion concentration dependence of

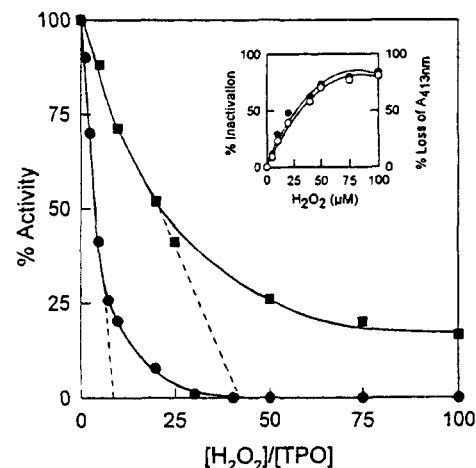


Figure 4. H_2O_2 dependence of inactivation of TPO by naringenin under aerobic and hypoxic conditions. TPO (1.0 μ M) was incubated with naringenin (150 μ M) in 0.1 M phosphate buffer (pH 7.4) at 25 ± 0.1 °C, and the enzyme activity was titrated by addition of limiting amounts of hydrogen peroxide (■). Minimal oxygen conditions (●) were maintained by flushing with nitrogen and keeping the solutions under nitrogen atmosphere during the reaction. Partition ratios were estimated by extrapolating the linear portion of the curves to the abscissa (42 and 8 μ M). Inset: Correlation between H_2O_2 added and the inactivation of TPO by naringenin (●) and loss of Soret absorbance (○).

Table 3. Inactivation of TPO by Kaempferol in the Presence and Absence of Alternate Substrates^a

reaction conditions	activity remaining (% of control)
TPO	99.2 \pm 3.17
TPO + H_2O_2	97.4 \pm 5.86
TPO + KAE	96.3 \pm 4.89
TPO + KAE + H_2O_2	18.2 \pm 8.96
TPO + I ⁻	96.8 \pm 4.59
TPO + PYG	99.6 \pm 3.92
TPO + KAE + I ⁻ + H_2O_2	63.8 \pm 8.46
TPO + KAE + PYG + H_2O_2	94.3 \pm 7.29

^a TPO (1.0 μ M) was incubated with 5.0 mM iodide ion, 150 μ M kaempferol (KAE), 200 μ M pyrogallol (PYG), and 200 μ M hydrogen peroxide (H_2O_2) in the combinations indicated at 25 ± 0.1 °C in 0.1 M phosphate buffer (pH 7.4) for 4 min, aliquots were withdrawn and diluted 1000- to 2000-fold, and the tyrosine iodination activity was measured. The values are mean \pm SD ($n = 6$).

Table 4. Inactivation of Various Peroxidases by Naringenin/Quercetin^a

peroxidase	% inactivation (naringenin)	% inactivation (quercetin)
LPO	73.2 \pm 2.36	61.4 \pm 6.32
TPO	82.7 \pm 3.18	73.3 \pm 4.96
MPO	48.5 \pm 1.45	51.8 \pm 6.32
CPO	24.8 \pm 9.83	17.1 \pm 10.53
CcP	24.6 \pm 15.43	61.0 \pm 8.67
HRP	2.0 \pm 8.25	43.7 \pm 7.93

^a Peroxidases (1.0 μ M) except CcP were incubated with naringenin or quercetin (150 μ M) and H_2O_2 (200 μ M) for 3 min at 25 °C in 0.1 M phosphate buffer (pH 7.4). In the case of CcP, 5.0 μ M of enzyme was incubated with 250 μ M test compound. Aliquots were withdrawn and diluted 1000-fold, and residual guaiacol oxidation activity was determined. The values given are mean \pm SD ($n = 4$). The activity in the absence of test compound, but containing 5% ethanol, was taken as 100%.

TPO-catalyzed tyrosine iodination was measured in incubations with myricetin and naringin. For both compounds, the inhibition was noncompetitive with respect to iodide ion concentration since V_{max} , but not K_m , was affected. The Dixon plots shown in Figure 5A,C

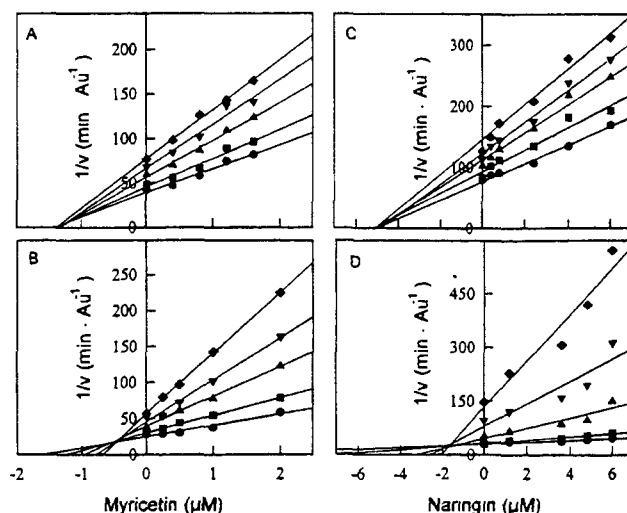


Figure 5. Dixon plots showing the inhibition of TPO-catalyzed tyrosine iodination by myricetin and naringin. (A) The incubation system contained 2.5 nM TPO, 150 μ M tyrosine, 12.5–100 μ M (\blacklozenge – \bullet) iodide ion, 25 μ M H_2O_2 , and 0–1.6 μ M myricetin. In (B) 2.5 nM TPO, 150 μ M tyrosine, 150 μ M iodide ion, 10–160 μ M H_2O_2 (\blacklozenge – \bullet) and 0–2.5 μ M myricetin were present. (C) 1.25 nM TPO, 150 μ M tyrosine, 25 μ M H_2O_2 , 12.5–166.7 μ M (\blacklozenge – \bullet) iodide ion, and 0–6.0 μ M naringin were present in the incubation mixture. In (D), 2.5 nM TPO, 150 μ M iodide ion, 150 μ M tyrosine, 5–80 μ M H_2O_2 (\blacklozenge – \bullet), and 0–6.0 μ M naringin were present.

yielded an apparent value for the enzyme–inhibitor dissociation constant, K_i , of 1.4 and 4.8 μ M, respectively, for myricetin and naringin when measured in incubations containing 25 μ M H_2O_2 . These K_i values are ca. 5- to 16-fold lower than the respective K_m for iodide ion.

The H_2O_2 concentration dependence of TPO-catalyzed tyrosine iodination was also measured in incubations with myricetin and naringin. The Dixon plots (Figure 5B,D) of the reciprocal rate of tyrosine iodination as a function of flavonoid concentration yielded families of curves intersecting above the x -axis. This pattern is consistent with either competitive or linear mixed-type inhibition (21), and an apparent K_i value of 0.54 and 1.7 μ M, respectively, for myricetin and naringin was obtained when measured at 150 μ M iodide ion. Replots of the Dixon plot slopes revealed that myricetin was a mixed-type inhibitor and naringin was a competitive inhibitor with respect to H_2O_2 concentration. The K_i values are 20- to 63-fold lower than the K_m determined for H_2O_2 (34 μ M).

Inhibition of TPO-Catalyzed Tyrosine Iodination by Biochanin A. Figure 6 shows the time course of iodination of tyrosine in the presence of different concentrations of biochanin A when glucose/glucose oxidase was used to produce a steady state concentration of H_2O_2 . The presence of biochanin A in the reaction mixture resulted in initial blockade of iodination followed by a linear increase in MIT formation after the lag phase. The length of the lag phase was dependent on the concentration of biochanin A. However, the rate of MIT formation following lag phase was unchanged from the control rate. TPO-catalyzed iodination of biochanin A (0–50 μ M) was measured at fixed concentrations of iodide ion (250 μ M) and H_2O_2 (50 μ M). Reactions were started by the addition of H_2O_2 to the reaction mixture and monitored spectrophotometrically at 290 nm. Reciprocal velocities ($1/\Delta A$ per minute) were plotted against the reciprocal of biochanin A concentration, and K_m and V_{\max} were deter-

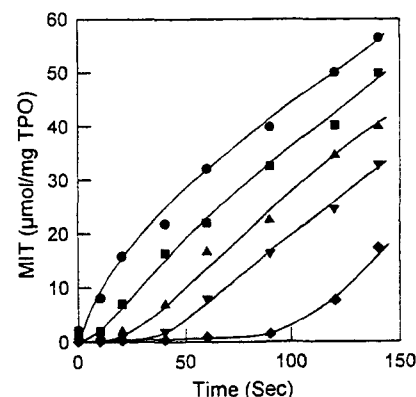
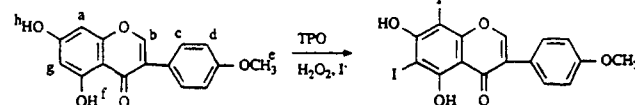


Figure 6. Time course of tyrosine iodination in the presence of biochanin A. The reaction was carried out in the presence of 2.5 nM TPO, 150 μ M iodide ion, 150 μ M tyrosine, 1.25 mM D-glucose, 10 nM glucose oxidase, and 0 μ M (\bullet), 4.0 μ M (\blacksquare), 8.0 μ M (\blacktriangle), 12.0 μ M (\blacktriangledown), or 20.0 μ M (\blacklozenge) biochanin A at 25 ± 0.1 °C. The reaction was started by addition of glucose oxidase to the reaction mixture. MIT formed was monitored by LC as described in Materials and Methods.

Scheme 1. Iodination of Biochanin A by TPO



mined using the Michaelis–Menten relationship to be 17.30 μ M and 0.0283 AU/min at a fixed concentration of iodide ion and H_2O_2 .

Characterization of Biochanin A Products Using ES/MS and NMR. TPO catalyzed the conversion of biochanin A into at least three products as determined by HPLC–UV. The peaks were collected off-line and analyzed by ES/MS in positive and negative ion modes. The major product (retention time 11.9 min) gave a deprotonated molecule at nominal mass 535 Da with prominent fragment ions at m/z 521 ($M - \text{CH}_2$) and 127 (I^-). In positive ion mode, the protonated molecule had a nominal mass of 537 Da, consistent with a diiodinated derivative. Both minor products were similarly analyzed and were consistent with isomeric monoiodinated [$(M - \text{H})^-$, m/z 409; $(M + \text{H})^+$, m/z 411]. Insufficient amounts of purified monoiodinated products precluded additional means of structure proof.

The structure of diiodinated product was further identified using ^1H -NMR. All proton resonances for biochanin A (see Scheme 1) were tentatively assigned as follows: 3.83 (s, 3H, H_a); 5.45 (bs, 1H, H_f); 6.28 (d, 1H, H_a , $J_{\text{H}_a, \text{H}_g} = 2.2$ Hz); 6.35 (d, 1H, H_g); 6.96 (d, 2H, H_d , $J_{\text{H}_c, \text{H}_d} = 6.7$ Hz); 7.43 (d, 2H, H_c); 7.84 (s, 1H, H_b); 12.90 (s, 1H, H_h). The assignments for H_c and H_d were confirmed by selective NOE irradiation experiments: NOE was observed for H_c upon irradiation of H_b and for H_d upon irradiation of H_c ; the resonances for H_a and H_g were assigned based on known effects of *o*-hydroxyl groups and the observed 2.2 Hz coupling. The assignment of H_f was based on the observed line broadening expected for a hydrogen bonded proton. H_h was assigned based on the expected acidity and its resulting extreme downfield resonance. The diiodinated product had similar proton resonances except that the two protons in the resorcinol moiety (H_a and H_g), and no others, were eliminated (data not shown).

Discussion

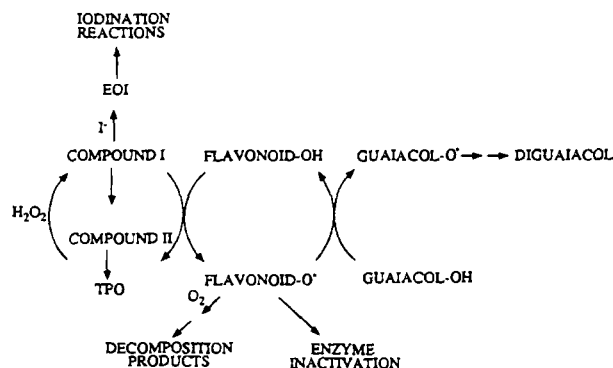
The flavonoids investigated in this study fit into several broad groups based on IC_{50} values for inhibition of TPO-catalyzed tyrosine iodination (see Table 1). While it is not valid to compare IC_{50} values for compounds with unknown inhibition mechanisms, the ability to differentiate grossly flavonoid potency was a useful screening procedure in the initial phase of this study. In previous work, we have demonstrated several mechanisms can be responsible for inhibition of TPO- and LPO-catalyzed reactions: suicide inactivation (19), rapid equilibrium (reversible) binding (22), and alternate substrate competition for the enzymatic iodinating intermediate (18, 23). These experimental approaches were used to determine inhibition mechanisms and structure-activity relationships for these typical dietary flavonoids.

When the more potent inhibitors were investigated further, it was seen that naringenin, quercetin, morin, and kaempferol caused irreversible inactivation of TPO (see Table 1, column 3). The kinetics of irreversible enzyme inactivation (see Table 2 and Figure 3), the altered visible absorbance spectrum of inactivated TPO (Figure 2), and the correspondence of spectral changes with loss of enzymatic activity (Figure 4) were consistent with mechanism-based inhibition as previously described for the action of resorcinol and derivatives on TPO and LPO (19). In the previously proposed mechanism, reactive resorcinol radicals are formed in the active site by compound I-mediated oxidation of the phenolic suicide substrate, and inactivation occurs by covalent binding of these radicals to catalytic amino acid radical(s) on compound II (19). This conclusion was supported by the observed binding of 10 mol of resorcinol/mol of LPO inactivated. The spectral changes observed for flavonoid-inactivated TPO (see Figure 2) are similar to those seen in resorcinol-inactivated TPO and LPO, suggesting a common mechanism of inactivation. At the present time, the cause for the observed spectral changes is not clear but may result from heme modification in addition to active site amino acid residues. The lack of commercial sources for radiolabeled flavonoids precluded further investigation of the covalent binding of flavonoids to TPO.

In agreement with our previous study, a free resorcinol moiety is present in all of the flavonoids (naringenin, quercetin, morin, kaempferol) that cause substantial inactivation (>40%) under the conditions (see Table 1). The smaller amounts of inactivation, observed for flavonoids that do not contain a resorcinol moiety (flavone, flavanone, baicalein, fisetin), were similar to the low level inactivation caused by the action of monophenolic radicals (e.g., guaiacol), as was observed in the previous study (19).

However, the presence of a resorcinol moiety in flavonoids was not sufficient to cause substantial TPO inactivation, as rutin, biochanin A, and myricetin were weak inactivators. Possible modifying factors for these compounds are as follows: the presence of a sugar moiety on the resorcinol hydroxyl group in naringenin completely blocked the inactivation observed for the aglycon, naringenin; the presence of a sugar moiety in the pyranone ring of rutin; the presence of a pyrogallol moiety in the 2-aryl substituent of myricetin appears to make this flavonoid a potent substrate and competitive inhibitor of TPO-catalyzed reactions; biochanin A, in which the aryl substituent is attached to the 3-position of the pyranone ring, is an alternate substrate inhibitor of iodination (see

Scheme 2. Proposed Formation and Reactions of Flavonoid Radicals



below); the change in going from a hydroxypyranone ring in kaempferol to a hydroxydihydropyran in catechin produced a marked reduction in IC_{50} and TPO inactivation; however, the same change from the hydroxypyranone in kaempferol to a dihydropyranone in naringenin produced a minimal change in inactivation potency.

Reversible (rapid equilibrium) inhibition of TPO-catalyzed tyrosine iodination was observed in the presence of myricetin and naringin. Figure 5 shows the Dixon plots for inhibition of tyrosine iodination in the presence of varying amounts of the two substrates, iodide ion (A, C) or H_2O_2 (B, D). The inhibition by myricetin and naringin was consistent with a noncompetitive mechanism with respect to iodide ion and linear mixed-type with respect to H_2O_2 (21). In both noncompetitive and linear mixed-type mechanisms, inhibition results from the binding of inhibitor and substrate to different sites or enzyme forms. A minimal mechanism consistent with these kinetics and the known properties of peroxidases is shown in Scheme 2. In this proposed mechanism, myricetin and naringin interact with TPO compounds I and II but not EOI (the enzymatic iodinating species) or native TPO. Further interpretation of the inhibition kinetics in this multisubstrate ping-pong system is beyond the scope of this discussion.

Figure 6 shows the effects of increasing amounts of biochanin A on TPO-catalyzed tyrosine iodination in the presence of a continuous source of H_2O_2 produced by glucose/glucose oxidase. As the concentration of biochanin A was increased, the length of the lag phase increased. Following the lag, tyrosine iodination resumed at the control rate. During the lag phase, or in the absence of tyrosine, biochanin A (λ_{max} 262, 322 nm) was converted to products with red-shifted UV absorption maxima (λ_{max} 280, 340 nm), and HPLC analysis demonstrated the formation of at least three products with altered UV spectra (data not shown). The identity of the major product was established by ES/MS and 1H -NMR as the diiodinated resorcinol derivative shown in Scheme 1. The two minor products observed by HPLC were found to be monoiodinated, presumably resulting from iodination of either position on the resorcinol ring. These data are consistent with alternate substrate inhibition of iodination as previously reported for inhibition of TPO-catalyzed iodination reactions by ethylenethiourea and N,N' -disubstituted benzimidazole-2-thiones (18, 23). In this mechanism, competition between tyrosine and the alternate substrate for the enzymatic iodinating species (EOI, see Scheme 2) results initially in complete blockade of tyrosine iodination because of the higher affinity for biochanin A. However, after the alternate substrate is

consumed, tyrosine iodination resumes at an unchanged rate.

Resorcinol-mediated inactivation was previously investigated for a series of peroxidases, and it was determined that TPO, LPO, and CcP were highly susceptible, but HRP and other peroxidases were resistant (19). These observations were consistent with the presence of catalytic amino acid radicals in the susceptible peroxidases but not in HRP. A similar trend was seen in the present study for naringenin, except that MPO was also significantly inactivated (see Table 4). This latter observation could be consistent with the suggestion that MPO also forms protein-centered radicals following reaction with H_2O_2 (24). Inactivation by quercetin was highest for TPO, LPO, and CcP, but significant inactivation of all peroxidases tested occurred, including HRP.

Most compounds tested inhibited the TPO-catalyzed oxidation of guaiacol with IC_{50} s of a magnitude similar to that seen for tyrosine iodination. However, low concentrations of myricetin and naringin ($<10 \mu M$) caused a stimulation of guaiacol oxidation, and the kinetics showed an increase in V_{max} (data not shown). These observations are consistent with preferential TPO-mediated oxidation, by either compound I or compound II, of the flavonoid to a phenoxyl radical that abstracts a hydrogen atom from guaiacol to form the guaiacol radical which produces the colored oxidation product, presumably a dimeric species (see Scheme 2, ref 25). Regeneration of the flavonoid completes a catalytic process that drives guaiacol oxidation at an increased rate due to higher enzymatic turnover of the flavonoid relative to guaiacol.

Other evidence for the participation of flavonoid radicals in TPO-mediated reactions is seen in Figure 4. It was observed that TPO inactivation by naringenin was enhanced when O_2 was removed from the incubation medium. Excluding O_2 decreased the partition ratio, the ratio between rate constants for suicide substrate turnover and inactivation (20), by ca. 5-fold from 42 to 8 μM . Similar results were observed with resorcinol (data not shown). These observations are consistent with a reaction between O_2 and the phenoxyl radicals formed by TPO-catalyzed oxidation that diverts the inactivating radical to a pathway leading to turnover of the suicide substrate and not enzyme inactivation (see Scheme 2). Similar reactions have been proposed for light emission that accompanies peroxidase-mediated metabolism of tetracycline and oxycams under aerobic conditions (26).

The inhibitory effects on TPO demonstrated for these dietary flavonoids are sufficient to explain the antithyroid effects reported in humans and experimental animals (12, 13). The different mechanisms identified here for TPO inhibition do, however, suggest differences in the potential hazards to humans consuming these compounds. In vivo, flavonoids that are TPO suicide substrates are likely to exert a long-lasting depression of thyroid hormone synthesis because de novo enzyme synthesis is required to restore lost activity. However, the inhibitory effects of flavonoids that are alternate substrate iodination inhibitors would be attenuated by TPO-catalyzed iodination to inactive products, and the effects of reversibly binding inhibitors would be attenuated by extrathyroidal metabolism and excretion. Therefore, intermittent or low-dose exposure to suicide substrates in the diet, but not alternate iodination substrates or reversible inhibitors, could have significant impact on thyroid hormone status. However, chronic exposure to any of these

flavonoid inhibitors, especially at high doses, could elicit prolonged blockade of thyroid hormone synthesis. Any compound that causes chronic inhibition of thyroid hormone synthesis is a *potential* thyroid carcinogen that acts by long-term stimulation of thyroid growth induced by elevated levels of thyroid stimulating hormone (TSH) (27). Although there is considerably less evidence of a role for TSH stimulation in the etiology of thyroid cancer in humans as opposed to experimental animals, three case-control studies in the U.S. consistently showed thyroid cancer was strongly associated with preexisting goiter and thyroid nodules (for a review, see ref 27). However, studies relating iodide intake levels and human thyroid cancer in endemic goiter regions of the world are equivocal (27). Nevertheless, our results do suggest that the consumption of foods rich in flavonoids, especially those that *inactivate* TPO, has the potential to induce goiter and in this manner may be involved in the etiology of human thyroid cancer.

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Breast Feeding and Insulin-Dependent Diabetes Mellitus in Children

P. Fort, MD, FACN, R. Lanes, MD, FACN, S. Dahlem, MD, B. Recker, RN,
M. Weyman-Daum, MS, RD, M. Pugliese, MD, FACN, and F. Lifshitz, MD, FACN

Department of Pediatrics, North Shore University Hospital, Manhasset and Department of Pediatrics, Cornell University Medical College, New York

We have evaluated the hypothesis of a protective effect of human milk on the development of insulin dependent diabetes mellitus (IDDM). We studied the feeding histories of 95 diabetic children and compared them with controls consisting of their non-diabetic siblings and a pair matched group of nondiabetic peers of the same age, sex, geographical location, and social background. The incidence of breast feeding in diabetic children was 18%. This was similar to the control group. The duration of breast feedings was also similar among all three groups. There was no difference in the age of introduction of solid food between diabetic and nondiabetic children. Twice as many diabetic children, however, received soy containing formula in infancy as compared to control children. The mean age of onset of IDDM was not related to the type of feeding during infancy. The incidence of positive thyroid antibodies was two and one half times higher in formula-fed diabetic children than in breast-fed ones. In our studies we were unable to document any relationship between the history of breast feeding and subsequent development of IDDM in children.

Key words: breast feeding, insulin dependent diabetes mellitus, soy containing formula, antithyroid antibodies

Real as well as potential medical and psychological benefits of breast feeding in early life have been well recognized [1,2]. Recently, a link between the incidence of insulin-dependent diabetes mellitus (IDDM) in children and the frequency of breast feeding has been reported [3]. It appeared that diabetic children were much more likely to have been formula-fed than breast fed or breast fed for a shorter period of time than nondiabetic control children. The authors suggested that breast feeding may be protective of subsequent development of IDDM during childhood. However, patients in these studies were not pair-matched with nondiabetic children. This would appear important, as the incidence of breast feeding has varied considerably over the years and in different locations. We have evaluated the hypothesis of protective effects of human milk on the development of IDDM in our population of diabetic children using their nondiabetic siblings and a pair-matched group of nondiabetic peers of the same age, sex, geographical location, and social background as controls.

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Address reprint requests to Pavel Fort, M.D., Department of Pediatrics, North Shore University Hospital, Manhasset, NY 11030.

TABLE 1. Results of Breast Feeding Studies

	IDDM children	Siblings of IDDM children	Controls
No. of subjects	95	194	95
No. of breast-fed subjects (%)	17 (18%)	35 (18%)	17 (18%)
Duration of breast feeding (months)	4.6	4.2	3.3
Onset of solids (months)	3.1	2.9	2.8

MATERIALS AND METHODS

Ninety-five children with IDDM who have been followed in our Diabetes Center at North Shore University Hospital and whose parents agreed to participate in the studies were evaluated. The mean age \pm SD of the diabetic patients was 14 10/12 \pm 5 6/12 years and the age \pm SD of onset of IDDM was 8 1/12 \pm 4 2/12 years. The parents of all children were given a questionnaire asking the following questions: 1) history of breast feeding in early life and if positive for how long; 2) if formula fed, the type of formula; and 3) the age at which solids were introduced to the diet. There were 194 siblings of diabetic children who did not have IDDM and whose feeding histories could be retrieved using a similar questionnaire. In addition, diabetic children and/or their parents were asked to forward a similar questionnaire to at least one friend of the same age, who originated and continued to live in the same geographical area with the same social background, and who did not have IDDM. There were 95 such children who were then matched for our diabetes patient population and who served as controls. This enabled us to obtain a well-matched, nondiabetic population of children, who were not only of similar age, but were from and have lived in the same area as their diabetic counterparts. There were 11 diabetic children who could not be matched or who changed their address; these patients were not included in the statistical analysis. The data were analyzed by a sign test and chi-square test.

RESULTS

The results of the study are summarized in Table 1. Seventeen out of the 95 IDDM children (18% incidence) were breast fed from early infancy, for various periods of time. Both the incidence and duration of breast feeding were similar to that seen in their siblings and in the nondiabetic matched controls (18% in the siblings and 18% in the controls). There was also no difference between the age at which solid food was introduced into the diet between IDDM and control children. Although the majority of formula-fed children were given either Enfamil or Similac, there were almost twice as many IDDM children, both breast fed and non-breast-fed, who received soy-containing formula in infancy as compared to nondiabetic controls. However, these differences were not statistically significant. The mean age of onset of IDDM was not related to the type of feeding during infancy. Thus, those who were breast fed were diagnosed to have IDDM at a similar age as those IDDM children who were formula fed (6 10/12 \pm 4 3/12 vs 8 4/12 \pm 4 2/12 years). The incidence of positive thyroid antibodies was 6% in breast-fed and 15% in formula-fed IDDM children; this difference was not statistically significant.

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DISCUSSION

The incidence of breast feeding during infancy in our population of IDDM children was 18%, which was not different from the incidence seen in nondiabetic age-, residence-, and sex-matched controls. Thus, in our studies we were unable to document any relationship between the history and duration of breast feeding and subsequent development of IDDM in children. These findings do not agree with the report of Borch-Johnsen et al, whose data showed a higher incidence and/or a longer duration of breast feeding among nondiabetic children [3]. The discrepancy between the above and our findings could be reflective of a different population of diabetic children. Apparently, Borch-Johnsen's patients were not matched for age, residence, and social background.

The notion of viral etiology IDDM has some theoretical merits. Breast milk contains various antibodies such as immunoglobulin A, cytotoxic T-lymphocytes, and HLA-DR molecules, all of which play a role in fighting infection [5]. If we assume a viral etiology of Type 1 IDDM, human milk could play a protective role for the development of IDDM. If the theory of protective effects of breast feeding on the development of IDDM in children would be valid, one may expect the increased incidence of other autoimmune disorders, such as Hashimoto's thyroiditis, among those IDDM patients who were not breast fed. In our studies, however, we were unable to document any significant difference in the incidence of antithyroid antibodies between breast-fed and formula-fed IDDM patients, although the incidence of antithyroid antibodies was two times higher in formula-fed IDDM children. Of interest were our findings that children with IDDM may have a history of more frequent feedings with soy-based formulae in infancy as compared with nondiabetic controls. Although indications for use of soy-containing formulae in infancy were not clearly defined in our patient population, it is generally agreed that such formulae are given to infants with gastrointestinal alterations while on milk feedings. Since these alterations may develop after a viral infection of the gastrointestinal tract [4], it could be postulated that in IDDM patients there is a higher incidence of gastrointestinal alterations during infancy than in the matched nondiabetic population. In conclusion, our studies failed to confirm the previously reported findings that the breast feeding in early life may have a protective effect on the subsequent development of IDDM in children. Since patient population may differ from one area to another, further studies along these lines are needed.

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Breast and Soy-Formula Feedings in Early Infancy and the Prevalence of Autoimmune Thyroid Disease in Children

P. Fort, MD, N. Moses, MNS, RD, M. Fasano, MS, RD, Teresia Goldberg, MS, RD, and F. Lifshitz, MD

Department of Pediatrics, North Shore University Hospital-Cornell University Medical College, Manhasset, New York

Key words: autoimmune thyroid disease, breast feedings, soy-containing formula

It has been suggested that feeding practices in infancy may affect the development of various autoimmune diseases later in life. Since thyroid alterations are among the most frequently encountered autoimmune conditions in children, we studied whether breast and soy-containing formula feedings in early life were associated with the subsequent development of autoimmune thyroid disease. A detailed history of feeding practices was obtained in 59 children with autoimmune thyroid disease, their 76 healthy siblings, and 54 healthy nonrelated control children. There was no difference in the frequency and duration of breast feeding in early life among the three groups of children. However, the frequency of feedings with soy-based milk formulas in early life was significantly higher in children with autoimmune thyroid disease (prevalence 31%) as compared with their siblings (prevalence 12%; $\chi^2 = 7.22$ with continuity factor; $p < 0.01$), and healthy nonrelated control children (prevalence 13%, $\chi^2 = 5.03$ with continuity factor; $p < 0.02$). Therefore, this retrospective analysis documents the association of soy formula feedings in infancy and autoimmune thyroid disease.

INTRODUCTION

It has been suggested that feeding practices in early infancy may affect the development of various autoimmune disorders later in life. For example, a link between the prevalence of insulin-dependent diabetes mellitus (IDDM) in children and breast feeding during infancy was suggested [1,2]. Although we were unable to confirm such an association in our population of children with IDDM, we did notice a higher incidence of antithyroid microsomal antibodies in children with IDDM who were formula-fed as compared to those who were breast-fed as infants [3]. In addition, IDDM children who were not breast-fed were more likely to receive soy-containing formulas in early infancy than nondiabetic control children [3].

Since thyroid alterations are among the most frequently encountered autoimmune conditions in children [4], we studied whether feeding practices in early life were associated with the subsequent development of autoimmune thyroid disease (ATD). Specifically, we attempted to assess the prevalence of breast feedings which may have a protective role in the development of

ATD later in life, and the type of milk formula feeding in early infancy which could be associated with a high incidence of ATD later in life.

MATERIALS AND METHODS

The subjects of the study were 59 children, females, and 19 males, with ATD who were being followed by the Division of Pediatric Endocrinology, Metabolism, and Nutrition at North Shore University Hospital-Cornell University Medical College, Manhasset, New York. Fifty-two children had autoimmune thyroiditis (Hashimoto's thyroiditis), with or without hypothyroidism, and seven had Graves' disease. The mean age of patients (\pm SD) at the time of evaluation was 14.7 ± 6.4 years. The mean age (\pm SD) at which the diagnosis of ATD was established was 9.6 ± 4.6 years. In all patients the diagnosis of ATD was made by the presence of goiter detected on physical examination and confirmed by laboratory assessment, which included measurement of serum T_4 , T_3 RIA, TSH, antithyroid antibodies, and thyroid stimulating immunoglobulin

Address reprint requests to Pavel Fort, M.D., Department of Pediatrics, North Shore University Hospital, 300 Community Drive, Manhasset, New York 11030.

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Table 1. The Prevalence of Breast Feedings in Early Infancy in Patients with Autoimmune Thyroid Disease (ATD), Their Healthy Siblings, and Other Healthy Children^a

	Patients with ATD	Healthy siblings	Healthy controls
Number of studied children	59	76	54
Number of children breast fed (%)	20 (34)	26 (34)	19 (35)
Duration of breast feeding (months)	5.2 ± 3.7	5.6 ± 3.2	8.7 ± 4.5
Solids started (months)	3.7 ± 3.3	3.0 ± 3.0	3.7 ± 2.8

^aData as mean ± SD.**Table 2.** The Prevalence of Soy-Containing Milk Formula Feedings in Early Infancy in Children with Autoimmune Thyroid Disease (ATD), Their Healthy Siblings, and Other Healthy Children

	Patients with ATD	Healthy siblings	Healthy controls
Number of studied children [†]	59	76	54
Number of children fed soy-containing formula (%)	18 (31)***	9 (12)	7 (13)

[†]Includes breast-fed subjects.

*p < 0.01 vs healthy siblings; **p < 0.02 vs healthy controls.

well as thyroid scan and uptake with ¹²³I, when indicated.

A telephone interview by a nutritionist was conducted with all patients with ATD being followed in our center. The parents of the children with ATD were asked the following questions: (1) history of breast feedings and/or formula feedings in early life and its duration, (2) type of infant formula given, and (3) age at which solid foods were introduced. This information was obtained for all the patients and their siblings. Data were available on 76 such siblings, 35 females and 41 males, whose mean age (±SD) was 14.8 ± 6.3 years, none of whom was known to have any thyroid illness.

Additionally, the nutritionist obtained information on 54 nonrelated control children without apparent ATD. There were 23 females and 31 males with mean age (±SD) of 13.2 ± 5.6 years. This comparative control group consisted of 27 friends of the patients with ATD

who were of the same age, lived in the same geographical area, and did not have, to the best of their knowledge, any thyroid ailment. The remaining 27 children were being followed in our Pediatric Endocrine Ambulatory Center for familial and/or constitutional short stature; in these patients thyroid disease was ruled out by physical examination and measurement of thyroid function tests (T₄, TSH) and antithyroid antibodies.

The data were analyzed by 2 × 2 χ^2 tests to evaluate for the differences in feeding practices in infancy between the children with ATD as compared with their healthy siblings and healthy nonrelated control children. The threshold of significance was set at a p < 0.05 level.

RESULTS

The prevalence and duration of breast feeding in early infancy in patients with ATD, their healthy siblings, and

other control children are shown in Table 1. Both the prevalence and duration of breast feeding among patients with ATD were similar to that seen in their siblings and in the healthy control children. Also, there was no difference between the age at which solid food was introduced into the diet among the three study groups (Table 1).

The mean age at which ATD was diagnosed was 10.0 ± 4.7 years in the breast-fed group as compared with 7.9 ± 4.0 years in the non-breast-fed group of patients; however, this difference was not statistically significant. Also, there were no significant differences in the initial values of serum T_4 , TSH, and titre of antithyroid antibodies (antithyroid globulin and microsomal) between those breast-fed and those not breast-fed.

The majority of children with ATD were fed cow's milk formula during infancy. However, 18 out of 59 children (31%) received soy-containing formula (Table 2). The prevalence of soy-containing formula feedings was significantly higher among patients with ATD than that found in their healthy siblings (prevalence 12%, $\chi^2 = 7.22$ with continuity factor; $p < 0.01$), and than that found in healthy nonrelated control children (prevalence 13%, $\chi^2 = 5.03$ with continuity factor; $p < 0.02$). There were no differences in the prevalence of soy-containing formula feedings between the siblings of patients with ATD and healthy nonrelated control children.

DISCUSSION

Our data show that the prevalence and duration of breast feedings during infancy in our population of children with ATD were the same as those found in their siblings who did not have any thyroid ailment, and were also similar to those of healthy age-matched children. Thus, in our studies we were unable to document any relationship between the history and duration of breast feeding and subsequent development of ATD. These findings conform with our previously reported observation that the incidence of other autoimmune conditions in children, such as IDDM, is not associated with breast feeding in infancy [3].

However, the so-called protective effect of human milk on the subsequent development of an autoimmune process, such as IDDM [1,2], may not be related to the human milk per se but rather to type of feeding given during the critical period of maturation of the immune system in early life. We observed a significantly higher prevalence of feedings with soy-containing formulas in early infancy in patients with ATD as compared with their healthy siblings and healthy nonrelated control

children. In fact, a child with ATD was two to three times more likely to have received soy-containing formula in early life than a child of similar age without an thyroid ailment. In contrast, the prevalence of feeding with soy-containing formula in infancy in the sibling and control groups was similar to that reported in the general pediatric population of the United States [5]. Thus, appears that when breast milk was substituted by soy formula feedings in early life there would be an association with ATD whereas when cow's milk feedings were used, no such an association existed.

Although the precise reason for the introduction of soy-containing formulas in patients with ATD could not be determined by the retrospective study, it is generally agreed that such formulas are given to infants with gastrointestinal or other alterations while on cow's milk formula feedings. Thus, one could postulate at least two hypotheses, in terms of the possible association between soy-containing formula feedings in early infancy and subsequent development of ATD in children. On the one hand, one could theorize that in children with ATD there is a higher prevalence of gastrointestinal alteration and/or cow's milk formula intolerance during infancy than in the healthy population. Indeed, an increased frequency of antibodies to certain serotypes of intestinal pathogens, such as *Yersinia enterocolitica*, has been reported in patients with ATD [6]. Thus, cow's milk intolerance, which is frequently associated with gastrointestinal alterations in early life [7], could be part of the long chain of events leading to subsequent development of an autoimmune process resulting in ATD. On the other hand, one could also postulate that in a genetically predisposed population of children with autoimmune diseases the soy-based infant milk formula may exert an adverse effect on the development of such conditions later in life. Indeed, it has been reported that soy-based formulas are highly immunogenic and may damage the intestinal barrier in infants with diarrhea [8]. A similar environmental effect on the development of autoimmune process has been shown in IDDM, development of which could be related, in certain geographical regions, to the intake of nitrosamines before the development of β cell autoimmunity [9].

The cause of autoimmune thyroid diseases, such as Hashimoto's thyroiditis and Graves' disease, is believed to be multifactorial, involving a genetic predisposition to develop an autoimmune response which may be triggered by an environmental insult [10]. From our data it would appear that the soy protein could be one of several environmental triggering factors. A high prevalence of antithyroid antibodies in patients with IDDM who were fed soy-based formula during infancy was also found in previous studies [3].

In conclusion, although we were unable to document any protective effects of human milk feeding in early life the subsequent development of ATD in children, we found that this population of patients had a significantly greater prevalence of a feeding history of soy protein-containing milk formulas in early infancy than healthy matched, both related and nonrelated, children. However, a more precise definition of the relationship between the ingestion of soy protein in infancy, heredity, and autoimmune disorders in a larger population of children should be pursued.

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MEDICAL INTELLIGENCE



OCCURRENCE OF GOITER IN AN INFANT ON A SOY DIET*

JERROLD D. HYDOVITZ, M.D.†

PITTSBURGH, PENNSYLVANIA

SEVERAL recent studies have established and explored the role of a soy-flour diet in the genesis of "high-uptake" goiter in the rat.^{1,2} Although it is a common practice to resort to the exclusive use of soy preparations for considerable periods in the management of milk allergy, no reports have appeared to date that implicate this nutrient in the production of goiter or hypothyroidism in the human patient. The sequence of events and data that evolved during an investigation into the possible cause of thyroid enlargement in a male infant and the subsequent personal communications from authors interested in the problem^{3,4} suggest that this phenomenon may indeed have its counterpart in the human being.

CASE REPORT

Fullness of the anterior aspect of the neck was first noted in B.J.,† a 4½-month-old-boy, by his mother who was unusually observant, as well as a registered nurse. No suspicious behavior or developmental patterns were reported by her, and progress compared favorably with that of his healthy twin siblings (5 years of age). Because of feeding difficulties that were attributed to milk allergy in the older siblings, B.J. was started on a commercial preparation containing skimmed milk, vegetable oils and vitamin and mineral supplements after the initial 3 weeks of breast feeding. This regimen was discontinued 1 week later, and replaced by a popular soybean formula, which served as the sole nutrient

except for vitamins A, C and D until the laboratory studies here reported were completed at 6 months of age.

Gestation had been uneventful except for a moderately elevated blood pressure, which was satisfactorily controlled coincident with the administration of exceedingly small doses of a compound containing *Rauwolfia serpentina*, protoveratrine and phenoxybenzamine hydrochloride from the 3d month to term. She was quite emphatic about not being aware of any suspicious finding in the patient's neck during the neonatal period despite frequent and searching general inspections of his person. There was no definite history of thyroid dysfunction in the immediate family. Residence during the pregnancy and after delivery was in an area not noted for its incidence of goiter.

Physical examination at 5 months of age demonstrated no abnormal findings except a thyroid gland 3 times normal size that was rubbery in consistence, apparently nontender and freely movable. No bruit was audible in the thyroid area. The appearance, behavior, skin texture, hair, eyes and periorbital structures, cry and so forth were entirely compatible with the euthyroid status. Osseous development was normal for chronologic age, and no epiphyseal dysgenesis was noted on x-ray examination. The serum protein-bound iodine concentration was in the low-normal range at 3.8 microgm. per 100 ml.; this determination was repeated 1 week later in the same laboratory and was 7.9 microgm. per 100 ml. The concentration of butanol-extractable iodine in this 2d sample of blood was 4.7 microgm. per 100 ml. (in another laboratory). Serum inorganic phosphorus and alkaline phosphatase values were normal. After the oral administration of 2 microcuries of carrier-free I¹³¹, 60, 68 and 73 per cent of this dose was detected in the neck with a scintillation counter at 5, 24 and 48 hours, respectively. The soy formula was continued during these studies. An attempt was made to assay the level of PBI¹³¹ for the activity of the administered dose of radioactive iodine, but because of technical difficulties the only interpretable data indicated that approximately 65 per cent of the total radioactive substance in the serum was not removed by several passages through an anion-exchange-resin column, and therefore may be considered to be protein bound. No measurements were made of urinary and fecal radioactivity after the tracer dose of I¹³¹, nor of the biologic half life or fecal content of labeled thyroxine.

Upon completion of these studies, the soy feedings were discontinued, and whole milk substituted. The mother reported detectable softening of the goiter within 1 week thereafter, and the thyroid gland was no longer palpable when the patient was examined 3 weeks after this. The stools, which had been abnormally frequent and had possessed a foul odor while he was receiving the soy preparation, became less frequent, bulky and offensive on the whole-milk diet. In addition, there was an apparent spurt in motor activity and dental development during the same immediate period. Repeat x-ray studies of the epiphyses 8 weeks after the initial ones, and 4 weeks after cessation of the soy feedings, revealed the existing centers to be larger, but no new ones to have developed. Repetition of the uptake study 72 days after the initial one and 67 days after cessation of the soybean extract feedings resulted in the thyroidal accumulation of 24 and 15 per cent of the administered radioactive iodine at 5 and 48 hours, respectively.

*From the Addison H. Gibson Laboratory, University of Pittsburgh School of Medicine.

†Clinical assistant professor of medicine and clinical associate, Addison H. Gibson Laboratory, University of Pittsburgh School of Medicine, major attending in medicine and chief, Division of Endocrinology, Magee-Women Hospital.

‡Referred by Dr. George Thiers, Pittsburgh, Pennsylvania

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DISCUSSION

The softening and subsequent disappearance of the enlarged thyroid gland that followed promptly upon the change in diet strongly suggests that the soy preparation was responsible for the production of the goiter in this particular instance. There is little objective evidence that any significant degree of hypothyroidism existed while the patient was receiving this nutrient, but the increased motor activity that was noted shortly after the soy feedings were stopped suggests that some slight deficiency in the production or utilization of normal thyroid hormones did actually coexist with the goiter. Since the serum protein-bound iodine and butanol-extractable iodine were determined in different laboratories, one is not justified in interpreting the relatively low concentration of the latter as evidence for the presence of an abnormal iodinated product of the thyroid gland. The standard technics for recording I^{131} uptake may result in artificially high values in infants of this size because of the collimation factors involved, scatter radiation and so forth. The greatly elevated neck count obtained in this patient at five hours, and possibly at twenty-four hours, after the administration of radioactive iodine might be subject to such a criticism; the extremely high value at forty-eight hours, however, can only reflect an abnormal thyroid avidity for iodine. Newborn infants demonstrate increased thyroidal uptakes of I^{131} ,⁵ but not after the first few weeks of life. The normal uptake of I^{131} by the thyroid gland at a later date, when the goiter was no longer present, is good evidence in favor of a temporary and reversible defect in thyroid function. This high-uptake type of goiter is an unusual occurrence with most known goitrogens, but has been observed in laboratory animals fed diets of soy flour, cellulose and bran.¹

There has been no definite demonstration that soy flour can induce goiter or hypothyroidism in the human being. The case reported above and the clinical experiences of others^{3,4} suggest, however, that such an occurrence is possible. Excessive fecal thyroxine excretion in association with goiter and an increased thyroid avidity for I^{131} have recently been reported in rats maintained on soy-meal diets.^{1,2} The production of thyroid enlargement in the rat and in the chick with the same type of regimen has been commented on previously by others.⁶⁻⁸ These changes can apparently be obviated by the addition of considerable amounts of iodine to the diet. The exact mechanism for this effect of soy meal in these animals is obscure, but the recent study by Beck² seems to have eliminated several likely possibilities: the increased biliary excretion of thyroxine; the addition of thyroxine or some other thyroid hormone to the intestinal tract from an extrabiliary source; the alteration of thyroxine complexes such as the glucuronide form so that reabsorption from the bowel is

compromised; and the presence of abnormal iodinated proteins in the feces. It has been suggested that this excessive loss of fecal thyroxine may be due to the accelerated rate of transport of the large fecal mass induced by such a diet. So far as this thesis is concerned, the same type of defect, in a lesser degree, has been observed in rats in conjunction with the use of diets high in bran or cellulose.^{1,9} Diminished time for the reabsorption of thyroxine from the intestinal lumen or the inefficient reutilization of the components liberated by the normal processes of thyroxine degradation may constitute an inordinate drain of tyrosine substrates and the inorganic iodide necessary for the synthesis of thyroid hormones. In line with this postulated abnormality in fecal transport, it is of interest to recall the mother's observation about the change in the character of the stools after the soy feedings were stopped.

An additional possibility that must be considered in the production of this disorder is the effect of a soy-flour factor on the performance of the intestinal mechanism involved in the reabsorption of thyroxine. A trypsin inhibitor has been demonstrated in soy-bean extracts,^{10,11} and amino acid deficiencies can be produced or accentuated by the addition of soy protein to the diet of chicks.¹² This soy factor evidently has the added capacity of interfering with the absorption of prehydrolyzed amino acids.¹³ Significant changes in the bacterial flora of the intestinal tract may also occur in association with soy and similar diets, and this facet warrants consideration of its role in the metabolism of thyroxine and iodine.

Admittedly, the enterohepatic circulation of thyroxine is not as active a feature in the metabolism of this hormone in the human being as it is in the rat. This aspect of the thyroxine cycle might vary significantly from the "normal" in some subjects, however, and consequently assume increased significance when one is confronted with further aberrations such as the addition of a diet high in soy meal. Also, a variety of subliminal defects (inborn or acquired) in the orderly synthesis of thyroid hormones, or their utilization, could result in barely adequate attainment of the euthyroid state; the superimposition of this type of diet-induced disturbance could then serve to produce overt evidences of thyroid dysfunction such as goiter or hypothyroidism or both. Fortuitous combinations of factors such as these may account for the well established, but peculiarly infrequent, goitrogenic effect of substances such as calcium iodide and cobaltous chloride.

It will continue to be necessary to consider these diverse concepts in ferreting out the causes for sporadic goiter, the elevated thyroidal uptakes of radioactive iodine in the presence of chronic hepatic disease and other currently inexplicable phenomena encountered in clinical thyroidology. The frequent examination of infants receiving soybean prepara-

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with particular emphasis on the size of the thyroid gland, might reveal an incidence of goiter previously unsuspected.

SUMMARY

The appearance of thyroid enlargement in an infant maintained on a soybean extract and the subsequent disappearance of his high-uptake goiter shortly after the elimination of this nutrient are reported. The data are compatible with the observations described in laboratory animals fed soy flour. The possible mechanisms involved in the production of this phenomenon are discussed.

Since this manuscript was submitted for publication, the same phenomenon has been described in another infant by Van Wyk and his associates.¹⁴ Also, attention has been called to a statement by Rawson and Rall¹⁵ initially noting this probable association in an additional case.

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SEVERE EXTRAPYRAMIDAL MOTOR ACTIVITY INDUCED BY PROCHLORPERAZINE*

Its Relief by the Intravenous Injection of Diphenhydramine

WILLIAM H. WAUGH, M.D.,† AND
JAMES C. METTS, JR., M.D.‡

AUGUSTA, GEORGIA

PHENOTHIAZINE derivatives are currently widely used as potent tranquilizing and antiemetic agents. Unfortunately, the more potent therapeu-

tically these drugs are, the more frequent are their toxic or untoward side effects.¹ Extrapyrimal motor reactions are very common side effects. Although these drug-induced dystonias or syndromes similar to Parkinson's disease may be dramatic and frightening, it has generally been held that even the severe reactions need not cause undue alarm, for they can be effectively controlled by sedation or by the administration of such drugs as trihexyphenidyl, bztropine, procyclidine and cycrimine.² However, these currently used drugs, as well as others such as ethopropazine and orphenadrine, are not generally available in a form suitable for parenteral injection if rapid effectiveness is desired.³

The purpose of this report is to emphasize the fact that the severe extrapyramidal reactions resulting from phenothiazine derivatives are potentially life threatening rather than merely dramatic and frightening and to call attention to the fact that the acute dyskinetic situation can be promptly controlled by intravenous injection of the antihistaminic drug, diphenhydramine, which is commercially supplied in a form for parenteral use and should be available in hospitals and dispensaries.

CASE REPORT

B.H.S., a 21-year-old woman, was admitted to the Eugene Talmadge Memorial Hospital on March 11, 1959, because of progressive uremic symptoms of 5 months' duration. The past history was not remarkable.

Physical examination disclosed a pale, alert woman with mild hypertension and slight periorbital and pedal edema. She was in no distress. Oliguria, isosthenuria and moderate proteinuria were present.

Examination of the blood showed a normochromic anemia (the hemoglobin was 6.8 gm. per 100 ml.), azotemia (the blood urea nitrogen was 177 mg. per 100 ml.) and renal acidosis (the carbon dioxide content was 17 milliequiv., the chloride 83 milliequiv., and the inorganic phosphate 13.5 milliequiv. per liter). The serum sodium was 137 milliequiv., and the potassium 4.9 milliequiv. per liter. The total serum protein content was 6.1 gm. per 100 ml., with a low-normal albumin concentration.

The oral intake of food brought about vomiting, for which 90 mg. of prochlorperazine was given in divided doses parenterally over the 1st 3 days in the hospital. A painful pericardial friction rub developed.

On the 4th hospital day hemodialysis was performed with a Kolff-Travenol twin-coil artificial kidney for 8½ hours. During this dialysis, the patient received a net transfusion of 1 liter of whole blood. The combined dialysis and transfusion procedure was carried out uneventfully, and a net loss of 1.6 kg. of body weight was experienced. Examination of the blood after dialysis showed a hemoglobin of 9.0 gm. per 100 ml., a urea nitrogen of 44 mg. per 100 ml. and a serum sodium of 140 milliequiv., a serum potassium of 4.4 milliequiv., a serum chloride of 98 milliequiv. and a serum calcium of 4.3 milliequiv. per liter. The carbon dioxide content of the serum was now 29 milliequiv., and the inorganic phosphate 4.5 milliequiv. per liter.

The dialysis alleviated the chest pain, and the friction rub soon disappeared. However, nausea and vomiting persisted, for which 4 10-mg. injections of prochlorperazine were given intramuscularly on both the 1st and the 2d day after dialysis.

About ½ hour after the 4th 10-mg. dose of prochlorperazine on the 2d day after dialysis, a fixed facial expression and motor hypophasia developed. Within the next several hours trismus, aphasia, spasm of the neck muscles, intermittent protrusions of the tongue and bizarre dystonic

*From the departments of Medicine and Physiology, Medical College of Georgia.

†Supported by a grant (SF-243) from the National Institutes of Health. ‡Senior research professor of physiology, Public Health Service Senior Research Fellow in Physiology and associate in medicine, Medical College of Georgia.

†Resident in medicine, Eugene Talmadge Memorial Hospital.

Phytoestrogens in Soy-Based Infant Foods: Concentrations, Daily Intake, and Possible Biological Effects (44229)

C. H. G. IRVINE,*¹ M. G. FITZPATRICK† AND S. L. ALEXANDER*

Animal and Veterinary Sciences Group,* Lincoln University, New Zealand, and Chemistry Department,† University of Auckland, Auckland, New Zealand

Abstract. Exposure to estrogenic compounds may pose a developmental hazard to infants. Soy products, which contain the phytoestrogens, genistein and daidzein, are becoming increasingly popular as infant foods. To begin to evaluate the potential of the phytoestrogens in these products to affect infants, we measured total genistein and daidzein contents of commercially available soy-based infant formulas, infant cereals, dinners, and rusks. We also assayed phytoestrogens in dairy-based formulas and in breast milk from omnivorous or vegetarian mothers. In most cases, the glucoside forms of the phytoestrogens were hydrolyzed before separation by HPLC.

Mean (\pm SEM) total genistein and daidzein contents in four soy infant formulas were 87 ± 3 and 49 ± 2 μ g/g, respectively. The phytoestrogen content of cereals varied with brand, with genistein ranging from 3–287 μ g/g and daidzein from 2–276 μ g/g. By contrast, no phytoestrogens were detected in dairy-based infant formulas or in human breast milk, irrespective of the mother's diet (detection limit = 0.05 μ g/ml). When fed according to the manufacturer's instruction, soy formulas provide the infant with a daily dose rate of total isoflavones (i.e., genistein + daidzein) of approximately 3 mg/kg body weight, which is maintained at a fairly constant level between 0–4 months of age. Supplementing the diet of 4-month-old infants with a single daily serving of cereal can increase their isoflavone intake by over 25%, depending on the brand chosen. This rate of isoflavone intake is much greater than that shown in adult humans to alter reproductive hormones. Since the available evidence suggests that infants can digest and absorb dietary phytoestrogens in active forms and since neonates are generally more susceptible than adults to perturbations of the sex steroid milieu, we suggest that it would be highly desirable to study the effects of soy isoflavones on steroid-dependent developmental processes in human babies. [P.S.E.B.M. 1998, Vol 217]

The use of soy-containing infant foods is increasing as the public has been made aware of the health promoting properties of soy. Even in 1986, approximately 25% of the liquid infant formulas sold in North America were soy-based (1). Although soy also contains antinutritive factors (2), these are largely eliminated by processing or counteracted by supplementation for infant feeding (3). By contrast, the isoflavone phytoestrogens, genis-

tein and daidzein, present in raw beans primarily as the glucosides, genistin and daidzin (4), are heat stable and show substantial carryover through regular processing methods (5). Recently, concern has been expressed that exposure to soy isoflavones may pose a developmental hazard to infants, particularly to the reproductive system (6–9). This is because the isoflavones may cause perturbations of the sex steroid milieu, which are poorly tolerated by neonates (7). *In vitro* experiments have shown that the soy isoflavones can bind to estrogen receptors and act as competitive agonists or antagonists to endogenous estrogens depending on relative concentrations and affinities (10–12). Moreover, they can influence endogenous steroid metabolism by inhibiting 17 β -hydroxysteroid oxidoreductase type 1, which is the enzyme responsible for converting relatively impotent estrone to the more potent estradiol and, to a lesser

¹ To whom requests for reprints should be addressed at Animal & Veterinary Sciences Group, PO Box 84, Lincoln University, Canterbury New Zealand. E-mail: irvinech@who.lincoln.ac.nz

extent, androstenedione to testosterone (13). Genistein can also inhibit protein tyrosine kinases, which phosphorylate intracellular proteins and are necessary for the action of insulin-like and epidermal growth factors (14, 15). For example, genistein blocks transforming growth factor- α induction of aromatase by inhibiting protein tyrosine kinase (16) and thus decreases the aromatization of androgens to estrogens. Therefore, there are several pathways by which soy isoflavones might affect sex steroid synthesis and activity *in vivo*.

The first step in evaluating the potential of the phytoestrogens in soy-based infant foods to affect the reproductive system of infants is to determine the isoflavone intake of infants. Because there are very few data available on the genistein, genistin, daidzein, and daidzin contents of typical infant foods (17), we measured their concentrations in: 1) several brands of soy- or dairy-based infant formulas commonly used in New Zealand and the United States; 2) breast milk from omnivorous or vegetarian women; and 3) several brands of infant cereal, dinners, and rusks. We were then able to calculate the daily phytoestrogen intake of infants fed breast milk or the commercial foods given according to the manufacturer's instructions.

Materials and Methods

Samples. Infant formula and other food items were commercially available and were purchased from a local supermarket. Four commonly used brands of powdered soy-based infant formula and one liquid "ready-to-feed" variety were used. All dairy-based formulas were powdered: three were cow's milk, and the fourth was goat's milk. The dinners were two brands of chicken and vegetable puree.

Milk samples from each of 11 breast-feeding mothers were frozen immediately following their collection. Two of these women were vegetarians. Although the diets of the mothers were not controlled, we monitored their intake of soy products for 48 hr before the breast-milk samples were collected. The soy consumption by the women over this 48-hr period was classed as follows: 1) No known soy consumed ($n = 6$); 2) soy consumption < 10 g ($n = 3$); 3) soy consumption between 10–50 g ($n = 1$); and 4) soy consumption > 50 g ($n = 1$).

Chemicals. All solvents (Mallinkrodt, ChromAR grade), hydrochloric acid, and acetic acid (BDH, AnalaR grade) were purchased from Lab Supply Pierce (Auckland, New Zealand). Genistein (Sigma Chemical Co., St Louis, MO) and daidzein (ICN Biomedicals, Aurora, OH) were used without further purification.

Extraction and Hydrolysis of Isoflavones. Genistein and daidzein were extracted from food items according to the method of Franke *et al.* (18). Samples (approximately 2.5 g for solid samples and 5 ml for liquid samples) were refluxed in 50 ml ethanol:hydrochloric acid (4:1) for 2 hr. The extracts were cooled and immediately passed through a 0.45- μ filter before 20 μ l was injected onto the HPLC system.

Instrumentation and Chromatographic Conditions. HPLC determinations were carried out on a Waters WISP 710B equipped with a 20 μ l injection loop and an ICI LC1200 UV/Vis detector (Waters, Milford, MA). A 3.9 \times 300 mm μ Bondapak C_{18} reversed-phase column (Waters) connected to a μ Bondapak C_{18} GuardPak (Waters) pre-column insert was used. Elution was carried out as described by Franke and Custer (19) (i.e., mobile phase 20:80 acetonitrile: 10% acetic acid for 15 min and then 70:30 acetonitrile: 10% acetic acid for 15 min at a flow rate of 0.8 ml/min). Analytes were monitored at 260 nm during each run.

The mean spike recovery of isoflavones was 93% from solid samples and 85% from liquid samples. The detection limits were 0.1 μ g/g for solid samples and 0.05 μ g/ml for liquid samples. Most commercial infant formulas and cereals were analyzed in triplicate; however, occasionally the batch analyzed differed between runs. The mean percentage coefficient of variation (i.e., $sd/mean \times 100$) of concentration estimates was 12.5%, which takes into account variation due to batch and to measurement error.

Isoflavone Analysis Without Prior Hydrolysis. To determine the distribution of isoflavones into glucosylated and aglucone forms, selected formula and cereal samples were extracted in 80:20 methanol:water for 4 hr (20) and submitted to HPLC analysis as described by Murphy and Wang (21).

Results

Glucosylated isoflavones were the predominant form in both soy-based infant formula and cereals (Fig. 1). Isoflavone concentrations in commercially available infant formula and food are shown in Table I. In this case, glucosylated isoflavones were hydrolyzed before the HPLC separation; therefore, results are expressed as total genistein and

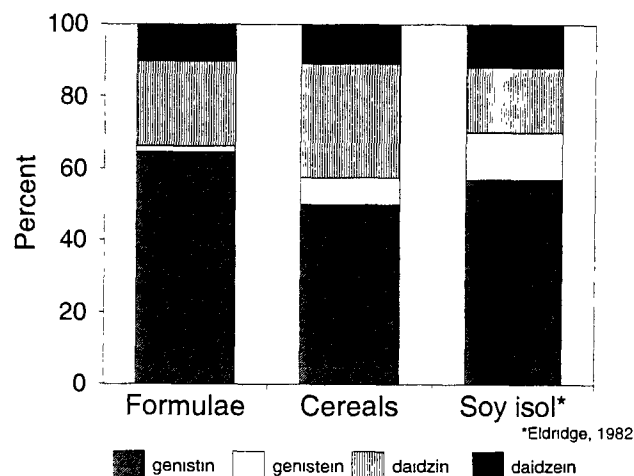


Figure 1. The percentage distribution of isoflavones into glucosides (genistin, daidzin) or aglucones (genistein, daidzein) in four soy-based infant formulas and three infant cereals. Shown for comparison is the mean isoflavone distribution in five soy protein isolates (soy isol; data from Ref. 20).

Table I. Concentrations (mg/kg) of Total Genistein and Total Daidzein in Commercial Infant Formula and Foods Commonly Fed in New Zealand

Product	Total genistein	Total daidzein
<u>Soy-based formulas</u>		
Formula A	92	55
Formula B	81	50
Formula C	91	48
Formula D	83	44
Soy isolate	514	248
<u>Infant foods</u>		
Cereal A	287	276
Cereal B	104	95
Cereal C	3	2
Dinner A	58	45
Dinner B	32	31
Rusks	<0.1	<0.1

Note. Shown for Comparison is the Isoflavone Content of Soy Protein Isolate (20). Published weights corrected for the molar conversion of glucosides into aglucones for comparison with the present data.

^b Most values are the mean of three analyses. The mean coefficient of variation was 12.5%, which includes variation due to measurement and to product batch.

total daidzein concentrations. The soy-based formulas were $13.8\% \pm 0.2$ protein according to the information on each product's label, with soy being the major protein source. The isoflavone concentrations in the soy formulas were $17.7\% \pm 0.5$ mean values in soy protein isolates (20), which is consistent with the formulas being simple dilutions of soy isolates.

The total genistein and daidzein concentrations in dairy-based infant formula were less than the detection limit of the analytical method (i.e., $< 0.1 \mu\text{g/g}$). Similarly, genistein and daidzein concentrations in all breast-milk samples were also less than the method's detection limit (i.e., $< 0.05 \mu\text{g/ml}$). By contrast, the ready-to-feed soy formula contained total genistein and daidzein concentrations of 18 and $15 \mu\text{g/ml}$, respectively.

We used the isoflavone concentrations measured in infant formulas and cereals to estimate the mean daily intake (i.e., total genistein plus total daidzein consumed) and the daily isoflavone dose per kg body weight basis received by infants when the products were fed as recommended by the manufacturer (Table II). For soy formula, the dose rate received by infants remained fairly constant between <1 and 4 months, with a mean ($\pm\text{SEM}$) value of $3.2 \pm 0.2 \text{ mg/kg}$ per day. There was little variability in intake due to the brand of formula chosen (Table I; also see the small SEM in Table II). By contrast, the isoflavone intake provided by cereals differed markedly with brand. For example, feeding 4-month-old infants one serving of Cereal A each day would increase the daily isoflavone dose by 28% (Table II).

Discussion

Our results show that considerable amounts of isoflavones remain after processing and formulation of most soy

Table II. Mean ($\pm\text{SEM}$) Daily Total Isoflavone (isoflav; total genistein + daidzein) Intake and Daily Isoflavone Dose Calculated on a Body Weight Basis Received by Infants Fed Soy-Based Infant Formula as Recommended by the Manufacturer

Age	Weight (kg)	Total isoflav (mg/day)	Isoflav dose (mg/kg/day)
<u>Soy-based formulas</u>			
<1 month	3	9.1 ± 0.7	3.0 ± 0.2
1 month	4	14.1 ± 0.6	3.8 ± 0.2
2 months	5	16.6 ± 1.1	3.3 ± 0.2
4 months	7 ^a	20.0 ± 2.0	2.9 ± 0.3
<u>Cereals</u>			
Cereal A ^b	7	5.6	0.8
Cereal B	7	2.0	0.3
Cereal C	7	0.05	0.01

Note. Formula means are based on the isoflavone contents of four infant formulas commonly used in New Zealand (See Table I). Also shown are the isoflavone intakes provided by three infant cereals. Because of the variability of their isoflavone contents, cereal values have not been meaned.

^a Weights based on data from Cruz *et al.*, (32).

^b Manufacturers recommend that cereal feeding should start when the infant is 4 months old. The isoflavone intake has been calculated on the basis of one 10-g cereal serving day.

infant foods. By contrast, the isoflavone contents of cow- or goat-based formulas were less than the method's detection limit of $0.05 \mu\text{g/ml}$. Likewise, isoflavones were not detectable in human breast milk regardless of the mother's soy consumption. Although endogenous steroids are present in human breast milk, total concentrations (i.e., conjugated plus free steroids) in most women are only 1%–5% those in plasma (22). Similarly, steroids in oral contraceptive pills are transferred into breast milk in small quantities, but the amounts are usually very low or insufficient to allow detection in the infants (23, 24). Moreover, in cattle, ovarian steroids used to induce lactation are not excreted through the milk in detectable concentrations (25). Therefore, it seems likely that the transfer of plasma phytoestrogens into milk may also be inefficient. In young vegetarian women, plasma total genistein and daidzein concentrations have been reported to be 118.5 nmol/l (i.e., approximately $0.04 \mu\text{g/ml}$) (26). Even if plasma phytoestrogens passed into milk with 100% efficiency, the resulting milk concentrations would be less than we would detect. On the other hand, the total isoflavone concentrations in soy infant formula diluted as used were at least 660-times higher than the maximum level possible in breast milk (i.e., our detection limit). This indicates that for infants, exposure to phytoestrogens *via* milk is much less significant than exposure *via* soy formula.

As in other soy products (4, 20, 21), the isoflavones in soy-based infant foods exist predominantly as glucosides, which are biologically inert (5). However, in adult humans, these glucosides are readily hydrolyzed in the acidic environment of the stomach and by intestinal bacteria (27, 28). Consequently, isoflavones are rapidly and efficiently ab-

sorbed and digested (29), with plasma concentrations of genistein and daidzein rising significantly after ingestion of soy products (27). Elimination of isoflavones occurs largely via the urine, mainly as glucuronide conjugates (28, 30). Fecal excretion is only 1%–2% of intake (30).

Although gut microflora appear during the first week of life (31), the ability of infants to digest ingested isoflavones has not been investigated before 4 months of age (32). An earlier abstract in which efficient digestion was reported at 2 months of age has not yet been confirmed (33). In a recent study, male babies, fed from birth on soy formula, were found to excrete significantly more daidzein and genistein in the urine at 4 months of age than did infants drinking cow's or mother's milk (32). This shows that 4-month-old infants can hydrolyze the soy isoflavones into active forms and absorb them (32). Furthermore, the small amount of phytoestrogen excretion by babies fed on cow's or mother's milk is consistent with our finding of undetectably low phytoestrogen concentrations in breast milks. Interestingly, urinary concentrations of equol, a more potent estrogenic metabolite of daidzein (10, 32), are similar and low in infants fed soy, cow's, or mother's milk (32). The absence of equol in the urine of soy-fed infants may be because the gut microflora, needed to produce it from daidzein, have not yet developed (32). Alternatively, it may simply reflect the variability in its production observed in adult humans (34 cf 26, 35, 30 cf 31, 36).

Our data show that as the infant's weight increases from 3–7 kg (32) the daily rate of intake of soy isoflavones provided by infant formula is held fairly constant at 3.2 ± 0.2 mg/kg when formula is fed as recommended by the manufacturers. Supplementing the diet of 4-month-old infants with a single serving of cereal each day can increase their daily intake by over 25%, depending on the brand chosen. Assuming efficient absorption as suggested by the work discussed above, would this isoflavone intake be sufficient to exert biological effects? Since species may differ in responsiveness to phytoestrogens, this question is best answered by considering data from humans. Unfortunately, few controlled studies have been reported on the effect of dietary soy on the human reproductive system. Two groups have used postmenopausal women to search for estrogenic effects of soy-supplemented diets (estimated intake = 1.2–2.5 mg total isoflavones/kg (27) assuming an average body weight of 65 kg) on the reproductive system. These have yielded contradictory results, with one group observing increased vaginal cell proliferation (37) and the other finding no significant changes in the vaginal epithelium, plasma gonadotropin, or sex hormone-binding globulin concentrations (27). Baird *et al.* (27) speculated that the soy isoflavones may act primarily as antiestrogens in the reproductive system. Therefore, their effects would be easier to detect in premenopausal women than in postmenopausal women whose endogenous estrogens are already very low (27). Supporting this deduction, Cassidy *et al.* found that in premenopausal women, a high soy intake significantly alters

reproductive hormones (38). That is: when young women consume 45 mg total genistein and daidzein (i.e., 0.73 mg/kg body weight) daily, the peri-ovulatory follicle-stimulating hormone (FSH) and luteinizing hormone (LH) surges fall to one-third and one-half, respectively, of control values, whereas follicular phase estradiol levels are raised significantly (38). These hormonal changes are accompanied by a lengthened follicular phase and/or delayed menstruation (38). Although treatment lasted 1 month, the effects persisted for up to 3 months (38).

The isoflavone dose rate given infants fed on soy-based formula is more than four times that shown to alter reproductive hormone secretion in cyclic women. Since neonates are generally more susceptible than adults to perturbations of the sex steroid milieu (7), what consequences might result from such a high isoflavone intake by infants?

It has long been known that modification of the sex steroid milieu in neonatal rodents alters reproductive axis function and sexual behavior and leads to structural changes in specific areas of the brain. The effects of neonatal steroid treatment, although irreversible, are often not manifested until the reproductive system is activated at puberty (39, 40). Moreover, there is only a limited window during development, the "critical period," when sex steroids can markedly influence neuronal structure and function. In humans unlike rodents, this critical period was thought to occur before birth (41, 42). However, recent theories on human sexual differentiation propose that there are several critical periods for development that occur not only prenatally but also during the early postnatal period (43). The timing of these critical periods seems to vary from tissue to tissue so that a temporary perturbation of the sex steroid environment may affect the development of only the one tissue that was passing through its critical period at that time (43). In extreme cases, this can lead to the development of sexual mosaics in which masculinized and feminized tissues coexist within the same body (43). Because the cerebral cortex develops late relative to other neural regions, the postnatal critical periods may be particularly important for cognitive development and other aspects of behavior that are mediated cortically (43).

In both humans and monkeys, the reproductive axis is active soon after birth. In males (44–47), plasma testosterone concentrations increase postpartum and are maintained at levels similar to those in adults for 20–90 days. In females, plasma estradiol concentrations approximately double during the first month of life (47). After this, gonadal steroid levels decline and remain at low basal levels until puberty (44–47). Observations in males strongly suggest that the postnatal period of raised testosterone secretion is a critical period for normal sexual development. For example, blocking the testosterone surge in male monkey infants significantly delays puberty (46). Once pubescent, treated monkeys have lower plasma LH and testosterone concentrations and reduced testicular volumes and sperm counts compared with normal controls (46). Moreover, there ap-

pears to be permanent impairment of the central nervous system pathway regulating gonadotropin-releasing hormone (GnRH) secretion (48), sexual behavior is compromised (49), and bone density is reduced (48). Interestingly, no adverse effects of blocking the postnatal testosterone surge were noted before puberty.

Although ethical considerations prevent intentional blocking of the postnatal testosterone surge in human infants, studies of boys with congenital hypogonadotropic hypogonadism suggest that early gonadal steroid deficiency may subsequently contribute to impaired testicular descent and maturation leading to oligospermia in the adult (46, 50). The postnatal androgen surge may also prime the urogenital tract by promoting early growth and by potentiating the maturational effects of testosterone at puberty (51). For example, boys born with micropallus related to androgen lack have inadequate androgen-mediated growth of the external genitalia if therapy is not commenced until puberty. Responses are normal if androgens are replaced during infancy (51). With regard to cognitive development: prepubertal androgen deficiency in boys results in impaired spatial perception that normally is more acute in men than women (52). Likewise, in monkeys, gender differences in maturation rate of learning ability (53) and performance of delayed visual discrimination tasks (54) can be manipulated by altering the postnatal sex steroid milieu.

The effect of modifying the sex steroid milieu in female primates has been little studied. This could be because the female's postnatal rise in plasma estradiol concentrations is much more subtle than the male's postnatal testosterone surge (47). However, it is still possible that these low levels of estrogen are needed for normal sexual development as has been clearly shown in female rats (55, 56).

While obliteration of the postnatal testosterone surge in primate males unquestionably impairs many aspects of sexual development, there is no experimental evidence that a high level of phytoestrogen intake by primate infants does or does not alter either the sex steroid milieu or sexual differentiation. Nevertheless, in neonatal rodents isoflavone administration during the critical period can alter brain structure and the adult regulation of LH secretion in a dose-dependent manner (57). Feeding infant female pigs on soy-ameal causes reproductive tract abnormalities (58). Likewise, other phytoestrogens given to female neonatal mice (59) or infant pigs (60) also affect reproductive tract anatomy. When adult, phytoestrogen-treated mice develop the premature anovulatory syndrome (61), whereas pigs show abnormal regulation of LH secretion for weeks after phytoestrogen exposure ceases (60). Furthermore, marked effects on postpubertal reproductive parameters have been shown in male and female rats nursed by phytoestrogen-fed mothers during early infancy (55). Early phytoestrogen exposure may also have beneficial effects. Mice injected with large doses of genistein soon after birth have reduced susceptibility to chemically induced tumor development when

adult (62). However, this treatment also impairs ovarian follicular development and cyclicity in the adult (63).

One reason for the lack of evidence linking isoflavone consumption with altered gonadal steroid-dependent developmental processes in human infants may be the probable delay in expression of the effects of early isoflavone exposure until after puberty. For example, another exogenous estrogen, diethylstilbestrol, was administered under controlled conditions to large numbers of women for over 20 years before its connection with postpubertal disorders in their offspring was observed, and several more years elapsed before the evidence of adverse effects was considered sufficiently convincing to cause withdrawal of the substance (7, 8, 64). Because of these observations and the increasing use of soy products as infant foods, it would be highly desirable to study the effects of soy isoflavones on steroid-dependent developmental processes in human babies.

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広 岡 良 文

名古屋大学環境医学研究所

村 田 善 晴

SMI ブリスドル (株) 北里バイオケミカルラボラトリー

富 樫 和 美

The Effects on the Thyroid Gland of Soybeans Administered Experimentally in Healthy Subjects

Ishizuki Thyroid Clinic

Yoshimochi ISHIZUKI

The Fourth Department of Internal Medicine, Aichi Medical University

Yoshifumi HIROOKA

The Research Institute of Environmental Medicine, Nagoya University

Yoshiharu MURATA

SMI-Bristol, Kitazato Biochemical Laboratories

Kazuyoshi TOGASHI

To elucidate whether soybeans would suppress the thyroid function in healthy adults, we selected 37 subjects who had never had goiters or serum antithyroid antibodies. They were given 30g of soybeans everyday and were divided into 3 groups subject to age and duration of soybean administration.

In group 1, 20 subjects were given soybeans for 1 month. Groups 2 and 3 were composed of 7 younger subjects (mean 29 y.o.) and 10 elder subjects (mean 61 y.o.) respectively, and the subjects belonging to these groups received soybeans for 3 months. The Wilcoxon-test and t-test were used in the statistical analyses. In all groups, various parameters of serum thyroid hormones remained unchanged by taking soybeans, however TSH levels rose significantly although they stayed within normal ranges. The TSH stimulation in group 3 revealed a more significant increase than that

$\frac{1}{2}$ the Cassidy
dose - over
twice as long

Translation of :

The Effects on the Thyroid Gland of Soybeans Administered
Experimentally in Healthy Subjects

Y. Ishizuki, Y. Hirooka, Y. Murata, K. Togashi

(Nippon Naibunpi gakkai Zasshi, 67, 622-629, 1991)

Japan Communication Service

PO Box 3857

Wellington

Tel: +64 4 232 8434

Fax: +64 4 232 4308

Mobile: 021 631 051

E-mail: 100246.2672@Compuserve.com.

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Introduction

It is said that soy beans contain a goitrogenic substance ¹⁾ and that the administration of soy beans to experimental rats, even for a short period, lowers serum T₄ levels ²⁾ and suppresses the ¹³¹I intake rate ³⁾. In the case of humans, there have been several cases in which the onset of goitres and hypothyroidism in infants fed with soy milk were reported. ^{3) - 8)} These writers reported that soy beans had slightly suppressed the thyroid function in chronic thyroiditis, and that they affected the thyroid gland in adults ⁹⁾. However, there has been no systematic study on whether soy beans in the normal diet suppress the thyroid function or not.

In this study, soybeans were administered to normal healthy adults, and it was investigated whether they affected thyroid function in adults or not. The study also examined whether the effects of diet on the thyroid function can be ignored or not, when reading the values of various parameters.

Subjects and methods

The subjects were selected from healthy working adults who had never had thyroid disorders or goitres, had no serum antithyroid antibodies, and were not on medications which influenced TBG fluctuation. Eight males and forty six females, 54 in total, aged between 22 and 76, were divided into 5 groups. Group 1 was the short duration group. Seven males and thirteen females, 20 in total, aged between 22 and 60 were given soy beans for 1 month. The long duration (3 months) group was divided into 2 groups (Groups 2 and 3) by age. Group 2 comprised younger females aged between 22 and 39 (average 29), and Group 3 comprised an older male (1) and females (9) aged between 46 and 76 (average 61). Control groups comprising the same age distribution, average age and number of subjects as Groups 2 & 3 were selected as Groups 4 & 5. Hence, Group 4 comprised 7 subjects, and Group 5 comprised 10 subjects.

Vinegared soy beans were prepared by pickling roasted soy beans (Produce of Takayama) in rice vinegar. 30 g of this preserve was administered orally every day, twice a day. Soy bean curd, miso (soy bean paste), and seaweed were given as usual without any restriction.

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13.

group 2, although inorganic iodide levels were lowered during the administration of soybeans. We have not obtained any significant correlation between serum inorganic iodide and TSH.

Hypometabolic symptoms (malaise, constipation, sleepiness) and goiters appeared in half the subjects in groups 2 and 3 after taking soybeans for 3 months, but they disappeared 1 month after the cessation of soybean ingestion.

These findings suggested that excessive soybean ingestion for a certain duration might suppress thyroid function and cause goiters in healthy people, especially elderly subjects.

結 言

大豆には甲状腺腫誘発物質¹⁾が含まれており、実験ラットに与えると、短期間でも血中T₄値が低下²⁾し、I¹³¹摂取率が抑制³⁾されるといわれている。人では豆乳で育てた乳児に甲状腺腫、甲状腺機能低下症が発症した数症例が報告⁴⁻⁶⁾されている。吾々は大豆が慢性甲状腺炎の甲状腺機能を長きに抑制する事を示し、成人甲状腺にも影響を及ぼす事を報告した。しかし常食の大豆が健康成人の甲状腺機能を抑制するかどうかについて、系統的に検討した報告がない。

そこでこの研究では健康者に大豆を投与し、成人甲状腺機能に変化を与えるかどうかを検討した。そして甲状腺機能検査値を判断する時、食事の影響を無視して判定して良いかどうか考察する事を研究目的とした。

対象並びに方法

対象には、甲状腺疾患の既往や甲状腺腫がなく、血中抗甲状腺抗体陰性で、TBG変動を招く薬剤服用がなく、通常の仕事に従事している人を健康者とし、22~76才の男8、女46計54例を選んだ。

5群に分けた。第1群は大豆1ヵ月投与の短期投与群で、22~60才の男7、女13例計20例である。大豆3ヵ月投与の長期投与群を年齢により更に2群に分けた。第2群は22~39才平均29才の女性若年群7例で、第3群は46~76才平均61才の男1、女9計10例の高年群である。第2、3群と夫々に同年令分布、同平均年齢、同数例を、対照群に選んだ。第4群が7例、第5群が10例である。

酢大豆は炒った大豆(高山産)を米酢に漬け、煮えたもので、30g/日を2分割し毎日経口投与した。三食、味噌、醤油摂取には制限を加えず、通常生活を続行させた。

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大豆投与実験は1989年8月から1年間に行い毎日摂取を怠ったものは除外した。

検査は大豆投与前、投与最終日、及び大豆中止3ヵ月以降日の午前中に施行し、問診、甲状腺触診と、採血を行った。−80℃で保存した血清を用い大豆前後をペアで同時測定した。甲状腺腫を認め例は超音波検査を行った。自覚症状は期間中の再三の問診から先ず全ての症状を取り上げた。その中から持続した症状のみを取り上げ、他病態によると判断される症状は除外した。大豆中止後に消失した症状を大豆効果と見なして採択した。対象例中4例は投与前検査が、また2例は大豆中止後の検査が出来なかった。

The administration of was continued for a year (sic) from August 1989, the subjects who neglected daily intake of soy beans were excluded.

Examinations were carried out before administration, on the last day of administration, and on a morning more than 3 months after the cessation of soy beans. Examinations comprised an interview, palpation of the thyroid gland, and blood tests. Serum was stored at minus 80 deg. C, and the serums before and after the administration of soy beans were measured simultaneously. Those in whom goitre was detected were examined by ultrasonic scan. All symptoms during the period were recorded. Only sustained symptoms were selected, and the symptoms which were assumed to be caused by other causes were excluded. The symptoms which disappeared after the cessation of the administration of soy beans were considered to be the result of the administration of soy beans, and so recorded. 4 subjects missed the pre-administration examination. 2 subjects missed the post-administration examination.

Serum T_4 , T_3 , FT_3 , gamma T_3 , and TBG were measured by RIA, and TSH was measured by the high sensitivity method. NEFA was measured by the enzyme method. CPK, LDH, GOT, and GPT were measured by the UV method. The TRH test used a 500 micro g venous injection method. The TSH value after 30 minutes was treated as TSH, and T_3 was measured at the same time. Serum total iodine was measured by the alkali incineration method. The inorganic iodide value was calculated by subtracting the iodine value in the T_4 value measured by RIA from total iodine. The t test, the Mann-Whitney method, and the order addition test using Wilcoxon, the X^2 test, and the direct probability method were employed in the statistical analyses.

Results

1) Thyroid function of the short duration group

Serum T_3 values, and T_4 values of Group 1 showed a tendency to drop after soy bean intake over one month, but the drop was not significant. FT_4 , FT_3 , gamma T_3 , TBG, the FT_4/T_4 ratio, and the FT_3/T_3 ratio showed no significant changes.

Inorganic iodide values showed no difference before and after the treatment, but TSH levels significantly increased after soy bean intake. ($P(U) < 0.01$ Table 1)

2) Thyroid function of the long duration group

Serum T_4 , T_3 , FT_4 , FT_3 , Inorganic iodide, the FT_4/T_4 ratio, and the FT_3/T_3 ratio in Group 2 of the long duration (3 months) group showed no significant changes, while TSH levels clearly increased ($P(U) < 0.01$), though the rise was slight. Serum T_4 , T_3 , FT_4 , FT_3 , Inorganic iodide, the FT_4/T_4 ratio, and the FT_3/T_3 ratio in Group 3 showed no significant changes before and after soy bean intake, while TSH levels clearly increased ($P < 0.05$) although they stayed within the normal range. Inorganic iodide levels in the older group were higher than those in the younger group, but the inorganic iodide values when

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TSH was raised by the intake of soy beans were lower than those before the intake of TSH. The TSH value after the treatment in Group 3 tended to be higher than that in Group 2 ($P(U) = 0.05$), and was clearly higher ($P < 0.02$) than that of Group 5 (Control), being on average 1.7 ± 0.2 micron U/ml. Δ TSH in Group 2 was 20.5 ± 2.6 micro U/ml, and was no different from that in Group 4, being on average 22.4 ± 2.9 micro U/ml. The post-TRH T_3 in Group 2 was 140 ± 10.3 ng/dl, which did not rise by TRH from the pre-TRH T_3 of 147.3 ± 6.7 ng/dl. Δ TSH in Group 3 was 30.3 ± 2.6 micro U/ml, and was clearly higher ($P < 0.05$) than Group 5, being on average 19.0 ± 2.2 micro U/ml, and also higher than that of Group 2 ($P(U) < 0.03$) (Fig. 1).

The T_3 in Group 3 at the time of the TRH test was 139.9 ± 3.8 ng/dl, and became 128.9 ± 10.2 ng/dl after 30 minutes, showing no increase.

8 subjects in Group 3 who showed a large fluctuation in TSH were studied after the cessation of the administration of soy beans. T_4 and T_3 showed a rising trend, and FT_4 and FT_3 clearly increased ($P(U) < 0.05$, < 0.025). The FT_4/T_4 ratio and the FT_3/T_3 ratio showed no significant fluctuation. However, TSH levels clearly dropped ($P(U) < 0.05$). The Δ TSH of seven subjects was on average 19.0 ± 4.7 micro U/ml, showing an obvious decrease ($P(U) < 0.01$), while inorganic iodide values showed no significant difference between before and after soy bean intake.

The correlation between the post-administration T_4 value and T_3 value was significant, the correlation coefficient being $+0.67$ ($N=37$, < 0.01), while there was no correlation between T_4 and TSH, and between inorganic iodine values and TSH, the correlation coefficient being $+0.07$. (Fig. 2)

3) Fluctuations of serum substances

Various substances which are considered to be affected by the thyroid function were studied. Serum albumin was not measured as it had already been accepted that it was not influenced by soy beans.¹²⁾ CPK, NEFA, GOT, AND GPT in Groups 1 and 2 showed no significant difference between before and after the intake of soy beans, and indices in Group 3 showed no significant difference between during the intake of soy beans and after the cessation of soy bean intake. LDH in Group 1 alone showed an increase owing to the intake of soy beans ($P < 0.001$). LDH in Group 3 during the intake of soy beans was higher than that in Groups 1 and 2, while there was no significant difference between the former and LDH in Group 1 during the intake of soy beans. (sic.) LDH in Group 3 clearly dropped after the cessation of soy bean intake ($P(U) < 0.01$). (Table 2)

4) Symptoms and goitre

20 subjects in Group 1 complained of symptoms of diarrhoea (7 subjects 35%), abdominal inflation (5 subjects 25%), constipation (4 subjects 20%), fatigue, lethargy, and oedema (2 subjects each 10%). 17 subjects in Groups 2 and 3 complained of symptoms of diarrhoea (1 subject, a decrease), constipation (9 subjects 52.9%), fatigue

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of 52.9%) and lethargy (7 subjects 41.2%), the latter three showing an increase. Symptoms indicating hypothyroidism were more noticeable than in Group 1 ($P = 0.039$, $P = 0.014$). All symptoms disappeared one month after the cessation of soy bean intake.

The goitre was a diffuse goitre of Degrees I & II enlargement with rubber-like hardness. It appeared in 3 subjects in Group 1, and 8 subjects (47.1%, 3 subjects are in Group 3) in Groups 2 & 3, indicating clearly that there was higher incidence in the long duration group. One subject (out of 3 subjects) with goitre in Group 1 developed subacute thyroiditis three weeks after the commencement of soy bean intake. Two subjects (out of 11 subjects) with goitre showed no change in size one month after the cessation of soy bean intake, while others showed a reduction in size, then the disappearance of goitre. The two subjects with no change received T_4 treatment, and then goitres reduced in size over 2 and 6 months. The treatment was then ceased. No subjects with goitre showed a hypoechogenic image on an ultrasonic scan.

CONCLUSION

The objectives of this test were firstly to use healthy subjects. Secondly, there was no change in diet except soy beans, since the effects of soy beans on the thyroid function were the purpose of this test.

The amount of vinegared soybean intake over 3 months induced a small goitre in half the subjects, though they were healthy. In addition, more cases of hypothyroidism were found in the long duration group than in the short duration group. Infant subjects in the Kipp report (4) showed a size reduction in goitre following the cessation of soy bean intake, but an enlargement of goitre 80 days after the recommencement of the administration of soy beans. Therefore, there is a possibility of the onset of goitre even in healthy adults after a large amount of soy bean intake over a long duration. Despite a high incidence of hypothyroidism, there was no drop in thyroid hormone levels, and TSH alone increased within the normal range. The rise in TSH was greater in the long duration group, and not so in the older group. The TSH response rose more significantly following the administration of TRH. Though the T_3 values did not rise following the administration of TRH, it was not necessarily due to the suppression of the thyroid function, as it was measured after a short time as 30 minutes. In the older group, there tended to be a rise in peripheral hormones and a drop in TSH and ΔT_4 three months after the cessation of soy bean intake, indicating the thyroid function was suppressed during the intake of soy beans. Moreover, regardless of age, goitres which appeared during the intake of soy beans, reduced in size, and the raised TSH level dropped after the cessation of soy bean intake. Based on these facts and the report (2) that the height of rat's thyroid follicles was raised by the intake of soy beans, it is reasonable to conclude that soy beans affected hormone synthesis rather than the secretion of thyroid hormones, and consequently increased TSH.

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In the case of chronic thyroiditis where the reserve tends to decrease, the intake of soy beans for more than 5 months clearly induced a drop in serum thyroid hormones, and a rise in TSH ⁹⁾. However, the subjects in this test were healthy adults with an ample reserve. The duration of the test was as short as within three months. These factors were considered to have contributed to the result that the level of hormone reserve did not drop as much as to lower the concentration of serum thyroid hormones. In healthy adults, serum T₄ and T₃ correlated well, gamma T₃ and TBG did not fluctuate, and the thyroid hormone levels did not drop. These indicated that the effect of soy beans on the suppression of thyroid hormone synthesis and the slowing down of Tg hydrolysis in the follicles was weak/mild, and a longer duration of administration was required to cause hypothyroidism severe enough to be clinically noticed. In a report where soy beans were administered for 5 days ⁸⁾, 2 subjects out of 14 showed a drop in PBI ¹³⁾ despite the PBI remaining constant (sic). The difference between the two indices was considered to be due to the loss of thyroid hormones in the faeces. There is an animal experiment ²⁾ where the addition of iodine to soy bean diet induced the disappearance of goitre which had occurred on a soy bean diet with iodine restriction. However, in the case of humans, goitre appeared with the normal intake of iodine, and a slight suppression of the thyroid function was detected. It was necessary to examine whether the drop in thyroid hormone and the rise in TSH in the elderly were caused by iodine or not. (Literal - Translator) The fact that the inorganic iodide levels in the older group were higher than those in the younger group indicated that the elderly had a higher intake of iodine than the young, and that soy beans did not inhibit the absorption of iodine. The higher intake of iodine in the older group was reflected on the high TSH levels before the administration of soy beans. Although the inorganic iodide levels after the administration of soy beans decreased compared with those before the administration, TSH increased, and Δ TSH was higher than that of the Control because of the intake of soy beans. However, the inorganic iodide levels did not correlate with TSH. This would indicate that the sporadic intake of a large amount of iodine in normal daily life is unlikely to affect the TSH fluctuation. There is a report in which older women showed a greater drop in serum T₃ and a greater rise in Δ TSH than young women, and it was concluded that this was due to lack of responsiveness of the thyroid gland caused by ageing ¹⁰⁾. In the case of chronic thyroiditis, the majority of the subjects aged in a wide range showed a high Δ TSH due to the intake of soy beans despite their inorganic iodine levels being in the normal range. The recovery of the thyroid function observed some period after the cessation of soy bean intake was not considered to be purely due to the age factor. Chronic thyroiditis shows a high sensitivity to iodine, and a high sensitivity to goitrogen in soy beans is also a possibility. However, the result of this experiment on healthy subjects indicates that soy beans may influence the thyrotropic hormone from the pituitary gland, rather than the suppression of thyroid functions due to iodine intake. It also indicates that the elderly are more susceptible than the young. Although there is a report ¹¹⁾ which states soy beans encourage the elimination of thyroid hormones into faeces, thereby lowering the function, this is debatable as both peripheral thyroid hormone and TSH levels fluctuated within the normal range.

— NOTE —

[Gann, 67, 763~765; October, 1976]

DEVELOPMENT OF MALIGNANT GOITER BY DEFATTED SOYBEAN WITH IODINE-FREE DIET IN RATS*¹

Shuichi KIMURA, Junichi SUWA, Michiko ITO, and Haruo SATO*^{2,3}

Department of Food Chemistry, Faculty of Agriculture, Tohoku University,*² and Department of Oncology, Research Institute for Tuberculosis, Leprosy and Cancer, Tohoku University*³

Hyperplastic goiters were observed frequently in female rats of Wistar strain when given defatted soybean under iodine deficiency for 6 to 12 months. The findings in the thyroid were those of malignant tumors in which the features of thyroid carcinomas were seen, accompanied with metastasis in the lungs of some animals. Enlargement of the thyroid was completely inhibited in rats when a small amount of iodine was added to the diet. The rôle of soybean factor(s) which causes enlargement of the thyroid is discussed in relation to the development of malignant goiter, together with pathological findings in the animals.

Enlargement of the thyroid gland by soybeans in the diet was first reported by McCarrison²⁾ in 1933. In spite of many work since then, the effective substance(s) in soybean responsible for the enlargement of thyroid has not yet been made clear. In 1961, Block *et al.*¹⁾ reported that the heated products of soybeans, which were being used in baby foods, did induce the enlargement of thyroid in rats, but it was prevented by giving increased amount of iodine in the diet. Recently, we reported that one of the soybean factors related to the enlargement of the thyroid was a soybean saponin.³⁾

This report deals with some findings in rats which were fed with defatted soybean under iodine deficiency for a long period (6~12 months). Changes found in the thyroid were those of malignant hyperplastic goiter with lung metastasis.

Materials and Methods

The animals used were female rats of the Wistar strain (body weight about 50 g) purchased from Nihon Rat Co. (Urawa). The iodine-deficient basal diet as a test diet used in the experiment was

as follows (detailed composition of the mixtures is given in Table I): Defatted soybean 40%, glucose 50%, salt mixture (Harper's mixture except that KI was omitted) 4%, water-soluble vitamin mixture 1%, fat-soluble vitamin mixture 1%, and soybean oil 4%. In the control diet, 0.015 mg. of iodine was added to 100 g of the test diet. The test diet was given to 30 rats and the control diet to 15. Most of the animals were sacrificed after feeding for 6 to 12 months and examined macro- and micro-scopically at autopsy.

Results and Comments

All the animals fed with the test diet revealed marked enlargement of the thyroid gland, without exception, which weighed about 100~300 mg/100 g body weight. In the rats of the control group fed with iodine-added diet, weight of the thyroid gland was about 10 mg/100 g body weight, showing no enlargement. Besides the goiter, tumor of the adrenal gland was observed in 3 out of 27 rats examined (11%), in one of which lung metastasis was found, and one mammary adenocarcinoma was seen in another rat. Although massive nodular lesions were frequently observed in the lungs at autopsy (16/27,

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*² Tsutsumidori-Amamiya-cho 1-1, Sendai 980 (木村修一, 諏訪純一, 伊藤道子).

*³ Hirose-machi 4-12, Sendai 980 (佐藤春郎).

Table I. Detailed Composition of Mixtures Used in Diet

Harper's salt mixture	%
CaCO ₃	29.29
CaHPO ₄ ·2H ₂ O	0.43
KH ₂ PO ₄	34.31
NaCl	25.06
MgSO ₄ ·7H ₂ O	9.98
Fe(C ₆ H ₅ O ₇)·6H ₂ O	0.623
CuSO ₄ ·5H ₂ O	0.156
MnSO ₄ ·H ₂ O	0.121
ZnCl ₂	0.02
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.0025
Harper's water-soluble vitamin mixture	%
Thiamine-HCl	0.059
Riboflavin	0.059
Pyridoxine-HCl	0.029
Ca pantothenate	0.235
Nicotinic acid	0.294
Vitamin K	0.006
Biotin	0.001
Folic acid	0.002
Cobalamin	0.0002
Inositol	1.176
Ascorbic acid	0.588
Lactose	97.551
Fat-soluble vitamin mixture	
Vitamin A	10,000 IU/kg diet
Vitamin D	1,000 IU
Vitamin E	100 mg
Choline chloride	2 g
p-Aminobenzoic acid	300 mg

59%), these were microscopically not metastases of tumor but inflammatory lesions such as pneumonia, bronchitis, and lung abscesses.

The enlarged thyroid appeared dark reddish and contained blood, like hemangioma. Some of the goiters formed a dumb-bell-shaped tumor by enlargement of both lobules and part of isthmus. Histologically, the thyroid tumors were diagnosed as carcinoma in which growth of follicular tissues showed either papillary, follicular, or trabecular pattern (Photos 1 and 2). Although most of goiters were encapsulated, invasive growth of hyperplastic follicles into the capsules was observed in many cases (Photo 3). The stroma was mainly composed of blood vessels and/or sinusoidal blood spaces together with various

amounts of fibrous and callosity-like tissues as described in human thyroid cancer.⁴⁾ Lung metastasis was seen in 2 out of 27 cases examined (Photo 4). These changes were not seen in any of the animals given iodine-added diet. It is well known that a goiter is induced by simple iodine deficiency, but it was noteworthy that hyperplastic goiters can be induced in rats in a high percentage by the administration of soybean factor(s) under iodine-deficient condition, together with accurate signs of malignancy such as invasiveness and metastasis formation in the lungs. This will be a useful model for experimental induction of thyroid cancer, although further studies are necessary to clarify whether the rôle of the soybean factor(s) is to promote changes induced by iodine deficiency or whether any carcinogenic substance(s) is present in the soybean factor(s).

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EXPLANATION OF PLATE

Photo 1. Thyroid tumor with a capsule, showing a feature of follicular adenocarcinoma in the lumens in which thin colloid substance is present. Some follicles are extended to form a cystic appearance. The stroma is rich in blood vessels, some of which are extended to form a sinusoid structure. Hematoxylin-Eosin stain.

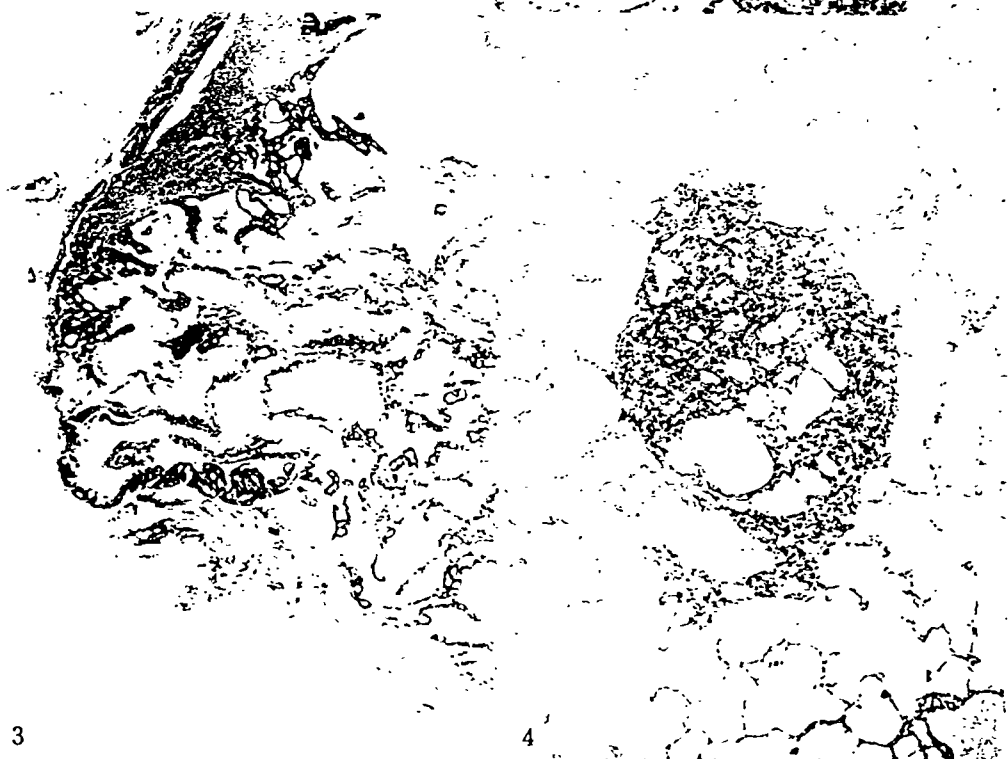
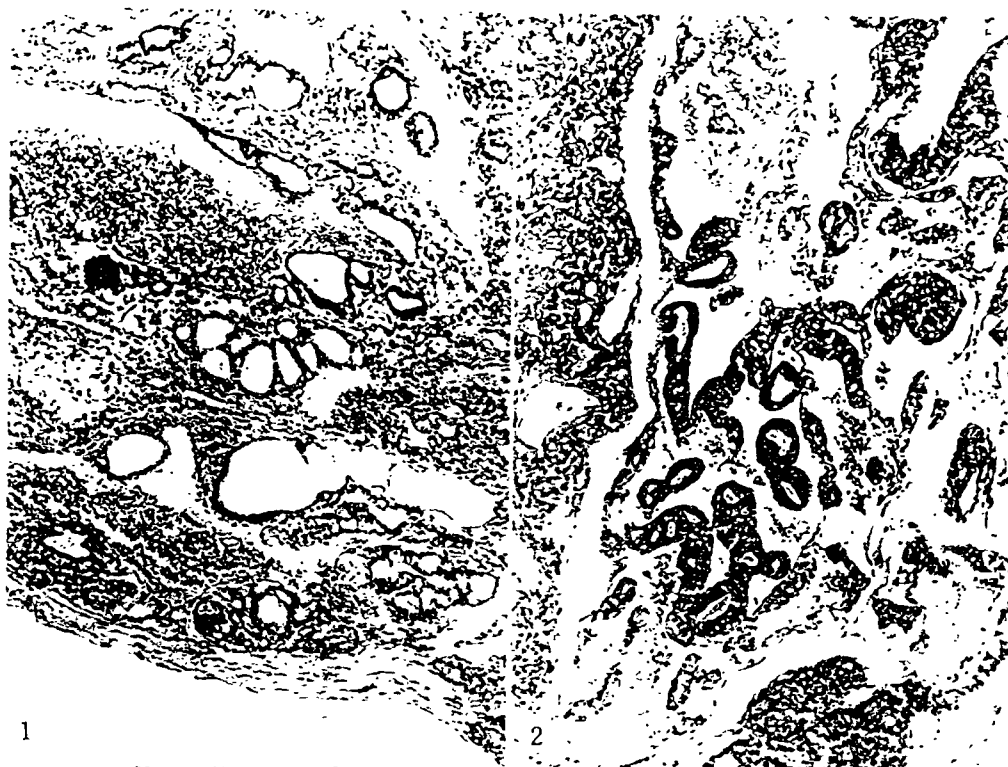
Photo 2. Thyroid tumor showing a feature of papillary and tubular adenocarcinoma with the stroma in which a large amount of sinusoidal blood vessels are seen. The goiter was soft and appeared dark reddish at autopsy, like a hemangioma. Hematoxylin-Eosin stain.

Photo 3. Invasive growth of thyroid tumor through the capsule. Hematoxylin-Eosin stain.

Photo 4. A metastatic lesion of thyroid tumor in the lung. Thin colloidal substance is seen in the follicles. Periodic acid-Schiff staining.

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MALIGNANT GOITER BY DEFATTED SOYBEAN IN RATS



The Effect of Prenatal Exposure to the Phytoestrogen Genistein on Sexual Differentiation in Rats (43832)

JILL R. LEVY,¹ KENNETH A. FABER, LINA AYYASH, AND CLAUDE L. HUGHES, JR.

Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, North Carolina 27710

Abstract. Exposure to naturally occurring estrogens during critical periods of development can alter morphologic and physiologic markers of sexual differentiation. The current experiment characterizes the effects of *in utero* treatment with genistein, an isoflavonoid phytoestrogen, on birth weight, anogenital distance (AGD) at birth, GnRH stimulated luteinizing hormone (LH) secretion, volume of the sexually dimorphic nucleus in the preoptic area of the hypothalamus (SDN-POA), puberty onset, and vaginal cyclicity. Pregnant Charles River CD rats were injected sc daily on gestation day 16–20 with either 25,000 µg genistein (G25), 5,000 µg genistein (G5), 5 µg diethylstilbestrol (DES), 50 µg estradiol benzoate (E), or corn oil alone for controls. Birth weights and anogenital distance was taken and exposed progeny were subsequently used in two experiments. In Experiment 1 intra-atrial catheters were placed in adult castrated rats, GnRH was given iv, serial blood samples were drawn and sera were assayed for LH by radioimmunoassay (RIA). Brains obtained by subsequent decapitation were saved for histology. In Experiment 2, females were monitored for timing of vaginal opening as a marker of puberty onset, and vaginal smears were taken to monitor cyclicity. G25-treated females and DES- and E-treated animals of both sexes had decreased weights at birth compared with controls. G5- and E-treated animals of both sexes and DES males had smaller AGD than controls. No significant differences in pituitary responsiveness to GnRH were found among treatment groups. There was a nonsignificant decrease in SDN-POA volume in G5-treated females while DES- and E-treated females had increased SDN-POA volume compared with controls. G5-treated females had delayed puberty onset, and DES-treated females had atypical vaginal cycles in comparison with controls. The results confirm that prenatal exposure to estrogens in the environment can influence sexual differentiation. Our previous experiments have demonstrated that castrate female rats exposed as neonates to genistein have decreased pituitary responsiveness to GnRH challenge and enlarged SDN-POA volume in comparison with controls. Prenatal genistein at these dosages did not significantly alter these markers. However, genistein did mimic other estrogens' effects on AGD and birth weight and had a unique influence on puberty onset. Not only are genistein's effects different from other estrogens, but dosage and timing of exposure during development appear to be important factors in genistein's ability to modify these endpoints.

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Estrogens and androgens play an important role in the sexual differentiation of animals. Evidence of hormonal influence is not only present in the reproductive tract but also can be seen

in reproductive physiology and the central nervous system (CNS). Compounds such as diethylstilbestrol (DES) and the naturally occurring estrogens in plants, phytoestrogens, that mimic endogenous estrogens can also influence development of these characteristics.

Development of the reproductive tract and external genitalia is influenced by endogenous and exogenous hormones. Exogenous estrogen administered *in utero* caused external genital feminization, persistence of Mullerian structures, and atrophy of Wolffian structures in male rat fetuses and hyperstimulation of Mul-

¹ To whom requests for reprints should be addressed at Division of Reproductive Endocrinology and Fertility, Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, NC 27710.

lerian structures in female rate fetuses (1). Normal variation in intrauterine exposure to androgens due to secretion from the testes of male siblings was found to lengthen the anogenital distance (AGD) (the length of tissue separating the anus and genitalla papilla) of neonatal females depending on the number of adjacent males *in utero*. This effect is additive, since females located between two male siblings had longer AGDs than females with only one adjacent male (2).

The perinatal environment has also been shown to affect neuroanatomy and neuroendocrinology. A morphological marker of sexual differentiation, the sexually dimorphic nucleus in the preoptic area of the hypothalamus (SDN-POA) which is normally larger in male rats than female rats is sensitive to hormonal influence during critical periods of development (3). Castration of neonatal males decreased SDN-POA size (3, 4) while exposure to aromatizable androgens or high-dose estrogens during the perinatal period resulted in increased SDN-POA in female rats (4, 5). Neuroendocrine effects included decreased adult basal and GnRH-stimulated luteinizing hormone (LH) secretion in female rats exposed to estrogen as neonates (6, 7, 8). In addition, exogenous estrogen exposure during the prenatal period delayed puberty onset in female guinea pigs in a dose-dependent fashion (9).

We have previously shown that female rats exposed as neonates to the phytoestrogen genistein had increased SDN-POA volume with results similar to females exposed neonatally to DES. In addition, adult castrate female rats given low-dose genistein as neonates had increased pituitary responsiveness to GnRH while high-dose genistein exposure blunted GnRH-stimulated LH secretion (8).

Nothing is known about *in utero* effects of genistein. Since genistein had similar effects to DES exposure during the neonatal period, we hypothesized that genistein would also influence markers known to be sensitive to intrauterine estrogen exposure. To determine the effects of genistein *in utero*, pregnant rats were treated during gestation day 16–20 and the progeny were compared with rats exposed prenatally to the reference estrogens DES and estradiol benzoate (E). Markers studied included birth weight, AGD, GnRH-stimulated LH secretion, puberty onset, and vaginal cyclicity.

Materials and Methods

Time-mated pregnant rats of the Charles River CD strain (Raleigh, NC) were purchased prior to 16 days gestation and maintained in air-conditioned quarters with Purina Laboratory Chow (Ralston-Purina, St. Louis, MO), water *ad libitum*, and a 14:10-hr light:dark cycle, with lights on from 0500 to 1900 hr EST. On Day 16 through 20 of gestation, injections were

given sc of either 25,000 µg genistein (Lot 44353; K and K laboratories, Division of ICN Biomedicals, Inc. Cleveland, OH) 5000 µg genistein, 5 µg DES (Lot #8836; Steraloids, Wilton, NH) 50 µg estradiol benzoate (1,3,5[10]-estratrien-33, 17B-diol 3 benzoate, Batch G160; Steraloids, Wilton, NH) or corn oil alone (Mazola 100% corn oil; Best Foods, CPC International Inc., Englewood, NJ). All injected doses were dissolved in 0.4 ml corn oil. Of note, two pregnant rats received sc injections of DES 5 µg on gestation day 15–20 and the resulting pups were used only in Experiment 2.

Day of delivery was defined as Day 1 if observed to occur prior to 1200 hr. All animals were weighed and the AGD was measured by vernier caliper on Day 1. All animals were identified by ink until Day 10, when ear marking was performed. Litters were then divided into two experimental groups.

In Experiment 1 animals were castrated under ketamine anesthesia (100 mg/kg body wt) and weaned on Day 21 of life. Animals were maintained in plexiglass cages. On Day 42 of life, right heart catheterization under ketamine anesthesia (100 mg/kg body wt) was performed. To control for the nonspecific effects of cannulation and fluid injection, animals were randomized to receive either saline alone (1 ml/kg) iv or GnRH (Factrel; Lot 3880235; Ayerst Laboratories, Inc., New York, NY) dissolved in saline (50 ng/kg body wt) iv 4 hr post cannulation. Blood samples of 0.3 ml volume were collected via the catheter immediately prior to and 5, 10, 15, and 30 min after injection of GnRH or saline. Blood volume was replaced with an equivalent volume of 10 U/ml heparinized saline each time. The sampling procedure was repeated 15 min after the last blood collection with animals receiving GnRH initially that had received saline injection and animals receiving saline that had initially received GnRH injection.

Blood samples were allowed to clot at room temperature and were centrifuged at approximately 1500g for 10 min. Sera were aspirated and frozen at –20°C for later radioimmunoassay (RIA).

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Serum LH concentration were measured by double-antibody RIA with rat LH supplied by NIDDK and the National Hormone and Pituitary Program (University of Maryland School of Medicine, Baltimore, MD). Second antibody (sheep, anti-rabbit) was graciously supplied by L. Tyrey, PhD (Duke University Medical Center, Durham, NC). Aliquots of serum (50 µl or less) were assayed in duplicate and the means were expressed in terms of NIDDKD-rLH-RP-3.

Intra- and interassay coefficients of variation for the measurement of LH in three serum pools were 1.8% and 8.2%, respectively. The assay sensitivity was 0.48 ng/ml.

Following the blood sampling, the animals were anesthetized for cannula removal and decapitated. The brains were promptly removed from the heads and

blocked. The block of tissue which included the optic chiasm and the hypothalamus was removed and placed in 10% formalin for a minimum of 2 weeks. Prior to frozen sectioning, the brains were placed in 30% sucrose and formalin solution for 48–72 hr. Sections were cut in the DeGroot plane (10) at 20 μ m thickness, mounted on slides, and stained with cresyl violet acetate. The slides were coded such the investigator performing light microscopy was blinded to the identity of the sample. The SDN-POA, as described by Gorski (3) with confirmation of the surrounding structures according to the stereotaxic atlas of Pellegrino (11), was traced with the use of a camera lucida. The cross-sectional area was computed with the use of a digitizing pad and planimetry computed software package (Sigmascan, Jandel Scientific, Corte Madera, CA). Volume of the SDN-POA was computed by adding together the traced areas and multiplying by the thickness of all sections.

In Experiment 2, animals were weaned on Day 21 of life. Females were separated from males and maintained in plexiglass cages as in Experiment 1. Females were monitored for day of vaginal opening. Subsequent to vaginal opening, female rats underwent daily vaginal cytology monitoring using staging criteria described by Everett (12). Smears were made by vaginal lavage with physiologic saline spread thinly on a clean glass slide. After fixation in 95% ethanol and removal of salt with deionized water, slides were then stained in 1% Toluidine Blue solution.

Individual birth weights, AGD measurements, SDN-POA volumes, and serum LH concentrations were all compared among treatment groups by one-way analysis of variance and Fisher's least significant difference test. Age at vaginal opening was compared among treatment groups by Kruskal-Wallis analyses and Mann-Whitney *U* tests. Vaginal smear cyclicity was compared qualitatively among groups.

Results

Disruption of Parturition and Resulting Progeny. Numbers of pregnant rats receiving treatments and numbers of viable progeny in each treatment group are recorded in Table I. Treatment of pregnant rats with DES and estradiol benzoate were associated with delayed parturition and increased rates of stillbirth and pup death before Day 10 of life, while these findings were not seen in animals treated with either dosages of genistein or corn oil treated animals. Pregnant rats were also treated with Estradiol Benzoate 100 μ g and 500 μ g during gestation day 16–20 but few viable pups were produced from these litters and these were not used in either experiment. Only one out of four mothers treated with estradiol benzoate 50 μ g on gestation day 16–20 produced viable pups. Those animals receiving estradiol benzoate 50 μ g *in utero* were

Table I. Number of Pregnant Rats Receiving Treatment and Number of Progeny in Each Treatment Group

Treatment	Number of pregnant rats	Total number of pups	
		Males	Females
Corn Oil	4	25	19
G25	4	23	18
G5	4	26	27
DES	4	28	16
E	1	9	3

used only in comparisons of birth weight, AGD, and SDN-POA.

Birth Weights in Newborn Rats. Mean birth weights are presented in Figure 1. G25-treated females ($P < 0.05$) and DES- and E-treated animals of both sexes ($P < 0.01$) had smaller birth weights than corn oil-treated animals of the same sex. Of note is that four males in the DES group died prior to weighing and were not used in this analysis.

AGD in Newborn Rats. Mean AGD measured on Day 1 of life are presented in Figure 2. G5- and E-treated animals of both sexes and DES-treated males had significantly shorter AGD than corn oil-treated animals ($P < 0.01$ for all aforementioned groups).

GnRH-Stimulated Secretion in 42-Day Castrate Rats. Mean LH concentration measured initially and at 5-, 10-, 15-, and 30-min intervals post GnRH or saline administration in females of the G25, G5, DES,

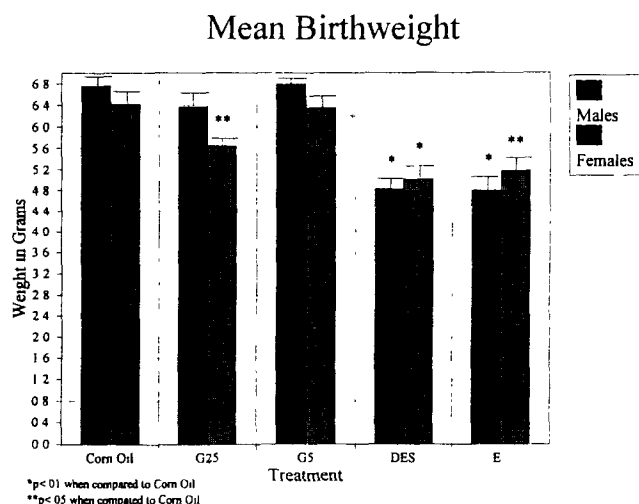


Figure 1. Mean birthweights in $g \pm$ SEM of rats exposed *in utero* on gestation day 16–20 to genistein 25,000 μ g (G25), genistein 5000 μ g (G5), diethylstilbestrol 5 μ g (DES), estradiol 50 μ g (E), or corn oil. Significantly decreased weights at birth were found in G25-treated females ($P < 0.05$), DES- and E-treated animals of both sexes ($P < 0.01$ for all DES animals and E males, $P < 0.05$ for E females). Number of animals within treatment groups were as follows: males—corn oil ($n = 25$), G25 ($n = 23$), G5 ($n = 26$), DES ($n = 24$), and E ($n = 9$); females—corn oil ($n = 19$), G25 ($n = 18$), G5 ($n = 27$), DES ($n = 16$), and E ($n = 3$).

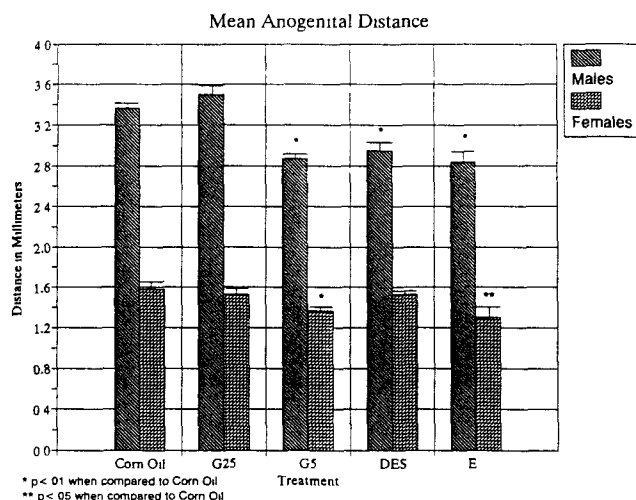


Figure 2. Mean anogenital distances (AGD) in millimeters \pm SEM of rats exposed *in utero* on gestation day 16–20 to genistein 25,000 μ g (G25), genistein 5,000 μ g (G5), diethylstilbestrol 5 μ g (DES), estradiol 50 μ g (E), or corn oil. Significantly smaller AGD at birth were found in G5- and E-treated animals of both sexes and DES males ($P < 0.01$ for G5-treated males and females, E- and DES-treated males, $P < 0.05$ for DES females). Numbers of animals within each treatment group were as follows: males—corn oil ($n = 25$), G25 ($n = 23$), G5 ($n = 26$), DES ($n = 28$), and E ($n = 9$); females—corn oil ($n = 19$), G25 ($n = 18$), G5 ($n = 27$), DES ($n = 16$), and E ($n = 3$).

and corn oil treatment groups are presented in Figure 3 a–d. Other than the expected differences between GnRH and saline treatments within groups, concentration of LH at baseline, peak, or at any given time interval was not significantly different among treatment groups for either sex.

SDN-POA Volume in 42-Day Castrate rats.

SDN-POA volume averages among treatment groups are presented in Figure 4 for females and Figure 5 for males. SDN-POA volume was significantly enlarged in DES- and E-treated females ($P < 0.01$) compared with corn oil treated females. There was an apparent but nonsignificant decrease in the SDN-POA volume of G5-treated females. Due to the high attrition rate of E-treated litters in the neonatal period, only three E-treated females were available for this part of the experiment. No significant differences were found among males of any treatment group.

Age at Vaginal Opening. Distribution of age at vaginal opening is presented in Figure 6. Corn oil-treated females were found to have vaginal opening consistently at 37 days (SD of 0). G5-treated females had significantly later onset of vaginal opening (mean, 38.8 days \pm SD 1.32) than corn oil-treated females ($P < 0.01$). There was no significant differences in timing of vaginal opening among other treatment groups.

Vaginal Smear Cytology. Length and regularity of cycles from vaginal opening until after 90 days of age were compared among treatment groups. Corn oil, G5, and G25 animals consistently exhibited estrous cycles usually between 3 and 5 days. DES animals

treated on gestation Day 15–20 were used in comparison of vaginal smear cyclicity. Three of the four DES-treated animals had marked cycle irregularity ranging from 2 to 12 days in length with a mean cycle length of 4.9 days. Prolonged cycles in DES were characterized by prolonged intervals of diestrus.

Discussion

The results of this study demonstrated that exposure to genistein *in utero* can influence markers known to be sensitive to estrogens. To our knowledge, this is the first demonstration of the effects of *in utero* exposure to the phytoestrogen genistein on hormone-sensitive characteristics. The effects of prenatal genistein are different from those associated with neonatal treatment with genistein, suggesting that timing of exposure or maternal and placental influences are important in the development of these characteristics. In addition, responses to genistein differ at low and high dosages possibly secondary to estrogenic agonist versus antagonist actions or estrogenic versus non-estrogenic metabolite effects. Finally, genistein effects differ from both DES and E, indicating that not all estrogens act alike and that each compound has its own profile of activity.

DES and estradiol were selected as comparison agents to genistein because of their known estrogenic properties. Genistein did not appear to adversely affect pregnancy, delivery, or survival in the neonatal period. However, there were some difficulties obtaining sufficient quantities of pups surviving past delivery and Day 10 of life in the litters exposed to DES and Estradiol *in utero*. Animals that were treated with estradiol benzoate at dosages of 100 μ g and 500 μ g had few pups surviving the perinatal period and were not used in these experiments. Cesarean sections were performed on some of the DES-treated pregnant rats who had not delivered by gestation Day 23, the typical length of gestation in rats, and evidence of fetal resorption was found. Similar results of prolonged delivery and increased perinatal mortality in pregnant rats treated with DES near gestation Day 18 were found by Zimmerman *et al.* who attributed uterine contraction failure to a concomitant depression in maternal steroid hormone levels. The relative incidence of stillbirth was reduced in their study by cesarean delivery indicating that DES induced higher pup mortality partly by disruption of labor (13).

Intrauterine androgen exposure is known to be associated with lengthened AGD. Likewise, AGD shortening in G-5, DES- and E-treated males is consistent with previous reports of “feminization” of external genitalia in male rats secondary to prenatal exposure (1). While estrogens mimic the actions of aromatizable androgens in masculinizing the CNS, at the external genitalia estrogens have the opposite effects of andro-

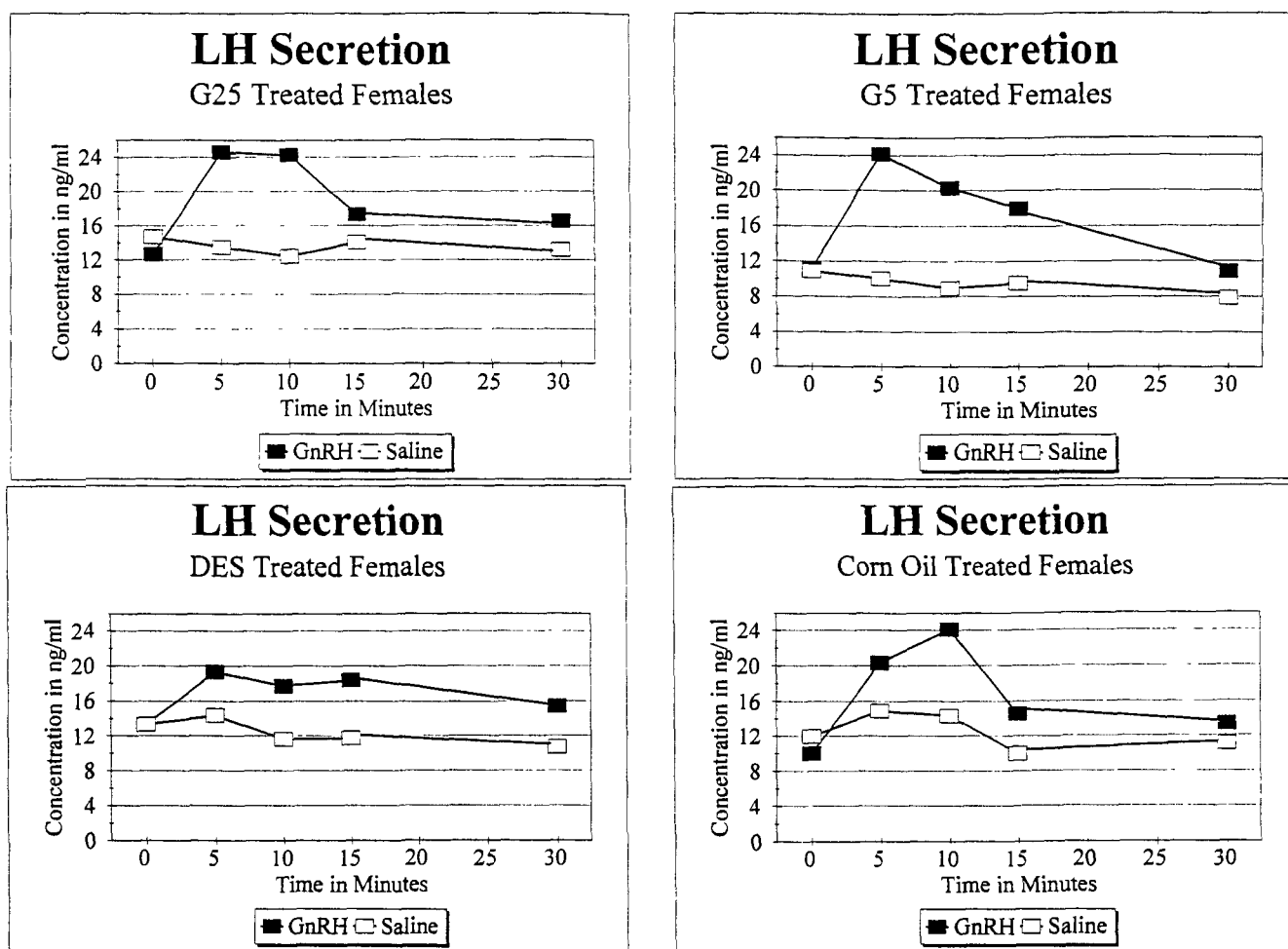


Figure 3. Mean serum LH secretion in ng/ml over time in female rats exposed *in utero* on gestation day 16–20 to (A) genistein 25,000 μ g (G25), (B) genistein 5000 μ g (G5), (C) diethylstilbestrol 5 μ g (DES), or (D) corn oil. Animals were given either GnRH or saline via intraatrial catheters at $t = 0$. No significant differences were found among treatment groups. Number of animals within each treatment group were as follows: G25 ($n = 9$), G5 ($n = 13$), DES ($n = 10$), and corn oil ($n = 7$).

gens. In addition, overall intrauterine growth as characterized by birth weight was decreased in female rats treated with higher dosages of genistein along with E and DES exposed animals of both sexes. This indicates that the shortened AGD length in the affected males are independent of the overall growth inhibition seen in both sexes.

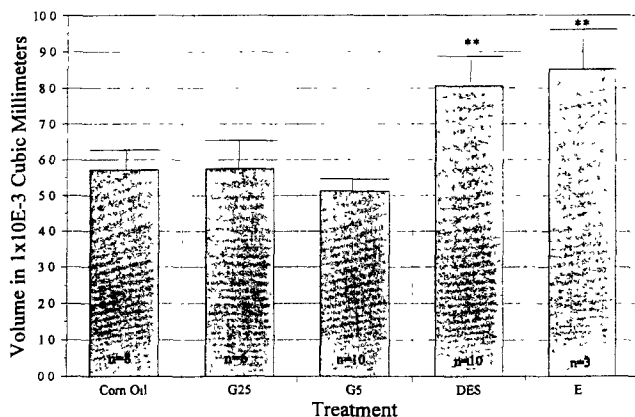
Our data on pituitary responsiveness to GnRH stimulation in genistein exposed rats demonstrates how both dosage and timing are important factors with respect to the effects of an estrogen. Adult rats treated as adults with 100 μ g/kg body wt iv genistein had blunted GnRH-induced LH secretion when compared with control animals (14). Later experiments demonstrated that neonatal treatment with 1000 μ g of subcutaneous genistein during Day 1–10 of life had blunted GnRH-stimulated LH secretion, while neonatal treatment with genistein dosages of 100 μ g enhanced GnRH-induced secretion (6). There was a lack of demonstrable differences in basal LH levels or GnRH-stimulated secretion among any of the treatment

groups in this study. It is possible that pituitary function development in rats may occur later than the prenatal period and is not consistently influenced by *in utero* exposure to estrogenic compounds. However, prenatal exposure to estrogens have been shown in this experiment to influence both indirect markers of pituitary function, vaginal opening, and vaginal cyclicity. An alternative explanation is that treatment dosages were not sufficient to induce consistent change GnRH-stimulated LH secretion.

A hormone sensitive period for differentiation of the SDN-POA in rats had been described to occur between gestation Day 16 and Day 5 of life (15, 16). SDN-POA volume increases associated with prenatal treatment with DES and E found in this experiment were consistent with these studies. Unlike our previous studies of neonatal genistein exposure that demonstrated increased SDN-POA volume in females (6, 8), prenatal genistein treatment did not cause any significant changes in SDN-POA volume. There was only a suggestion of a decreased SDN-POA volume with

Mean Sexually Dimorphic Nucleus Volume

Female Rats



**p < 0.05 when compared to Corn Oil

Figure 4. Mean volumes of the sexually dimorphic nucleus in the preoptic area (SDN-POA) of female rats exposed *in utero* on gestation day 16–20 to genistein 25,000 µg (G25), genistein 5000 µg (G5), diethylstilbestrol 5 µg (DES), estradiol 50 µg (E), or corn oil. SDN-POA volumes were significantly increased in DES- and E-treated females ($P < 0.05$) when compared with corn oil treated females.

Mean Sexually Dimorphic Nucleus Volume

Male Rats

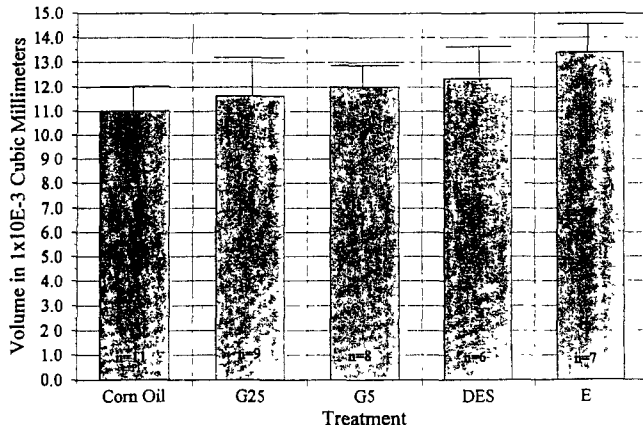


Figure 5. Mean volumes of the sexually dimorphic nucleus in the preoptic area (SDN-POA) of male rats exposed *in utero* on gestation day 16–20 to genistein 25,000 µg (G25), genistein 5000 µg (G5), diethylstilbestrol 5 µg (DES), estradiol 50 µg (E) or corn oil. There were no significant differences among treatment groups.

low-dose genistein, but this apparent difference did not attain statistical significance. If further studies demonstrate a reduction of the SDN-POA volume, then a low-dose antiestrogenic effect of genistein might be implied. It is difficult to assess whether high-dose genistein's relative inactivity in influencing most of the markers studied was due to differences in maternal metabolism or placental transport, some alteration of bioavailability due to concentration in the corn oil vehicle or expression of mixed agonist-antagonist

Age at Vaginal Opening

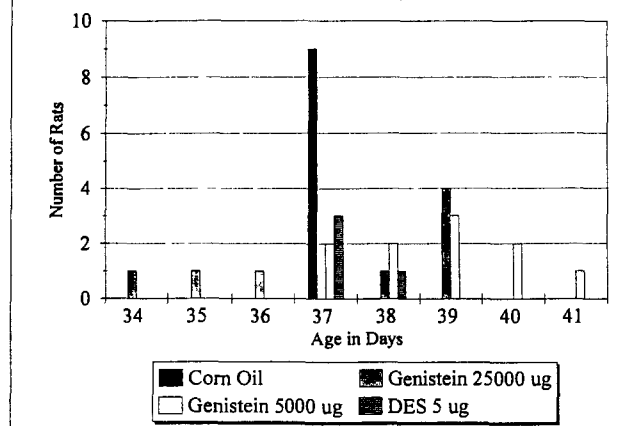


Figure 6. Frequency histogram of age in days at vaginal opening, a marker of puberty onset, of female rats exposed *in utero* on gestation day 16–20 to genistein 25,000 µg (G25), genistein 5000 µg (G5), or corn oil. Those animals treated with diethylstilbestrol 5 µg (DES) were exposed on gestation day 15–20. G5-treated females had later onset of vaginal opening when compared with corn oil controls.

effects. Of note is that while the SDN-POA volumes in male rats were found to be normally three to five times larger than those found in females (3), SDN-POA volumes in control animals in this experiment were consistent with those of gonadectomized animals in studies by Rhees *et al.* (16).

Low-dose genistein was the only treatment that delayed puberty marked by vaginal opening in comparison to the corn oil treatment group in this study. Previous research appears to be contradictory concerning the effect of perinatal estrogen exposure on puberty onset. Increased estrogen production by the ovary at puberty under the control of the hypothalamo-hypophyseal axis is believed to initiate vaginal opening. While neonatal estrogen exposure had been shown to cause precocious onset of puberty in mice (17), prenatal estrogen and testosterone exposure delayed onset of puberty in guinea pigs (18, 19). These latter studies suggest that testosterone *in utero* influences neuroendocrine mechanisms regulating puberty onset in part via conversion to estrogen. Prenatal exposure to estrogens (including genistein) may also cause a delay in puberty onset by an inhibitory or androgenizing effect on the hypothalamo-hypophyseal axis.

Genistein was not found to significantly disrupt vaginal smear cyclicity in this experiment. Persistent vaginal estrus or cornification, anovulation, and infertility during adulthood were shown to be characteristic responses to androgen and estrogen exposure in rodents during critical periods of neuroendocrine differentiation (less than 5 to 10 days of age) (19). Exposure to the phytoestrogen coumestrol also induced persistent vaginal estrus (20). Only DES-treated animals had

significantly disrupted cycles in our study, further highlighting the differences among estrogens. In addition, cycles in these DES-treated females were characterized by long periods of diestrus rather than estrus. There may be some differences in the type of ovulation disruption seen in this experiment. An alternative explanation is that the onset of persistent estrus may occur later in the rat's life cycle. The sampling period in this experiment may have been too short to detect this change.

One of the variables not controlled in this experiment was the amount of phytoestrogens present in the laboratory chow. The concentrations of phytoestrogens (including genistein) and how they varied between batches were not analyzed. In addition, the relative amounts of laboratory chow consumed by each pregnant rat and the resultant pups were not recorded. It is unclear how much impact this baseline exposure to phytoestrogens had on the resulting measures.

Differences in action were found among genistein, DES, and E in this study. Prenatal exposures to genistein also did not always have the same effects on pituitary responsiveness and SDN-POA volume as those associated with neonatal exposures demonstrated in our earlier (6, 7, 8, 14) studies. Dosage of genistein was an important factor with respect to the effects seen in our present and previous developmental studies. Genistein may have estrogen agonist and antagonist effects at high and low dosages and this relationship may not be simple or unidirectional. While this study suggests these effects are complex, genistein influences estrogen-dependent aspects of fetal and neonatal development by modifying both morphologic and neuroendocrine endpoints.

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The Effects of Phytoestrogens on Neonatal Rat Uterine Growth and Development (43861)

KEVIN L. MEDLOCK,*¹ WILLIAM S. BRANHAM,* AND DANIEL M. SHEEHAN*,†

Division of Reproductive and Developmental Toxicology, National Center for Toxicological Research, Department of Health and Human Services, Food and Drug Administration, Jefferson, Arkansas 72079-9502 and Departments of Biochemistry and Pharmacology and Toxicology,† University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205*

Abstract. Phytoestrogens found in clover, alfalfa, and soybeans have caused reproductive toxicity in several mammalian species. Other estrogens, such as diethylstilbestrol (DES), are developmental toxicants, reducing uterine estrogen receptor (ER) concentration, altering uterine growth, and eliciting reproductive tract abnormalities in the rat. The present study examines the effects of the phytoestrogens coumestrol and equol on the developing rat uterus. Various doses of these compounds were injected sc on postnatal days (PND) 1–5 or 1–10 to ascertain their effects on uterine weight and ER levels, and on PND 10–14 to determine their effects on uterine weight and gland genesis. Coumestrol (PND 1–5) was about 10^{-3} as potent as DES in increasing uterine weight (wet or dry) while equol increased dry weight only, with a potency of 10^{-5} that of DES. Although the 10 and 100 μg doses of coumestrol (PND 1–5 or 1–10) initially increased uterine wet weight, by PND 20 uterine weights either equaled or fell significantly below controls. The 100- μg dose of coumestrol (PND 1–5 or 1–10) reduced ER levels at all ages, while the 10- μg dose was not as effective. Equol (PND 1–5 or 1–10) did not affect ER levels. Premature uterine gland genesis occurred by PND 9 for the PND 1–5 100- μg coumestrol dose. When given on PND 10–14 (the critical period of gland genesis), 10 μg and 100 μg of coumestrol and 10 μg DES greatly increased uterine weight, while no effect was elicited by equol. Although coumestrol and equol inhibited uterine gland genesis in a dose-dependent manner, neither abolished gland genesis as did 10 μg of DES or tamoxifen. These data demonstrate that coumestrol elicits uterine biochemical and morphological toxicity much like DES. Equol decreased uterine gland number without increasing uterine wet weight or luminal epithelial hypertrophy, which is inconsistent with either an estrogenic or antiestrogenic action in the uterus.

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Phytoestrogens may act as estrogens or antiestrogens (1–5, 9, 13) while causing both direct and indirect toxicological effects (6–8, 10–12) on the reproductive tracts of mammals. Although the adverse effects of phytoestrogens on livestock have been studied since the mid 1940s (14), interest in the possible

benefits and risks of phytoestrogens in humans has recently increased (12). Most investigations of the effects of phytoestrogens on the reproductive tract, however, have been conducted in the rodent (1–4, 7–11, 16–18), where estrogenic potency depends on the particular phytoestrogen and endpoint. For example, miroestrol is equipotent to diethylstilbestrol (DES) or 17β -estradiol (E_2) (4), coumestrol is 200 times less potent than E_2 (18), and equol is at least 10^3 -fold less potent (17). Coumestrol-induced alterations in the reproductive tract of neonatally treated mice include persistent vaginal cornification, hemorrhagic ovarian follicles, and premature vaginal opening (8, 19). In mature mice, coumestrol caused decreased ovulation rates and an increase in embryo degeneration (11).

We have used the neonatal rat to determine estrogen effects on the ontogeny of various biochemical and

¹ To whom requests for reprints should be addressed at Division of the Reproductive and Developmental Toxicology, HFT-130, National Center for Toxicological Research, Jefferson, AR 72079-9502

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morphological parameters. These studies provide a basis for a comparative assessment of developmental insult to the reproductive tract for both estrogens and antiestrogens (20–25). Neonatal (postnatal days [PND] 1–5) estrogen exposure causes a slightly premature appearance of uterine glands but ultimately results in reduced uterine gland numbers (20–21). During the period of rapid uterine gland development (PND 10–14) estrogens cause a dose-dependent delay in the appearance of uterine glands (20, 21). This delay in the onset of uterine gland development may be related to the maintenance of estrogen-induced luminal epithelium hypertrophy (25). Antiestrogens also cause substantial and long-lasting luminal epithelium hypertrophy with little uterine weight gain. However, antiestrogens are potent inhibitors of uterine gland genesis (21, 22). Both estrogens and antiestrogens cause a persistent reduction in uterine estrogen receptor (ER) levels (22, 26).

In this report, we characterize the effects of coumestrol and equol on uterine weight gain, luminal epithelial cell height (LEH), gland genesis, and the short-term regulation of ER level in neonatal rats. As well, the persistence of these effects on uterine weight, ER levels, and glands is examined in older rats following neonatal dosing.

Materials and Methods

Animals. Offspring from date-mated Sprague-Dawley rats from the NCTR breeding colony were culled according to sex and the females randomly distributed to dams within 24 hr of birth. Groups of pups were injected sc, in the middorsal region, on PND 1–5 or 1–10 with various doses of coumestrol or equol in 10 μ l of sesame oil. Control animals were untreated. The animals were sacrificed on PND 5, 10, 15, 20, and 25. The number of animals used at each time point and treatment group ranged from 21 at PND 5 to three at PND 25.

Chemicals. Coumestrol and equol were purchased from Spectrum Chemical Mfg. Corp. (Garden, CA). DES was obtained from Research Plus Steroid Laboratories, Inc. (Denville, NJ). Tamoxifen (TAM) was a gift from Stuart Pharmaceuticals (Wilmington, DE). [3 H]-E₂ (sp act 92–115 Ci/mmol) was purchased from Dupont NEN Products (Boston, MA). All other chemicals were laboratory grade.

Uterotropic Dose-Response. Pups were injected daily on PND 1–5 with 0.001, 0.01, 0.1, 10, or 100 μ g DES; 0.01, 0.1, 1, 10, 50, 100, or 1000 μ g coumestrol; or 1, 10, 100, or 1000 μ g equol. On PND 5, the pups were weighed and killed by cervical dislocation after being lightly anesthetized with ether. The uteri were removed, weighed, placed on preweighed squares of aluminum foil, dried overnight at 70°C, and then reweighed.

ER Time Course. Pups were injected on PND 1–5

or 1–10 with 10 or 100 μ g coumestrol or 100 μ g equol. One hour before sacrifice, all animals were injected with 10 μ g DES to maximize ER binding in the nuclear fraction and thus avoid confounding of results due to alpha-fetoprotein contamination (27). Following sacrifice, the uteri were pooled and then homogenized in cold TE buffer (10 mM Tris, 1.5 mM EDTA, pH 7.4) at a concentration of 30 mg tissue/ml of buffer. The homogenates were processed as described previously with nuclear ER levels being determined by the [3 H]-E₂ exchange assay (24).

Uterine Gland Genesis. Time course study. Pups were injected on PND 1–5 with 10 or 100 μ g coumestrol or equol. They were sacrificed at PND 5, 9, 26, and 60 for comparison of effects with those reported previously for E₂, DES, and TAM (20–22). Uteri were carefully removed, stripped of connecting mesentery, and fixed in 10% neutral buffered formalin. They were processed using standard histological procedures, stained with hematoxylin and eosin, and sectioned at 4 μ m. At least six sections per animal were scored for uterine glands and measurements of LEH (25).

Dose-response study. Control pups and those given 1, 10, or 100 μ g of coumestrol; 10, 100, or 1000 μ g of equol; or 10 μ g of DES or tamoxifen were injected on PND 10–14. They were sacrificed on PND 14 and treated as described above.

Statistics. Statistical analyses were done using a two-way ANOVA and are presented as means \pm SEM with a level of significance of $P < 0.05$ (significance was determined by Duncan's multiple range test).

Results

Following dosing on PND 1–5 and evaluation on PND 5 the uterine wet weight dose-response curves for DES and coumestrol were parallel (Fig. 1A). There was a slight but not significant uterine wet weight gain at the highest dose of equol. DES was about 4×10^2 -fold more potent than coumestrol. Uterine dry weight dose-response curves for DES, coumestrol, and equol were all parallel in the ascending part of the curves (Fig. 1B). Equol induced a significant dry weight increase only at the highest dose with a potency about 10^{-5} of that for DES. For dry weight, DES was about 3×10^3 -fold more potent than coumestrol. Thus, coumestrol was about 8-fold more potent for wet weight gain than dry weight gain relative to DES.

Based on the dose-response curves, phytoestrogen doses were chosen for ontogeny studies after neonatal dosing. Again, both 10 μ g and 100 μ g coumestrol doses elicited significant uterine weight gain on PND 5 (Fig. 2A). By PND 10, only the 100 μ g coumestrol dose group still showed increased uterine weight. At PND 10, 20, and 25 for the 10 μ g coumestrol group, and PND 20 and 25 for the 100 μ g coumestrol and

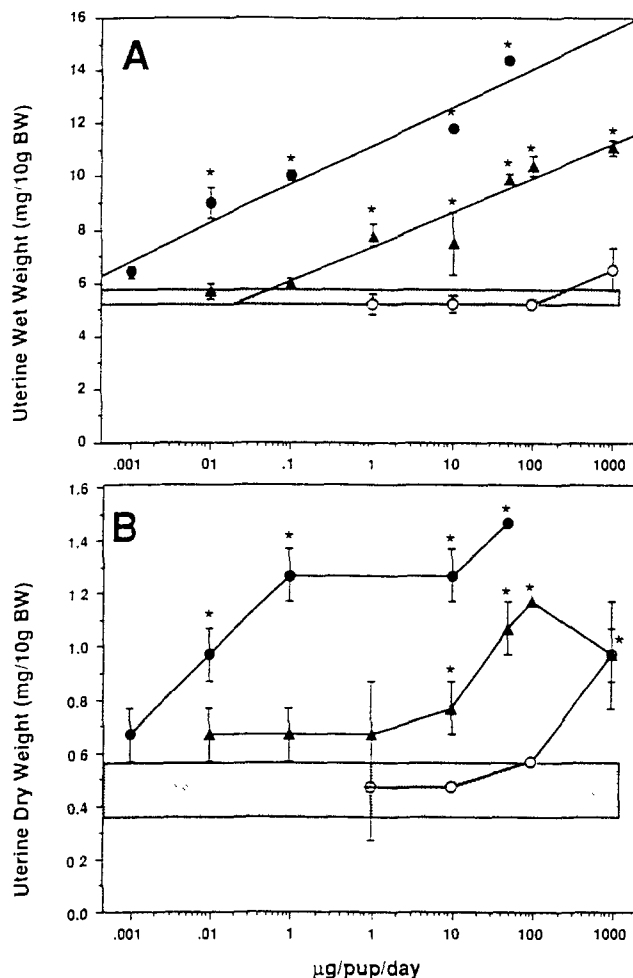


Figure 1. Uterine weight dose-response in rat pups either untreated or injected on PND 1–5 with DES (●), coumestrol (▲), or equol (○) and sacrificed on PND 5. (A) Uterine wet weight; (B) Uterine dry weight. Shaded area represents the untreated means \pm SEM. The data are present as means \pm SEM with $n \geq 7$. Asterisks indicate significant differences at $P \leq 0.05$.

equol groups, uterine weights were significantly lower than controls. Ten days of treatment (PND 1–10) significantly increased uterine weight in the 10 and 100 µg coumestrol dose groups (Fig. 2B). However, by PND 15, the 10 µg equol and coumestrol groups had uterine weights significantly lower than controls, while rats given 100 µg coumestrol had uterine weights significantly greater than controls. By PND 20, no differences were seen in any group, whereas by PND 25 uterine weights in the 100 µg coumestrol group were significantly lower than controls.

There was no effect of 100 µg equol at ages up to PND 25 on uterine ER levels when given on PND 1–5 (Fig. 3A). After 5 days of coumestrol treatment (Fig. 3A), the 100 µg dose reduced the ER level to 60% of controls. By PND 10, ER levels decreased even further, to 15%–20% of controls. Subsequently, ER levels remained 40%–70% below controls (Fig. 3A). The 10 µg dose of coumestrol caused a significant ER reduc-

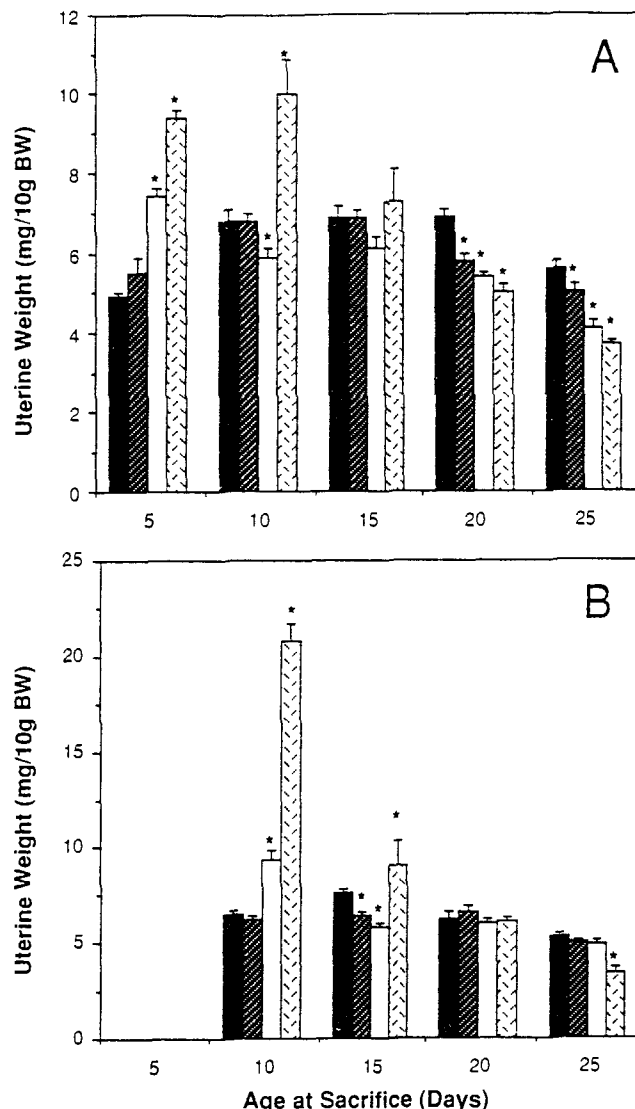


Figure 2. Uterine weight in untreated rat pups (■) or pups injected neonatally with 100 µg equol (▨), 10 µg coumestrol (□), or 100 µg coumestrol (▩) and sacrificed on PND 5, 10, 15, 20, and 25. (A) Pups were injected on PND 1–5; (B) Pups were injected on PND 1–10. The data are presented as means \pm SEM with a $n \geq 18$. *Significant difference at $P \leq 0.05$.

tion on PND 10, 15, and 25. After 10 days of exposure (PND 1–10), 100 µg equol reduced ER significantly, but at later ages, ER remained at control levels (Fig. 3B). By contrast, both coumestrol doses severely reduced the ER levels to 20%–40% of controls on PND 10 (Fig. 3B). At later ages, the ER level in the 10 µg coumestrol dose group progressively increased, until by PND 20–25 it was not different from controls. In rats given 100 µg coumestrol, the ER level remained at 20% of control on PND 15 after which it increased with time to a value about two thirds of controls.

Following PND 1–5 dosing, no uterine glands were observed at PND 5 in either the controls or any treated pups (Table I). Equol treatment had no effect on gland number at any age. However, at PND 9, there was a

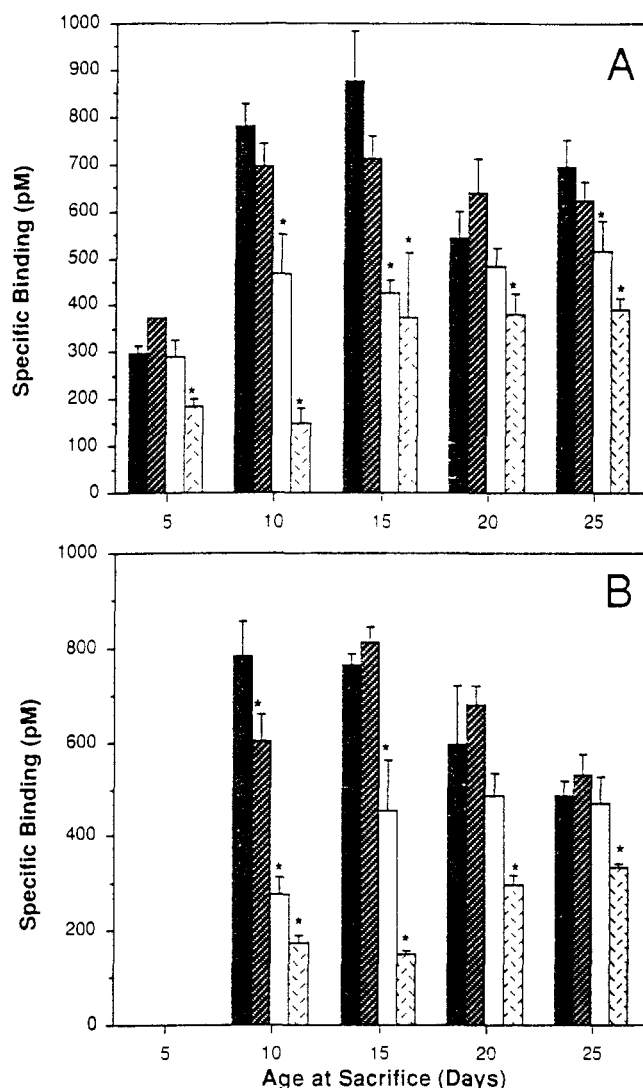


Figure 3. Uterine ER levels in nontreated rat pups (■) or pups injected neonatally with 100 µg equol (▨), 10 µg coumestrol (□), or 100 µg coumestrol (▤) and assayed on PND 5, 10, 15, 20, and 25. (A) Pups were injected on PND 1-5. (B) Pups were injected on PND 1-10. The data are presented as means ± SEM with a *n* ≥ 3. *Significant difference at *P* ≤ 0.05.

significant increase in glands at the 100 µg coumestrol dose (Table I). By PND 26, the gland numbers for all treatments were essentially the same as the controls (Table I). While gland numbers increased from PND 26 to PND 60, there were no significant differences between any of the groups. LEH was elevated only on PND 5 in the high-dose coumestrol group.

After PND 10-14 treatment, only the 10 and 100 µg doses of coumestrol significantly increased uterine weight (Fig. 4A). While the antiestrogen TAM increased uterine weight slightly but significantly at the 10 µg dose, the same dose of DES tripled uterine weight (Fig. 4A). Both coumestrol and equol caused a reduction in gland number, and the equol effect was dose dependent (Fig. 4B). Coumestrol appeared to be 10-100 times more potent than equol with respect to

inhibition of gland genesis, although the maximum reduction (to 25% of controls) was the same for both compounds (Fig. 4B). Both the 10 µg TAM and DES doses dramatically reduced gland genesis. With the exception of TAM, the effect of all treatments on LEH was very similar to that seen for uterine weight (Fig. 4A and C). The 10 µg TAM dose increased LEH as much as the 10 µg DES dose. No dose of equol increased LEH (Fig. 4C). The 10 and 100 µg doses of coumestrol increased LEH to double the control value, although both were significantly lower than TAM or DES (Fig. 4C).

Discussion

The data reported here demonstrate that the phytoestrogen coumestrol is estrogenic when examined at several ages during the postnatal development of the rat, using uterine growth and morphological and biochemical endpoints. By contrast, equol demonstrated characteristics inconsistent with any estrogen or antiestrogen previously studied in this system (20-25).

In immature rats, the uterotrophic potency of coumestrol and equol compared with E_2 or DES is 0.5% and 0.1%, respectively (17, 18). We have examined a variety of estrogens and antiestrogens with respect to uterine weight gain following treatment on PND 1-5 (20-24). Our wet weight data are in general agreement with the estimated potency of coumestrol but not equol in immature animals. If the difference in uterotrophic potency between coumestrol and equol is the same in neonates as in immature rats, then at the 100 µg/pup dose, we would have expected an average uterine weight of 9 mg instead of the actual value of 5 mg. We can estimate equol potency for uterine growth by using dry weight data, which suggests a potency that is 30-fold less than coumestrol or about 10^{-5} -fold less than DES. The former should be compared with the 5-fold lower potency of equol in immature rats (17, 18). These findings demonstrate an age-dependent alteration in relative equol potency. Pharmacokinetic differences between the neonatal and the immature rat (i.e., changes in metabolism and/or in excretion pathways) might explain this finding.

Comparison of parallel segments of the dose-response curves for wet and dry weight for coumestrol and DES shows that coumestrol is about 8-fold less potent than DES in causing dry weight gain compared with wet weight gain. Thus, wet and dry uterine weights give different estimates of relative potency for both coumestrol and equol.

Following PND 1-5 treatment, we observed a small but significant decrease in uterine weight on PND 20 and 25 with both coumestrol and equol. A similar effect has been previously reported for other neonatally administered estrogens (20). For this delayed estrogenic effect, equol appears to be about 10-

Table I. Effect of Treatment on PND 1–5 with Equol or Coumestrol on Uterine Glands and LEH

Treatment	5		9		26		60	
	Glands/ section	LEH	Glands/ section	LEH	Glands/ section	LEH	Glands/ section	LEH
Control	0.00	12.56 ± 0.82	0.00	23.11 ± 0.39	4.44 ± 0.21	15.52 ± 1.48	6.39 ± 0.42	18.29 ± 1.82
100 µg equol	0.00	14.43 ± 0.31	0.00	23.86 ± 0.40	3.61 ± 0.34	15.67 ± 0.46	7.35 ± 0.74	21.89 ± 4.00
100 µg coumestrol	0.00	30.52 ± 1.36 ^a	1.67 ± 0.34 ^a	24.24 ± 1.00	3.87 ± 0.49	12.36 ± 0.44	5.75 ± 0.92	21.86 ± 6.48

^a Significantly greater than control.

fold less potent than coumestrol (compare 10 µg coumestrol with 100 µg equol; Fig. 2A). The decrease in uterine weight may be due to an alteration in the hypothalamo-hypophyseal-ovarian axis resulting in lowered estrogen secretion (28). Alternatively, the uterine weight reduction might be due to reduced responsiveness to estrogens since earlier work has shown that rats treated with DES on PND 1–5, ovariectomized and then implanted with an E₂ implant for a week gave only a 70% uterine weight response compared with the control response. This implies a persistent impairment of uterine responsiveness by the neonatal DES treatment.

The coumestrol data imply that coumestrol has a longer half-life than equol or DES. The pattern of uterine weight gain and loss for the 100 µg coumestrol dose differed from that reported earlier for 10 µg DES in which there was a 3-fold increase over controls on PND 5 which dropped by approximately 50% on PND 10 and was significantly lower than controls on PND 15 (20). The 100 µg coumestrol effect was more persistent than DES in that normalized uterine weight at PND 10 was the same as on PND 5. Perhaps coumestrol is removed more slowly than DES due to metabolic differences, retention in fat depots, or other pharmacokinetic differences.

PND 1–10 treatment was less sensitive than PND 1–5 in eliciting later uterine weight reduction. This apparent lowered responsiveness of the PND 1–10 dosing schedule compared with PND 1–5 may be due to the fact that on PND 25, examination is 20 days post-dosing in the 5-day regimen, while it is 15 days post-dosing for the PND 1–10 schedule.

The effect of equol on PND 1–5 in subsequently lowering uterine weight on PND 20 and 25 is not reflected in lowered ER levels. Coumestrol, however, elicited dose-dependent, persistent decreases in uterine ER. This decrease is not due to the uterine weight loss, per se, since the same weight of control or treated tissue (30 mg/ml) is used in the ER assay. On PND 1–10, coumestrol again induced a dose-dependent, persistent decrease in ER, while a late uterine weight decrease was seen only on PND 25 in the high-dose coumestrol group. Thus, ER reduction appears to be a

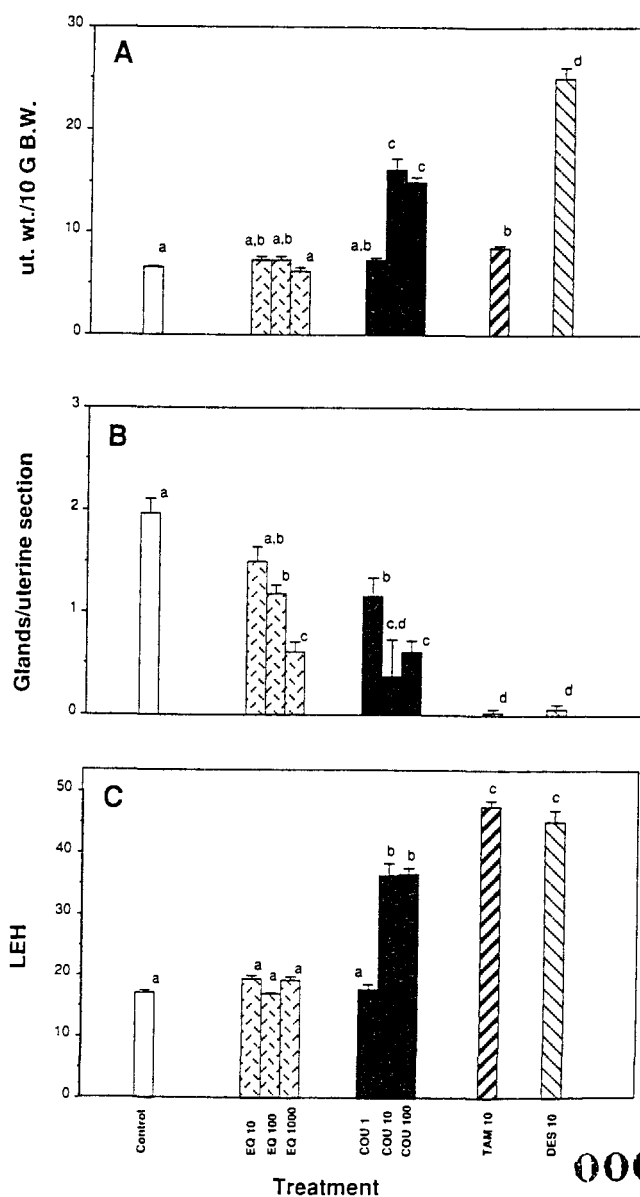


Figure 4. Dose-response for normalized uterine weight (A), gland number (B), and luminal epithelial cell height (LEH) (C) in rat pups injected on PND 10–14 and sacrificed on PND 14. Treatments included untreated controls; 10, 100, or 1000 µg/pup of equol, 1, 10, or 100 µg/pup of coumestrol or 10 µg/pup of tamoxifen or DES. The data are presented as means ± SEM with a *n* ≥ 7. Values with different letters are statistically different from each other at *P* ≤ 0.05

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more sensitive measure of early coumestrol exposure than is uterine weight reduction.

Coumestrol given on PND 1-5 induced an increase in uterine LEH and premature gland genesis, but had no effect on later gland levels. Equol was without effect. Potent estrogens such as E_2 , DES, and ethynylestradiol induce premature gland genesis but long-term gland numbers are lowered (21, 25). In this study, controls showed the same ontogenic pattern of LEH and gland number as previously reported (25).

Dosing with estrogens and antiestrogens during the critical period of gland genesis (PND 10-14) has a more profound effect on inhibition of gland development than dosing on PND 1-5, a period prior to gland appearance (22, 25). However, antiestrogens, such as TAM, and estrogens, such as DES, have distinctly different properties during this period of development. While DES greatly increases uterine weight, TAM only slightly increases it. Both inhibit gland appearance and increase LEH. These effects were replicated when TAM and DES were used as positive controls in this study. Coumestrol behaved like an estrogen, increasing uterine weight and LEH, and inhibiting gland appearance. Equol, however, had no effect on uterine weight or LEH, but inhibited gland appearance in a dose-dependent manner. Equol appears about 100-fold less potent than coumestrol for this endpoint. Thus equol shows a different pattern of effects on the developing uterus during PND 10-14 than either estrogens or antiestrogens. This finding is consistent with the earlier described effect of equol following treatment on PND 1-5 in increasing uterine dry weight but not wet weight, and having a persistent effect on uterine weight out to PND 25, without lowering ER levels during the same developmental period. Coumestrol, on the other hand, behaves like DES in the types of effects observed. These effects could be explained by different levels of sensitivity to estrogens for different endpoints.

It is unclear why equol shows a pattern of effects during development that are different from estrogens or antiestrogens. Earlier studies on equol suggested that it is an estrogen, albeit a weak one (17). Possibilities for such a different pattern for equol include dissociation of noncausally linked estrogen-regulated events (i.e., wet weight from dry weight [29]), an additional mechanism of toxicity beyond its weak estrogenic activity, an additional site of action (i.e., at a different cell type, tissue, or receptor) or pharmacokinetic and/or metabolic differences leading to altered pharmacodynamics.

This study has demonstrated the developmental potency and toxicity of the phytoestrogens, coumestrol, and equol. Coumestrol exhibits properties found with estrogens generally, while equol possesses a pattern of developmental outcomes that is not shared by

either estrogens or antiestrogens. The findings with equol require more detailed studies of its pharmacological and toxicological properties to understand its mechanism of action.

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Effects of Coumestrol and Equol on the Developing Reproductive Tract of the Rat

(43833)

KEVIN L. MEDLOCK,^{*,1} WILLIAM S. BRANHAM,^{*} AND DANIEL M. SHEEHAN^{*,†}

Department of Health and Human Services, Food and Drug Administration, National Center for Toxicological Research, Division of Reproduction and Developmental Toxicology, Jefferson, Arkansas 72079-9502 and Department of Biochemistry and Department of Pharmacology and Toxicology,† University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205*

Abstract. The phytoestrogens, coumestrol and equol, are weakly estrogenic. Here, we have examined their ability to induce responses in the neonatal rat uterus. Potent estrogens such as diethylstilbestrol (DES) and 17 β -estradiol which initially double uterine weight on postnatal Day (PND) 5 when given on PND 1–5 subsequently reduce both uterine growth and gland development at later ages. In this study, Sprague-Dawley pups were treated neonatally (PND 1–5) with various doses of coumestrol and equol, and sacrificed at different ages to determine alterations in biochemical and morphological endpoints. Other rats were injected with the same compounds during the critical period of gland genesis (PND 10–14) to examine their effects on gland development. At the 100 μ g coumestrol dose, on PND 1–5, premature gland development and increased uterine weight were observed. However, at later ages, uterine weight was significantly lowered and there was a severe suppression in the estrogen receptor (ER) levels. Equol lowered uterine weight at the later ages but did not affect ER levels. When given on PND 10–14, both coumestrol and equol caused a dose-dependent inhibition of gland genesis though not as severe as either DES or tamoxifen. Coumestrol was about 10³ more potent than equol as an estrogen and behaved much like DES with respect to its effects on uterine weight, glands, and ER levels. At the doses used in this study, equol failed to demonstrate either estrogenic or anti-estrogenic activity.

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Investigations of the effects of phytoestrogens on the rodent reproductive tract have shown that coumestrol is 10⁻¹ to 5 \times 10⁻³ times and equol is 10⁻³–10⁻⁵ times as potent as either estradiol (E₂) or diethylstilbestrol (DES) (1–3). Coumestrol-induced pathologies in the reproductive tracts of mice include persistent vaginal cornification, hemorrhagic ovarian follicles, and premature vaginal opening in neonatally treated animals and decreased ovulation rates and an increase in embryo degeneration in animals treated as adults (4–6). Despite the fact that equol is the primary

metabolite excreted in the urine of humans and animals ingesting soy products, little is known about its potential toxicity in the developing reproductive tract (7).

Both estrogens and antiestrogens have a toxic effect on the developing reproductive tract of the neonatal rat (8–14). Exposure to DES on PND 1–5 causes early uterine weight gain and premature gland genesis; however, by PND 60 both uterine weights and gland numbers are reduced to levels below controls (8, 14). Tamoxifen (TAM), a triphenylethylene antiestrogen with partial estrogen agonist activity, given on PND 1–5, causes a reduction in uterine weight equivalent to that seen for E₂ and nearly abolishes gland development, while E₂ reduces gland numbers to 75% of controls at PND 26 (10, 13, 15). During the period of rapid uterine gland development (PND 10–14), estrogens cause a dose-dependent delay in the appearance of uterine glands which may be related to the maintenance of estrogen-induced luminal epithelial cell hy-

¹ To whom requests for reprints should be addressed at Division of Reproductive and Developmental Toxicology, HFT-130, National Center for Toxicological Research, Jefferson, AR 72079-9502.

pertrophy (LEH) (8, 9, 13, 14). Antiestrogens also cause substantial and long-lasting LEH and are potent inhibitors of uterine gland genesis (9, 10, 15).

In view of the several and varied developmentally toxic effects elicited by both weak and potent estrogen agonists and antagonists, we have examined uterine toxicity of phytoestrogens in the developing rat. In this paper, we report the effects of coumestrol and equol on uterine weight gain, LEH, gland genesis, and ER levels in neonatal rats. We also examine the persistence of these effects following neonatal dosing.

Materials and Methods

Offspring from date-mated Sprague-Dawley rats from the National Center for Toxicological Research (NCTR) breeding colony were culled according to sex and the females randomly distributed to foster dams within 24 hr of birth. Groups of pups were injected sc in the mid-dorsal region on PND 1–5 or 10–14 with 1, 10, or 100 µg of coumestrol; 10, 100, or 1000 µg of equol; or 10 µg of TAM or DES in sesame oil. Control animals were untreated. Rats treated with coumestrol and equol on PND 1–5 were sacrificed at various time-points. Groups were sacrificed on PND 5, 10, 15, 20, and 25 to determine how persistent the effects of these compounds were on uterine weight and ER levels. Other groups were sacrificed on PND 5, 9, 26, and 60 to assess premature gland genesis and any long-term effects on either gland numbers or LEH. Finally, additional groups were sacrificed on PND 8, 9, and 10 to further define the temporal aspects of premature gland genesis, LEH alterations, and uterine weight changes and to compare them with a dose of DES known to elicit premature gland genesis. Animals injected on PND 10–14 were sacrificed on PND 14 to determine the effects of the phytoestrogens on uterine weight, gland number, and LEH when given during the critical period of gland genesis and to compare these compounds to doses of an estrogen (DES) and an antiestrogen (TAM) known to abolish gland genesis.

For ER determinations, all animals were injected with 10 µg DES 1 hr before sacrifice to maximize the ER binding in the nuclear fraction (16). Following sacrifice, the uteri were pooled and then homogenized in cold TE buffer (10 mM Tris, 1.5 mM EDTA, pH 7.4) at a concentration of 30 mg/ml of buffer. The homogenates were processed as described previously with nuclear ER levels being determined by the [³H]-E₂ exchange assay (12).

For measurement of gland number and LEH, the uteri were carefully removed, stripped of connecting mesentery and fixed in 10% neutral buffered formalin. They were then processed using standard histological procedures, stained with hematoxylin and eosin, and sectioned at 4 µm. At least six sections per animal

were scored for uterine glands and measurements of LEH (13).

Coumestrol and equol were purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA). DES was obtained from Research Plus Steroid Laboratories, Inc. (Denville, NJ). TAM was a gift of Stuart Pharmaceuticals (Wilmington, DE). [³H]-E₂ (sp act 92–115 Ci/mMol) used in the receptor exchange assay was purchased from Dupont NEN Products (Boston, MA). All other chemicals were laboratory grade.

Statistical analyses were done using a two-way analysis of variance (ANOVA) and are presented as means ± SEM with a level of significance of $P \leq 0.05$ (significance was determined by Duncan's multiple range test).

Results

Following dosing on PND 1–5, both the 10 and 100 µg dose of coumestrol elicited significant uterine weight gain on PND 5; however, by PND 10, only the 100 µg dose group had uterine weight above controls (Fig. 1A). At PND 10, 20, and 25 for the 10 µg coumestrol group and PND 20 and 25 for the 100 µg coumestrol and equol groups, uterine weights were significantly lower than controls (Fig. 1A). However, by PND 60 there was no significant difference between the treatment groups and the controls (data not shown). Relative to control level, the 10-µg dose of coumestrol given on PND 1–5 caused a significant reduction in the uterine ER level by PND 10 which was still reduced on PND 25 (Fig. 1B); ER levels remained constant from PND 10 to 25. The 100-µg dose of coumestrol significantly reduced ER levels relative to control uteri on PND 5–25, although the levels observed on PND 15–25 were double that seen on PND 5 and 10 (Fig. 1B). Equol (100 µg) had no effect on ER levels at any age.

Only the 100 µg coumestrol dose had a significant impact on uterine morphology. On PND 5, coumestrol more than doubled the LEH, which had not dropped by PND 8 although there was a slight but significant increase in gland number (Fig. 2 and 3, B and C). Even though the gland number continued to increase on PND 9, no further increase was seen on PND 10 despite a continued decrease in LEH. This differs from the effect elicited by 10 µg DES which caused slight but significant increase in gland number (equivalent to that of coumestrol) on PND 8, and a further increase to Day 10 (Fig. 3B). While equol (100 µg) did not elicit premature gland genesis on PND 8–10 (Fig. 3B), LEH was significantly higher than in controls on PND 9 and 10 (Fig. 3C). Equol also had no effect on uterine weight, whereas the 100-µg dose of coumestrol and the 10-µg dose of DES both elicited a significant increase

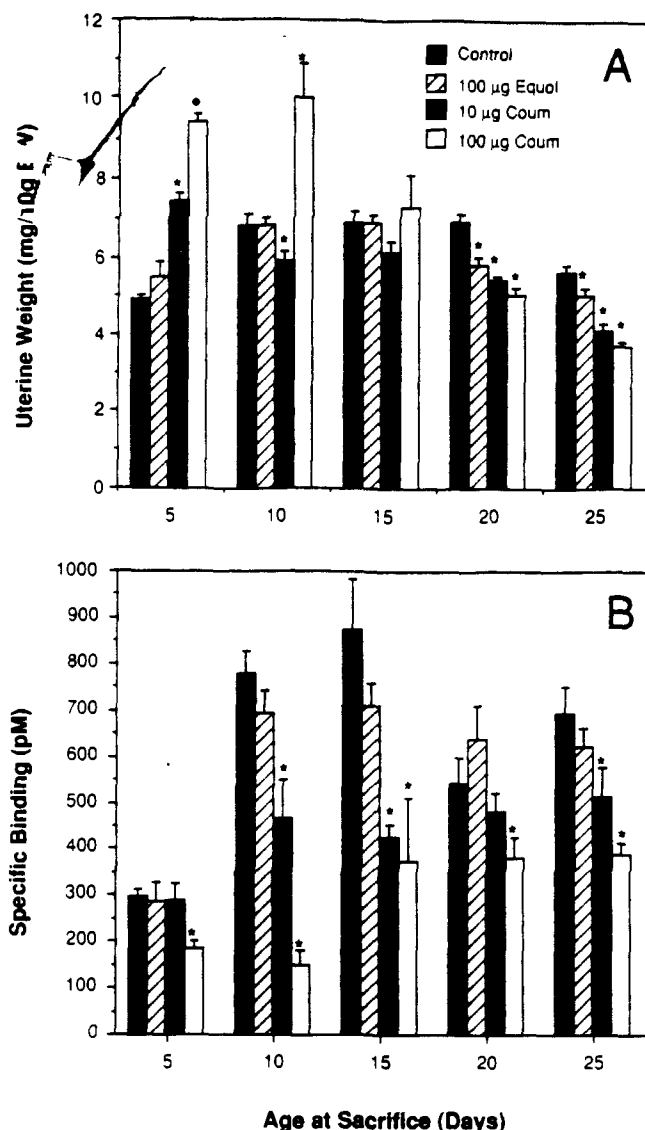


Figure 1. Uterine weight (A) and ER levels (B) in untreated rat pups or pups injected on PND 1–5 with 100 µg equol, 10 µg coumestrol, or 100 µg coumestrol and sacrificed on PND 5, 10, 15, 20, and 25. The data are presented as means ± SEM with $n \geq 18$ (A) or $n \geq 3$. Asterisks indicate significant differences at $P \leq 0.05$.

in uterine weight on PND 8–10 with the coumestrol group significantly higher than the DES group (Fig. 3A).

When given on PND 10–14, the 10-µg and 100-µg doses of coumestrol doubled uterine weight (Fig. 4A). The 10-µg dose of the antiestrogen TAM caused a slight but significant increase in uterine weight while the same dose of DES tripled uterine weight (Fig. 4A). Although equol had no effect on uterine weight (Fig. 4A), there was a dose-dependent decrease in gland number (Fig. 4B). Coumestrol also exhibited a reduction in glands, but there was no difference between the two higher doses (Fig. 4B). Coumestrol appeared to be 10–100 times more potent than equol with respect to inhibition of gland genesis, although the maximum reduction (25% of controls) was the same for both com-

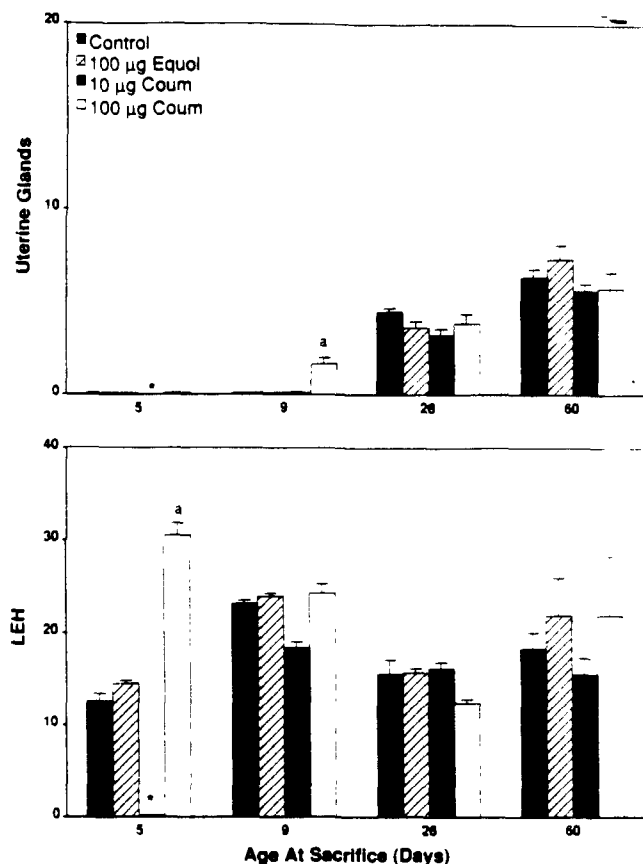
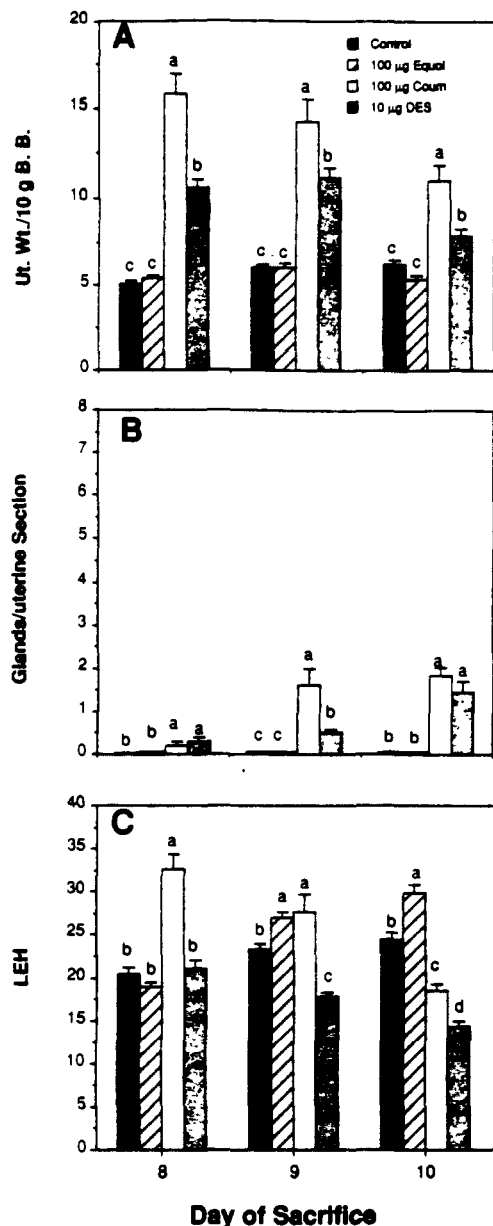


Figure 2. Gland number (glands/uterine section) (A) and LEH (B) in untreated pups or pups injected PND 1–5 with 100 µg equol, 10 µg coumestrol, or 100 µg coumestrol and sacrificed on PND 5, 9, 26, and 60. The data are presented as means ± SEM with $n \geq 6$. Asterisks indicate that measurements were not made at this dose. Values significantly different from controls are indicated by the letter a at $P \leq 0.05$.

pounds (Fig. 4B). Glands were essentially abolished by the 10-µg doses of DES and TAM, which also caused identical increases in LEH (Fig. 4, B and C). Compared with controls, the 10- and 100-µg doses of coumestrol doubled LEH, although both were significantly lower than TAM or DES (Fig. 4C). LEH was not increased by any dose of equol (Fig. 4C). With the exception of TAM, the effect of all treatments on LEH was very similar to that seen for uterine weight (Fig. 4, A and C).

Discussion

Coumestrol is clearly estrogenic, although its potency depends on the endpoint measured and the time and conditions under which it is measured. Comparison of normalized uterine weight on PND 10 in this study shows that the 10-µg dose of DES falls between the 10- and 100-µg doses of coumestrol when given on PND 1–5. However, we have shown earlier that DES is about 400 times more potent than coumestrol when given on PND 1–5 and measured on PND 5 (3). Measurement of premature uterine gland genesis on PND 9



Day of Sacrifice

Figure 3. Temporal response for (A) uterine weight, (B) gland number, and (C) LEH in rat pups either untreated or injected with 10 µg DES, 100 µg coumestrol, or 100 µg equol on PND 1–5 and sacrificed on PND 8, 9, and 10. The data are presented as means \pm SEM with $n \geq 7$. Values with different letters are statistically different from each other at $P \leq 0.05$.

and 10 also shows that DES is less than 10 times more potent than coumestrol. These data would suggest a long half-life for coumestrol which is supported by a recent report in the literature (17). It is interesting that 10 µg DES given on PND 1–5 results in a severe reduction in gland number (40% of controls) on PND 60 (14), while 100 µg coumestrol does not cause any reduction in gland number on PND 60. This may be a result of potency differences between DES and coumestrol, or it may indicate that these compounds interact differently with other growth factors or that they act differently at the genomic level. The latter

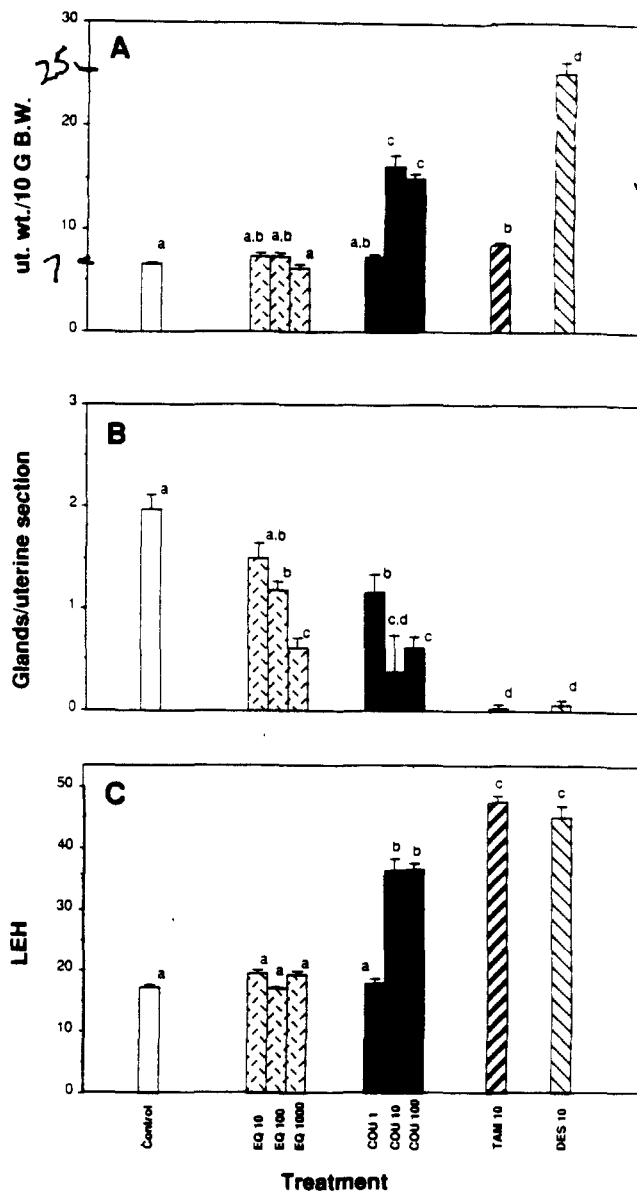


Figure 4. Dose response for uterine weight, gland number, and LEH in rat pups injected on PND 10–14 and sacrificed on PND 14. Treatments were untreated controls; 10, 100, or 1000 µg equol; 1, 10, or 100 µg coumestrol, or 10 µg TAM or DES. The data are presented as means \pm SEM with $n \geq 7$. Values with different letters are statistically different from each other at $P \leq 0.05$.

possibilities are currently under investigation. Comparison of the effects of coumestrol given on PND 10–14 on uterine growth and morphology with the effects of DES and TAM given on the same days again demonstrates that coumestrol is a weak estrogen although its potency is uncertain.

As with DES (8), there is no apparent correlation between uterine growth and ER levels following neonatal administration of either phytoestrogen. Coumestrol initially causes an uterine weight increase while severely reducing ER levels. However, by PND 20, uterine weight is significantly lower than control and

ER levels have increased although still below the control levels. Equol significantly lowers uterine weight on PND 20 and 25 but does not affect ER levels. The data presented in this study would suggest that there are no long-term adverse effects following neonatal exposure to the concentrations of phytoestrogens used as are seen for 10 µg DES (14).

The preponderance of the data presented in this study suggests that equol does not act entirely as either an estrogen or an antiestrogen. When given on PND 1–5, equol lowers uterine weight (as does DES) on PND 20 and 25 and causes a significant increase in LEH on PND 9 and 10. Treatment with equol on PND 10–14 has no effect on uterine weight or LEH at any dose. DES (10 µg) given on PND 10–14 caused a significant increase in both uterine weight and LEH, while TAM (10 µg) had no effect on uterine weight and significantly increased LEH. Lastly, equol causes a dose-dependent decrease in gland number although not nearly as severe as either DES or TAM. We have reported earlier that equol had an estrogenic potency 10^{-5} times that of DES (3). It would seem, therefore, that if equol is an estrogen, it is an extremely weak one.

This study has characterized several aspects of the developmental toxicity of the phytoestrogens, coumestrol and equol. Coumestrol exhibits properties generally found with estrogens while equol possesses a pattern of developmental outcomes that is not shared by either estrogens or antiestrogens. The findings with equol require more detailed studies of its pharmacological and toxicological properties to understand its mechanism of action.

The authors gratefully acknowledge the contributions of Mary Lee Leamons for her technical assistance in conducting the ER assay; Loetta Bradford and Diann Smith for manuscript preparation; and the Information Management Branch, National Center for Toxicological Research, for preparation of the figures. The authors also express appreciation to Vera Campbell, Hazel Carpenter, Howard Durrett, Charles Law, Marsha Law, Vickie Thompson, and Alice Crumpton for their care and attention in animal maintenance and hormone injections.

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The Effect of Neonatal Exposure to Diethylstilbestrol, Coumestrol, and β -Sitosterol on Pituitary Responsiveness and Sexually Dimorphic Nucleus Volume in the Castrated Adult Rat (43834)

B. REGISTER, M. A. BETHEL, N. THOMPSON, D. WALMER, P. BLOHM, L. AYYASH AND C. HUGHES, JR.¹

Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, North Carolina 27710

Abstract. The neonatal hormone environment influences the sexually differentiated patterns of development. Estrogens, derived from intracerebral aromatization, promote male pattern development of the central nervous system. The purpose of this study was to determine the effects of neonatal exposure to environmental estrogens on luteinizing hormone (LH) secretion and development of the sexually dimorphic nucleus of the medial preoptic area (SDN-POA) in castrated adult rats. Neonatal rats of both sexes received injections of either corn oil, 0.1 μ g diethylstilbestrol (DES), 3 μ g β -sitosterol (B1), 30 μ g β -sitosterol (B2), 0.1 μ g coumestrol (C1), 1 μ g coumestrol (C2), or 10 μ g coumestrol (C3) on Day 1–10 of life and were castrated on Day 21. Right heart catheters were placed on Day 42, and GnRH (50 ng/kg) was administered. Blood was sampled for LH at 0-, 5-, 10-, 15-, and 30-min intervals. All doses of β -sitosterol and coumestrol elicited increased basal levels of LH in females. In males, B1, B2, C2, and C3 increased basal levels of LH. The GnRH-induced LH increase was prevented in females treated with diethylstilbestrol and 10 μ g of coumestrol. Males in all treatment groups exhibited GnRH-induced LH surges. The animals were sacrificed by decapitation on Day 49. Volumes of the SDN-POA of the groups were compared. Treatment with the agents did not result in significantly increased SDN volume in females; nor was there a difference in SDN size among the male groups. These data show that exposure to environmental estrogens early in development alters both postpubertal pituitary response to GnRH and basal LH secretion in females and alters only basal LH secretion in males. No significant enlargement (i.e., masculinization) of the SDN-POA was exhibited.

[P.S.E.B.M. 1995, Vol 208]

The exposure of the central nervous system (CNS) to sex steroid hormones during development influences the formation of a sexually dimorphic brain. Males and females show developmental differences in neuroanatomy, changes in reproductive physiology, and changes in sexual behavior. A

well-studied sex difference is the differential release of gonadotropins from the anterior pituitary. Males have a tonic release pattern while females exhibit cyclical release (1). Males also exhibit structural CNS differences that seem to impact male typical behavior. The sexually dimorphic nucleus (SDN) is enlarged in the male and seems to have a role in promoting male mating activities (2). Lesions of this area in adult male gerbils interfere with open field scent marking and are associated with decreased mating behavior (3). The SNB, a nucleus in the caudal spinal cord which innervates the muscle responsible for penile reflexes, is also significantly enlarged in the male (4). In rats, each of these sexually dimorphic characteristics differentiate

¹ To whom requests for reprints should be addressed at Box 3418, Duke University Medical Center, Durham, NC 27710.

Estrogenic Flavonoids: Structural Requirements for Biological Activity (43830)

RICHARD J. MIKSICEK¹

Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794-8651

Abstract. A systematic survey of polycyclic phenols has been performed to identify members of this chemical group with estrogenic activity. Twelve compounds were found to be able to stimulate the transcriptional activity of the human estrogen receptor expressed in cultured cells by transient transfection. These natural estrogens belong to several distinct, but chemically related classes including chalcones, flavanones, flavones, flavonols, and isoflavones. Selected examples of estrogenic flavonoids were further analyzed to determine their biological potencies and their relative affinities for binding to the estrogen receptor. These data are interpreted with respect to the molecular structure of polycyclic phenols required for hormonal activity as nonsteroidal estrogens.

[P.S.E.B.M. 1995, Vol 208]

A considerable diversity has been described of compounds that share with steroidal estrogens an ability to activate the estrogen receptor. Studies from a number of laboratories have shown that estrogenic activity is exhibited by several members of the large flavonoid family of plant secondary metabolites. It has long been appreciated that genistein and a number of related isoflavones (biochanin A, daidzein, and formononetin) are inherently estrogenic (1-7). A previous report from this laboratory recently expanded the family of phytoestrogens to include a number of other multiply hydroxylated flavonoids (8).

Flavonoids occur naturally in all plant families and can be isolated from most plant tissues, including leaves, stems, roots, flowers, and seeds (3, 9). The biological roles played by flavonoids in plants are not fully understood and do not easily account for the large chemical diversity of this family. Individual members of this group are thought to serve as natural fungicides (phytoalexins), chemical deterrents against insect and animal herbivores, regulators of plant hormones, and UV protectants (9). Since the major flower

pigments are flavonoids, these compounds also play an important role supporting the reproductive success of plants that depend upon insects for pollination.

Plant flavonoids are biosynthetically derived from chalcones (10) and can be divided into several structurally related groups (Fig. 1). While most of the flavonoids found in plants are present as glycosides, only the nonconjugated (aglycone) forms appear to exert estrogen-like activity in animals. However, the aglycones can readily be released from their sugar components by acid hydrolysis (11) to reveal their latent biological activity. It is the uncharged members of this family (chalcones, flavanones, flavones, flavonols, and isoflavones) rather than the intensely colored and highly charged anthocyanidin pigments that are of interest with respect to their estrogenic activity. This study represents an extension of a previous report documenting the hormonal activity of plant flavonoids (8) and was intended to systematically analyze the structure/activity profile of estrogenic flavonoids.

Materials and Methods

Chemical Reagents. The chemicals used in this study were obtained from the following sources: Sigma Chemical Co. (St. Louis, MO) (17 β -estradiol, flavone, flavanone, phloretin, chrysin, hesperetin); Aldrich Chemical Co. (Milwaukee, WI) (4-hydroxy-4'-methoxychalcone, 3-hydroxyflavone, 7-hydroxyflavone, 7,8-dihydroxy-flavone, myricetin, biochanin

¹ To whom requests for reprints should be addressed at Department of Pharmacological Sciences, School of Medicine, State University of New York at Stony Brook, Stony Brook, NY 11794-8651.

A); Steraloids, Inc. (Wilton, NH) (dienestrol, hexestrol); Fluka Chemical Corp. (Ronkonkoma, NY) (kaempferol, kaempferide, [\pm]catechin, apigenin); and ICN Biomedicals, Inc. (Costa Mesa, CA) (genistein). A number of compounds were generous gifts from R. Bednar, Merck Sharp & Dohme (West Point, PA) (4-hydroxychalcone, 4,4'-dihydroxychalcone, isoliquiritigenin, naringenin chalcone, galangin, fisetin, quercetin, morin hydrate, naringenin, taxifolin). The remaining flavonoids were acquired from Indofine Chemical Co. (Somerville, NJ). Purity of the chemicals tested for their estrogenicity was confirmed by thin layer chromatography on silica G plates (Machery-Nagel, Düren, FRG) developed in chloroform:acetic acid (9:1, v/v) or toluene:ethyl acetate:formic acid (9:7:4, v/v). These flavonoids were generally used without further purification. ICI-164,384 was a kind gift from A. Wakeling, ICI Pharmaceuticals.

Cell Culture and Transfection. The biological activity of potentially estrogenic flavonoids was determined using a transient transfection assay in HeLa cells. A wild-type, recombinant estrogen receptor cDNA was expressed from the plasmid pER-18 (Y. Wang and R. J. Miksicek, unpublished plasmid. Structures are available upon request) and stimulation of the transcriptional activity of this receptor was assessed using the estrogen-responsive reporter plasmid pERE-TK-CAT (12). Conditions used for cell culture and transfection have been previously described (7, 8). Hormones and chemicals were added directly to culture dishes at the final concentrations indicated, as $1000\times$ stock solutions prepared in 80% ethanol. Chloramphenicol acetyl transferase (CAT) activity was measured as described by Gorman *et al.* (13) and was calculated as pmoles of chloramphenicol acetylated per min per mg of cytosolic protein. Data were compiled from over thirty independent transfection experiments and were analyzed for significance using Student's *t*-test.

Competition Binding Analysis. The relative affinities of hormonally active flavonoids were determined by their ability to compete for binding of 17β -[3 H]estradiol to estrogen receptor expressed in COS-7 cells using a high-copy expression vector (pCMV-ER; Wang and Miksicek). Transfection, preparation of cellular extracts, and competition binding analysis were performed as previously described (7, 8). Binding reactions were incubated for 2 hr at room temperature with 10 mM 17β [2,4,6,7,16,17(N)- 3 H]estradiol (170 Ci/mmol; New England Nuclear) in the presence of increasing concentrations of unlabeled competitor, followed by the addition of dextran-coated charcoal (DCC) and further incubation for 15 min at 4°C on ice.

Hormone remaining bound to the receptor was defined as radioactivity resistant to adsorption by DCC.

Results

Previous studies from this laboratory have described the use of a transient transfection assay in cultured HeLa cells to analyze the hormonal activity of known and suspected estrogens (7, 8). This assay is based upon expression of the recombinant human estrogen receptor and assessment of its transcriptional activity using an estrogen-inducible reporter plasmid. Assays of this type have been instrumental in dissecting the structural organization of the steroid receptors since they are highly sensitive, relatively rapid, and readily amenable to experimental manipulation. Using co-transfection assays with appropriate control plasmids, we have previously shown that induction of a chloramphenicol acetyl transferase (CAT) reporter gene by estrogen requires both co-expression of the estrogen receptor and the presence of a specific DNA-binding site for this receptor within the promoter of the reporter plasmid (8). This assay is responsive to physiological concentrations of 17β -estradiol, cross reacts with a wide variety of known steroidal and nonsteroidal estrogens, and is sensitive to inhibition by known estrogen antagonists (7, 8). Data using this assay to measure the activity of selected estrogens is given in Table 1.

To broaden our studies on the estrogenic activity of naturally occurring plant flavonoids, we have utilized this assay to assess the ability of additional chemically defined phenolic aglycones to activate the human estrogen receptor. Compiled results from these studies are presented in Table II through V for four independent series of hydroxyflavonoids. For simplicity, each of these compounds was tested at a single concentration (10^{-6} M) based on the activities of other known phytoestrogens in this system (7, 8). As shown in Table II, substantial estrogenic activity was displayed by 4,4'-dihydroxychalcone, 2',4,4'-trihydroxychalcone (isoliquiritigenin), and 2',4,4',6'-tetrahydroxydihydrochalcone (phloretin) ($P < 0.005$). These data also indicate that 2',4,4',6'-tetrahydroxychalcone (naringenin chalcone) may possess a low level of activity ($P < 0.05$), but that 4-hydroxychalcone and 4-hydroxy-4'-methoxychalcone are apparently inactive. The biological activity of these chalcones is consistent with previous reports describing that phloretin (14) and several synthetic derivatives of chalcone (15) display estrogenic and antifertility effects when tested in animal models.

Among a large series of flavones tested (Table III), six compounds exhibited significant estrogenic activity ($P < 0.005$): 3,4',5,7-tetrahydroxyflavone (kaempferol), 4',5,7-trihydroxyflavone (apigenin), 4',6-dihydroxyflavone, 4',5-dihydroxyflavone, 6-hydroxyflavone, and 4'-hydroxyflavone. However, the

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Table I. Hormonal Activity of Steroidal and Diphenylethylene Estrogens

Compound	Concentration (nM)	n	CAT activity ^a	
			Mean ± SE	Range
Ethanol	n/a ^b	15	82 ± 12	34–214
17β-Estradiol	5	15	964 ± 117 ^c	435–2037
ICI-164,384 ^d	100	5	43 ± 13	9–73
Dienestrol	10	6	914 ± 218 ^c	371–1766
Hexestrol	10	5	800 ± 180 ^c	474–1453

Note. HeLa cells were co-transfected with plasmids encoding the estrogen receptor and an estrogen-inducible CAT reporter plasmid, followed by treatment for 2 days with the indicated compound. CAT enzymatic activities were measured for the number of transfections (n) indicated and are expressed as the mean ± SE, with the maximum and minimum values indicated as the range.

^a CAT activities are given as pmoles/min · mg protein.

^b n/a = not applicable.

^c Significantly different from the ethanol vehicle at the level of $P < 0.005$ as determined by Student's *t*-test.

^d ICI-164,384 is a pure antagonist of the estrogen receptor developed by Wakeling and colleagues (17)

Table II. Estrogenic Activity of Chalcone Derivatives

Chalcone	Trivial name	Concentration (μM)	n	CAT activity ^a	
				Mean ± SE	Range
Ethanol vehicle	—	n/a ^b	15	82 ± 12	34–214
4-Hydroxy	—	1	3	152 ± 99	44–350
4,4'-Dihydroxy	—	1	6	471 ± 156 ^c	163–1218
4-Hydroxy-4'-methoxy	—	1	1	86	n/a
2',4,4'-Trihydroxy	Isoliquiritigenin	1	7	994 ± 219 ^c	300–1801
2',4,4',6'-Tetrahydroxy	Naringenin Chalcone	1	8	156 ± 35 ^d	41–301
2',4,4',6'-Tetrahydroxydihydrochalcone	Phloretin	1	7	402 ± 68 ^c	124 ± 711

Note. Experiments were performed and analyzed as described in Table I to compare the biological activities of the indicated hydroxychalcones.

^a CAT activities are given as pmoles/min · mg protein.

^b n/a = not applicable.

^c Significantly different from the ethanol vehicle at the level of $P < 0.005$ as determined by Student's *t* test.

^d Significantly different from the ethanol vehicle at the level of $P < 0.05$ as determined by Student's *t* test.

magnitude of the transcriptional responses induced by the latter two mono-hydroxylated flavones was much less than that of the others. 3',7-dihydroxyflavone and 3',4',5,7-tetrahydroxyflavone (luteolin) also appeared to show activity that was weak but significantly above that of the ethanol control ($P < 0.05$). In contrast, 14 related compounds, including flavone itself were essentially devoid of activity at the concentration tested.

Only two of the flavanones tested reproducibly activated the estrogen receptor in this transfection system (Table IV). These were 4',7-dihydroxyflavanone and 4',5,7-trihydroxyflavanone (naringenin). Inactive members of this group included 3',5,7-trihydroxy-4'-methoxyflavanone (hesperetin), 3,3',4',5,7-penta-hydroxyflavanone (taxifolin), and 3,3',4',5,7-flavan pentol ([+/-] catechin), as well as flavanone itself. The results shown in Table V confirm previous reports (1–7) that four isoflavones (genistein, daidzein, biochanin A, and formononetin) support statistically significant ($P < 0.005$) estrogenic responses. They also agree with the published finding that the 4'-hydroxylated isoflavones (genistein and daidzein) are inherently more active than their 4'-methoxylated counterparts (biochanin A and formononetin) (2, 7). In contrast,

single determinations using 3',4',7-trihydroxyisoflavone and 4',6,7-trihydroxyisoflavone suggest that these compounds are without activity. Together, the data summarized in Tables II through V indicate that at least 12 structurally related flavonoids share with 17β-estradiol and the diphenylethylene estrogens (Table I) an ability to stimulate the transcriptional activity of the estrogen receptor at least 4-fold above its basal level (Table VI). They also reveal a highly consistent hydroxylation pattern among polycyclic phenols with estrogenic activity, considering that Positions 4, 2', and 4' of chalcone are equivalent to Positions 4', 5, and 7, respectively, of the flavonoid ring system (Fig. 1).

Most phytoestrogens display their hormone-like activity over a concentration range of 0.1 to 10 μM (7, 8). To ascertain if this is also true for the estrogenic flavonoids, an experiment was undertaken to examine the concentration dependence of the transcriptional stimulatory activity of four selected dihydroxyflavones. The dose/response curves shown in Figure 2 indicate that 4',6-dihydroxyflavone is the most potent of this subset, with an EC₅₀ of approximately 0.1 μM. In comparison, 4',5- and 3',7-dihydroxyflavone require progressively higher concentrations for half-maximal

Table III. Estrogenic Activity of Flavone Derivatives

Flavone	Trivial name	Concentration (μM)	n	CAT activity ^a	
				Mean \pm SE	Range
Ethanol vehicle	—	n/a ^b	15	82 \pm 12	34–214
Flavone	—	1	4	52 \pm 11	35–85
3-Hydroxy	—	1	3	92 \pm 9	81–110
4'-Hydroxy	—	1	2	237 ^c	149–324
6-Hydroxy	—	1	2	287 ^c	203–370
7-Hydroxy	—	1	4	93 \pm 22	55–155
3',6-Dihydroxy	—	1	2	161	93–230
3',7-Dihydroxy	—	1	3	162 \pm 30 ^d	107–211
4',5-Dihydroxy	—	1	3	389 \pm 95 ^c	234–563
4',6-Dihydroxy	—	1	2	427 ^c	420–434
5,7-Dihydroxy	Chrysin	1	1	50	n/a
7,8-Dihydroxy	—	1	6	72 \pm 19	17–146
3',4',7-Trihydroxy	—	1	1	42	n/a
3,5,7-Trihydroxy	Galangin	1	4	72 \pm 17	42–119
4',5,7-Trihydroxy	Apigenin	1	6	544 \pm 157 ^c	179–1169
4',7,8-Trihydroxy	—	1	1	68	n/a
3,3',4',7-Tetrahydroxy	Fisetin	1	4	135 \pm 34	73–228
3',4',5,7-Tetrahydroxy	Luteolin	1	2	168 ^d	114–221
3,4',5,7-Tetrahydroxy	Kaempferol	1	5	858 \pm 143 ^c	321–1171
3,5,7-Trihydroxy-4'-methoxy	Kaempferide	1	3	130 \pm 31	95–193
3,3',4',5,7-Pentahydroxy	Quercetin	1	3	75 \pm 28	25–123
2',3,4',5,7-Pentahydroxy	Morin	1	3	67 \pm 11	46–80
3,3',4',5,5',7-Hexahydroxy	Myricetin	1	4	74 \pm 18	48 \pm 12

Note. Experiments were performed and analyzed as described in Table I to compare the biological activities of the indicated hydroxyflavones.

^a CAT activities are given as pmoles/min \cdot mg protein.

^b n/a = not applicable.

^c Significantly different from the ethanol vehicle at the level of $P < 0.005$ as determined by Student's t test.

^d Significantly different from the ethanol vehicle at the level of $P < 0.05$ as determined by Student's t test.

Table IV. Estrogenic Activity of Flavanone Derivatives

Flavanone	Trivial name	Concentration (μM)	n	CAT activity ^a	
				Mean \pm SE	Range
Ethanol vehicle	—	n/a ^b	15	82 \pm 12	34–214
Flavanone	—	1	3	40 \pm 13	21–66
4',7-Dihydroxy	—	1	5	903 \pm 202 ^c	450–1396
4',5,7-Trihydroxy	Naringenin	1	7	574 \pm 235 ^c	174 \pm 1930
3',5,7-Trihydroxy-4'-methoxy	Hesperetin	1	1	86	n/a
3,3',4',5,7-Pentahydroxy	Taxifolin	1	3	97 \pm 28	44–139
3,3',4',5,7-Flavan pentol	[+/-]Catechin ^d	1	4	97 \pm 16	67–140

Note. Experiments were performed and analyzed as described in Table I to compare the biological activities of the indicated hydroxyflavanones.

^a CAT activities are given as pmoles/min \cdot mg protein.

^b n/a = not applicable.

^c Significantly different from the ethanol vehicle at the level of $P < 0.005$ as determined by Student's t test.

^d It should be noted that catechin is a flavan rather than a flavanone.

activity, while 3',6-dihydroxyflavone shows little stimulatory activity even at 10 μM . By analogy to the flavanones tested at 1 μM (Table IV) it can be predicted that 4',7-dihydroxyflavone will also display estrogenic activity between 0.1 and 1 μM ; unfortunately, this flavone was not available for testing.

It is generally assumed that nonsteroidal estrogens exert their stimulatory effect on the estrogen receptor by binding to the same site as that occupied by steroidal estrogens such as 17 β -estradiol. Supporting this

presumption, we have previously shown that a variety of estrogenic flavonoids including 4,4'-dihydroxychalcone, 2',4,4'-trihydroxychalcone, 4',7-dihydroxyflavanone, 4',5,7-trihydroxyflavanone, 4',5,7-trihydroxyflavone, and 4',5,7-trihydroxyisoflavone can compete with 17 β -[³H]estradiol for binding to the human estrogen receptor in extracts prepared from COS-7 cells that over express this receptor (8). Inhibition of estradiol binding required the competing flavonoid to be present at concentrations ranging from

Table V. Estrogenic Activity of Isoflavone Derivatives

Isoflavone	Trivial name	Concentration (μM)	n	CAT activity ^a	
				Mean \pm SE	Range
Ethanol vehicle	—	n/a ^b	15	82 \pm 12	34–214
4',7-Dihydroxy	Daidzein	1	4	469 \pm 74 ^c	256–589
7-Hydroxy-4'-methoxy	Formononetin	1	3	287 \pm 96 ^c	177–478
3',4',7-Trihydroxy	—	1	1	86	n/a
4',5,7-Trihydroxy	Genistein	1	8	988 \pm 296 ^c	412–2839
4',6,7-Trihydroxy	—	1	1	63	n/a
5,7-Dihydroxy-4'-methoxy	Biochanin A	1	10	331 \pm 77 ^c	33–683

Note. Experiments were performed and analyzed as described in Table I to compare the biological activities of the indicated hydroxyisoflavones.

^a CAT activities are given as pmoles/min \cdot mg protein.

^b n/a = not applicable.

^c Significantly different from the ethanol vehicle at the level of $P < 0.005$ as determined by Student's t test.

Table VI. Flavonoids with Highly Significant Estrogenic Activity

Chalcones
4,4'-Dihydroxychalcone
2',4,4'-Trihydroxychalcone (isoliquiritigenin)
2',4,4',6'-Tetrahydroxydihydrochalcone (phloretin)
Flavones
4',5-Dihydroxyflavone
4',6-Dihydroxyflavone
4',5,7-Trihydroxyflavone (apigenin)
Flavonols
3,4',5,7-Trihydroxyflavone (kaempferol)
Flavanones
4',7-Dihydroxyflavanone
4',5,7-Trihydroxyflavanone (naringenin)
Isoflavones
4',7-Dihydroxyisoflavone (daidzein)
4',5,7-Trihydroxyisoflavone (genistein)
5,7-Dihydroxy-4'-methoxyisoflavone (biochanin A)

Note. This table summarizes the polycyclic phenols analyzed in Tables II through V able to support a 4-fold or greater stimulation of the transcriptional activity of the human estrogen receptor. All of these responses show significance at the level of $P < 0.005$.

10^{-7} to 10^{-5} M depending on the flavonoid in question and was not observed for nonestrogenic flavonoids such as flavone and flavanone.

To broaden these results, competition binding analysis was also used to examine the relative binding affinities of a wider range of hydroxyflavonoids. The results shown in Figure 3 confirm that 4',5-dihydroxyflavone shares with 4',7-dihydroxyflavanone and 4',7-dihydroxyisoflavone (daidzein) the ability to compete with 17 β -estradiol for binding to the estrogen receptor, but only when present in a 1,000- to 10,000-fold molar excess over this steroid. A slightly higher affinity was displayed by 4',6-dihydroxyflavone, consistent with its greater potency in a transient transfection assay (Fig. 2). In contrast, little if any inhibition of estradiol binding was observed for 3',6-dihydroxyflavone, 4'-hydroxyflavone, 6-hydroxy-

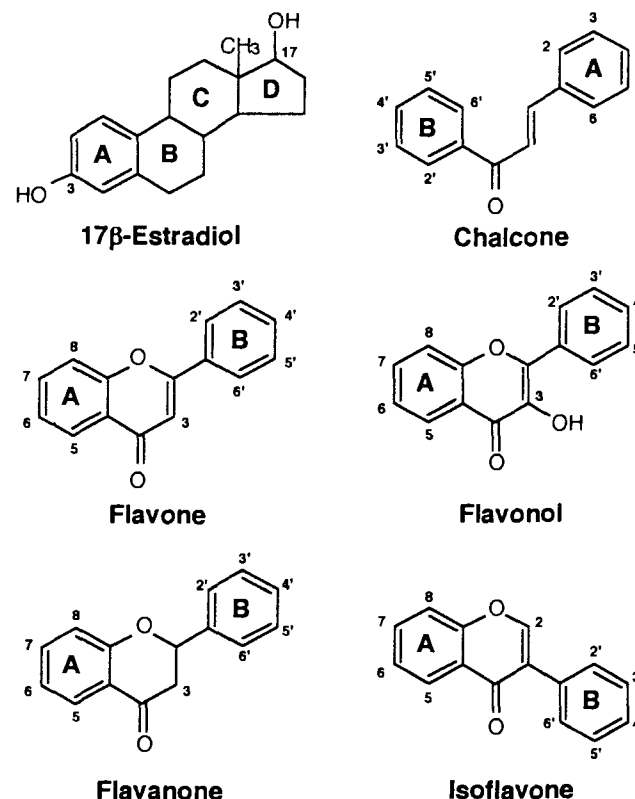


Figure 1. Structures of 17 β -estradiol and classes of polycyclic phenols analyzed in this study. Also shown are the conventional numbering schemes to indicate the hydroxylation patterns of the flavonoids characterized.

flavone, or 7-hydroxyflavone even when these compounds were present at a 10,000-fold excess.

These data support the conclusion that estrogenic hydroxyflavonoids exert their biological effect by interacting directly with the estrogen receptor. Extrapolating from the reported dissociation constant (K_d) of 17 β -estradiol (0.3 nM), they also infer that these hydroxyflavonoids display affinities for the estrogen receptor on the order of 0.3 to 10 μM . The relationship between the estrogenic potency of selected dihydroxy-

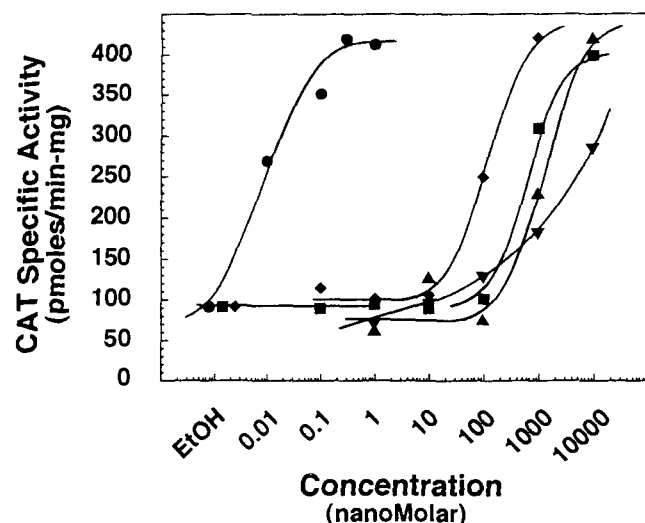


Figure 2. Concentration dependence of the transcriptional stimulatory effect of estrogenic flavonoids. HeLa cells co-transfected with the human estrogen receptor gene and the pERE-TK-CAT reporter plasmid were treated with increasing concentrations of 17 β -estradiol (●), 4',6-dihydroxyflavone (◆), 4',5-dihydroxyflavone (■), 3',7-dihydroxyflavone (▲), or 3',6-dihydroxyflavone (▼). CAT specific activities were measured in cell extracts following 48 hr of continuous hormone treatment. Shown are individual determinations from a representative experiment.

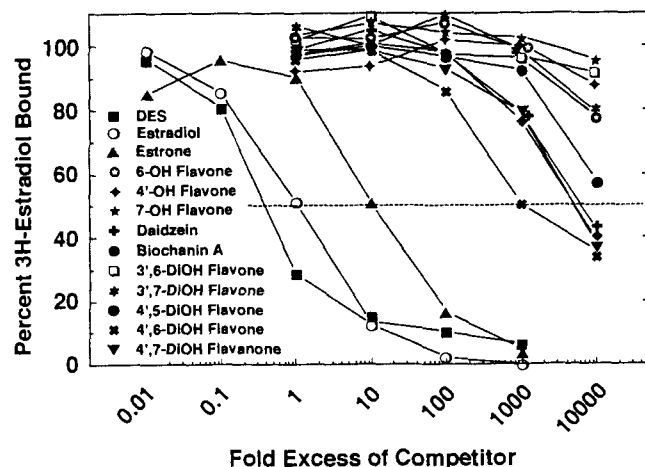


Figure 3. Competition binding analysis of estrogenic flavonoids. Extracts from COS-7 cells over expressing the human estrogen receptor were incubated for 2 hr at room temperature with 10 nM 17 β -[³H]estradiol in the presence of increasing concentrations of the unlabeled ligands shown. 17 β -[³H]Estradiol remaining bound to the estrogen receptor was determined as radioactivity resistant to adsorption by DCC.

flavones and their relative affinity for the estrogen receptor is shown in Figure 4. Slight differences in the uptake of these compounds or their metabolic stability may account in part for deviations of this relationship from linearity.

Discussion

The results of this study present the first systematic analysis of structure/activity relationships among

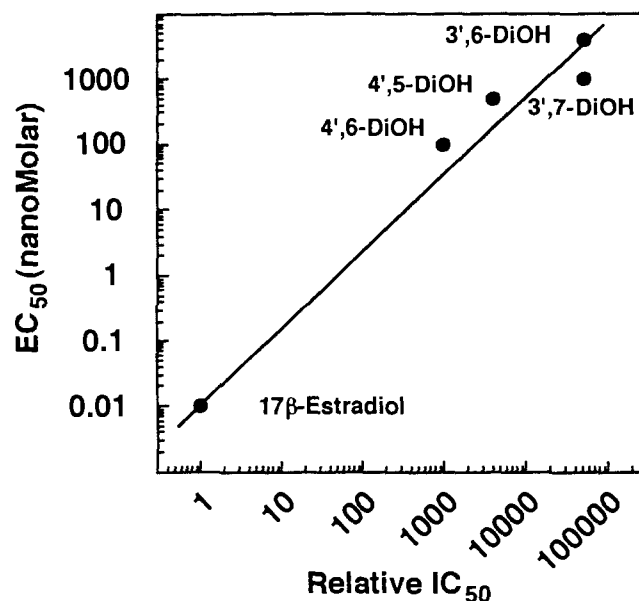


Figure 4. Correlation of estrogenic activity of dihydroxyflavones with their relative affinity for the estrogen receptor. Concentrations required for half-maximal activation of the estrogen receptor (EC₅₀ values, extrapolated from Fig. 2) were plotted against the molar excess of ligand required for 50% competition of [³H]estradiol binding to the estrogen receptor (relative IC₅₀ values, from Fig. 3) for 17 β -estradiol and the four dihydroxyflavones indicated. The line represents a least squares fit to these data (coefficient of correlation, $r = 0.983$).

estrogenic flavonoids. Summarized in Table VI are members of this family that show a highly significant ability ($P < 0.005$) to stimulate the transcriptional activity of the human estrogen receptor as assessed using a transient transfection assay in cultured cells. It is immediately evident from this compilation that the structural features that are most important with respect to estrogenic activity include the diaryl ring structure common to all flavonoids (Fig. 1) and a minimum of one hydroxyl substituent on each of these aromatic rings. Despite obvious differences in the central bridge connecting the phenolic A and B rings when the chalcones, flavones, flavanones, and isoflavones are compared there is a remarkably strong consensus in the optimal pattern of hydroxylation that gives rise to estrogenic activity. Thus, compounds with hydroxyl substituents in Positions 4' and 7 of the flavan or isoflavan nuclei (equivalent to Positions 4 and 4', respectively, of chalcone) are invariably estrogenic. An additional hydroxyl group in Position 5 of the flavones or isoflavones (equivalent to the Position 2' of chalcone) are not only tolerated, but may in some cases increase estrogenic activity. While the apparent tolerance for 4'-methoxylation in biochanin A might be interpreted as an exception to the generalizations made above, it should be noted that a previous study (16) concluded that the hormone-like activity observed for biochanin A *in vivo* is likely to result from its conver-

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sion to the much more potent compound genistein. This is supported by the finding that the relatively low affinity of biochanin A for estrogen receptor (Fig. 3) (2, 3, 7) does not adequately account for its estrogenic activity in cultured cells.

Among the large series of flavones analyzed, it is apparent that some flexibility exists with respect to the hydroxylation pattern of Ring A. Both 4',5-dihydroxyflavone, 4',6-dihydroxyflavone, and 4',7-dihydroxyflavone (extrapolating from the behavior of the analogous flavanone) exhibit substantial estrogenicity. In contrast, there appears to be less tolerance for changes in the hydroxylation pattern of Ring B comparing the progressively reduced activities of 4',6- and 3',6-dihydroxyflavones (Fig. 2). The activity exhibited by 3,4',5,7-tetrahydroxyflavone (kaempferol) indicates that hydroxylation at Position 3 is not detrimental to activity provided that the remaining pattern of hydroxylation is favorable and suggests that additional flavonols (3-hydroxyflavones) may be estrogenic. However, hydroxylations that create catechols (e.g., 7,8-dihydroxyflavone, 4',7,8-trihydroxyflavone, 3,3',4',7-tetrahydroxyflavone, 3,3',4',5,7-pentahydroxyflavone, and 3,3',4',5,5',7-hexahydroxyflavone) or that increase the number of hydroxyl substituents above 4 appear to abolish estrogenic activity. In addition, 4'-methoxylation of chalcones, flavones, flavanones, and isoflavones invariably reduces or eliminates the estrogenic activity of these compounds relative to their 4-hydroxy counterparts.

This study should serve to greatly broaden our understanding of natural plant products that possess biological activity as estrogens. There has been a recent resurgence of interest in plant estrogens both with respect to their beneficial effects and their potential to serve as human toxicants. Until recently, there has been a perception that common crop plants that represent significant sources of dietary estrogens are almost invariably members of the legume family. An important implication of this report is to question this presumption since many of the estrogenic flavonoids described above show a much broader species distribution than estrogenic isoflavones such as genistein and daidzein. Ultimately, it will be necessary to assess the distribution and content of these flavonoids among crop species that represent a significant portion of the human diet. More importantly, little is known at present about the bioavailability, absorption, metabolism, and excretion of estrogenic flavonoids in man. A

thorough understanding of the beneficial and potentially deleterious effects of dietary estrogens such as the flavonoids will require questions such as these to be addressed.

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the daily collection, dialysis and reinfusion of the lymph of our patient was continued until February 22, 1965. His remarkably good clinical improvement persisted until February 12, 1965, when the insidious onset of weakness, anorexia, lethargy and a rise in blood pressure to 180/118 developed. By February 20, nocturnal hallucinations and a coarse tremor had developed. A deficiency of magnesium was suspected, and he was given 2.0 gm. magnesium sulfate parenterally on February 20 and 21. His response to these small doses was judged to be excellent, but on February 22, 1965, he suddenly had a severe grand-mal seizure and died.

Post-mortem examination revealed no immediate cause of death; therefore, we concluded that an insidious magnesium depletion developed as a result of the continuous dialysis of lymph for 78 days. This led to a severe convulsive seizure, with an anoxic period, causing ventricular fibrillation. Serial lymph levels for magnesium were obtained retrospectively and revealed a fall from 3.8 milliequiv. per liter initially to 0.48 milliequiv. per liter at the time of death.

If lymph collection for dialysis are only performed for 6 hours a day or for 2 days a week, we believe that the depletion of trace elements can be avoided without seriously decreasing the efficiency of the procedure.

The dialysis coils and the artificial kidney used in this project were supplied by the Travenol Company, Morton Grove, Illinois. The Silastic bags and catheters were provided by Dow Corning Center for Aid to Medical Research, Midland, Michigan. The analyses of lymph and urine for mercury were performed by Dr. Joseph Nash, director, Toxicology Laboratory, Department of Pharmacology, University of Texas Medical Branch. The analyses of lymph and blood

coagulation factors were performed by Dr. D. Mason Guest, professor and chairman of physiology and director of the Blood Coagulation Research Laboratory, University of Texas Medical Branch.

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THYROID REFRACTORINESS IN AN ATHYREOTIC CRETIN FED SOYBEAN FORMULA*

ALDO PINCHERA, M.D.,† MARGARET H. MACGILLIVRAY, M.D.,‡ JOHN D. CRAWFORD, M.D.,§ AND ALLAN G. FREEMAN, M.D.¶

BOSTON

GOITER and hypothyroidism have occasionally been reported in infants fed soybean diets.¹⁻⁵ In these patients both goiter and hypothyroidism have disappeared when soybean was eliminated. Since 1959 a widely used commercial brand of soybean has been supplemented with iodide,⁶ and to our knowledge no further cases of soybean-induced goiters have been observed.

*From the departments of Medicine and Pediatrics, Harvard Medical School, and the Medical Service (Thyroid Unit) and the Children's Service, Massachusetts General Hospital.

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†Clinical and research fellow in medicine, Harvard Medical School and Massachusetts General Hospital (work was done during the tenure of an international postdoctoral research fellowship of the National Institutes of Health; permanent address, Istituto Patologia Medica, University of Rome, Italy).

‡Clinical and research fellow in pediatrics, Harvard Medical School and Massachusetts General Hospital; trainee fellow of the National Heart Institute, National Institutes of Health, United States Public Health Service (HTS-1539).

§Associate professor of pediatrics, Harvard Medical School; chief, Pediatric Endocrine Unit Children's Service, Massachusetts General Hospital; United States Public Health Service senior fellow (GSF-14).

¶Attending physician, Elliot Community Hospital and pediatrician, Keene Clinic, Keene, New Hampshire.

The athyreotic cretin described below showed persistent hypothyroidism while being fed a soy formula, in spite of receiving large doses of desiccated thyroid. Since laboratory animals on soy diet acquire goiter associated with fecal wastage of thyroxine⁷⁻⁹ we proposed that refractoriness of this infant to exogenous thyroid might have been caused by impaired absorption of the hormone. Accordingly, the intestinal absorption of I¹³¹-labeled L-thyroxine was studied in conjunction with other measures of thyroid function while the patient was receiving soy formula and after its withdrawal. We are not aware of previous studies on intestinal absorption or fecal excretion of thyroid hormones in human beings receiving soybean diets.

CASE REPORT

K R (M G H 122 39-29), the product of a full-term uncomplicated pregnancy and delivery, weighed 3062 gm. (6 pounds, 12 ounces) and was 48 cm. long at birth. The neonatal period was unremarkable. During the 1st 3½

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months weight gain was satisfactory, but linear growth progressively deviated from the 10th percentile at birth to less than the 3d percentile.

At 3½ months of age severe hypothyroidism was evident. Notable clinical features were cretinous facies, dry skin, poor head control and an umbilical hernia. The diagnosis was confirmed by a protein-bound iodine (PBI) of 1.9 microgm per 100 ml (total iodine, 2.4 microgm per 100 ml). The bone age was less than that of a full-term newborn infant. Tri-iodothyronine (T_3) 4 microgm 3 times a day, was started. Over the next 3 weeks this was gradually increased to 20 microgm per day. A prompt improvement was observed, with increased alertness and general activity. The electrocardiograph showed an increase in voltage. At this time T_3 was withdrawn and replaced by a preparation of desiccated thyroid* (30 mg. per day), which was maintained throughout the period of observation (Fig. 1).

From 3½ to 5 months of age the infant was fed whole milk. In this period he had an episode of otitis media on the right side and frequent bouts of wheezing and nasal congestion. At 5 months he was clinically euthyroid on thyroid (45 mg. per day), and the PBI was 3.6 microgm. per 100 ml. Symptomatology suggestive of milk allergy prompted a trial with soybean formula†. Stools, which had been normal before soy feedings, became loose, bulky and foul. He also became hypothyroid again, and the dosage of thyroid was increased to 45 mg. alternating with 60 mg. daily. As shown in Figure 1, he was maintained on the soy formula for the next 7 weeks with the exception of a 2-week period of cow's milk, during which the stools became formed and of normal size. Despite these unusually high doses of thyroid in the range of 240 mg per square meter of body-surface area per day the PBI at the end of the trial with cow's milk was only 4.6 microgm. per 100 ml; the total serum iodine was 13.8 microgm. per 100 ml, reflecting the frequent use of an iodide-containing cough suppressant. The most striking clinical features on the soy formula were the virtual cessation of linear growth and clinical hypothyroidism. He also had frequent minor bouts of gastroenteritis and otitis media, and appeared irritable and unhappy.

Ten days before admission the soy formula was reinstituted, and the loose, foul, bulky stools returned.

On admission at the age of 6½ months he was a pale, irritable, alert infant with features suggesting partially treated hypothyroidism. These included slight facial puffiness and depressed nasal bridge, pale, dry and slightly mottled skin and cool extremities. No thyroid tissue was palpable. Semilunar ridges were present on the fingernails indicating the onset of thyroid replacement. The previously noted umbilical hernia was no longer present. Developmentally, he was within normal limits. He could sit with little support, had good head and trunk control and could reach for and transfer objects.

The hemoglobin was 10.1 gm. per 100 ml., and the white-cell count 10,400, with 32 per cent neutrophils, 47 per cent lymphocytes, 5 per cent monocytes and 16 per cent eosinophils. Urinalysis was within normal limits. Microscopical examination of the stools gave no suggestion of fat or of protein malabsorption. A sweat sodium value of 27 milliequiv. per liter was further evidence against fibrocystic disease of the pancreas. The serum calcium was 9.6 mg., the serum total protein 4.7 gm., and the serum cholesterol 178 mg. per 100 ml. Skin testing revealed a marked reaction to egg antigen. Milk precipitins were not demonstrable in a serum sample.

Laboratory evaluation confirmed the clinical impression of persistent hypothyroidism with refractoriness to thyroid replacement. The PBI was 2.7 microgm., the butanol-extractable iodine (BEI) 2.4 microgm., and the total iodine 4.2 microgm. per 100 ml. The erythrocyte uptake of I^{131} -

labeled T was 7.9 per cent (normal, 12 to 16 per cent). The bone age was 1 month. Although the growth curve suggested some thyroid function in the early months of infancy, there was no significant thyroid uptake of radioactive iodine. Radioactivity over the neck area 6, 24 and 48 hours after the oral administration of I^{131} (0.5 microcurie) was only 5, 3 and 2 per cent of the dose respectively after subtraction of the nonthyroidal-tissue background obtained by counting over the thigh. The intravenous injection of I^{131} (1 microcurie) similarly revealed no significant thyroid uptake at 3, 20, 60 and 120 minutes. Because the patient was clinically and chemically hypothyroid at the time these studies were performed they probably indicate an absence of thyroid tissue rather than suppression of iodine uptake by administered thyroid.

The saliva-serum ratio of I^{131} 4 hours after the oral administration of radioactive iodide was 29, reflecting normal ability of the salivary gland to concentrate iodide. This distinguishes the patient from a previously described cretin with a defect in the iodide-concentrating mechanism.¹⁰

The uptake study of orally administered I^{131} was repeated after TSH stimulation while the patient was on a whole-milk diet. Again, there was no evidence of uptake by the gland.

The reports of fecal hormonal iodine wastage in laboratory animals fed soy^{1,2} prompted a study of thyroxine absorption in this infant. The investigation was performed while he was receiving a soy diet and repeated after its withdrawal.

METHODS

A solution of 10 microgm. of sodium L-thyroxine (T_4) in saline solution containing 3 microcuries of I^{131} -labeled L- T_4 ‡ was fed to the patient by mixing with the diet. The I^{131} -labeled thyroxine administered was assessed for purity by chromatography and found to contain 92 per cent of total activity as T_4 , the remainder being iodide. The patient was kept on a metabolic bed, and feces and urine collected and measured as indicated in Table 1. A serum sample was drawn at twenty-four hours. Feces were weighed, dissolved in distilled water and homogenized. Total I^{131} content of the urine, serum and feces was measured in duplicate in a well-type scintillation counter. Corrections were made for the residue in the diet container. The fecal thyroxine-like radioactivity — that is, BEI¹³¹ — was isolated by acid butanol extraction followed by alkaline washing, a modification of the method of Man et al.¹¹ being used. Serum PBI¹³¹ was measured when radioactivity was sufficient to permit this determination.

Study 1. The soy diet had been started ten days before and continued throughout this study. Thyroid replacement was maintained at a level of 60 mg. daily.

After Study 1 was completed, the infant was discharged on a cow's milk formula and unaltered thyroid replacement.

Study 2. The patient was re-evaluated after a two-week period of cow's milk feeding. This diet and thyroid (60 mg) were maintained throughout this study. At the time of Study 2 the PBI was 3.5 microgm., the BEI 3.3 microgm., and the total

*In the form of Armour thyroid, Armour Pharmaceutical Company, Kankakee, Illinois.

†In the form of Mull-Soy, Borden Company, Pharmaceutical Division, New York City.

‡Abbott Laboratories, Oak Ridge, Tennessee.

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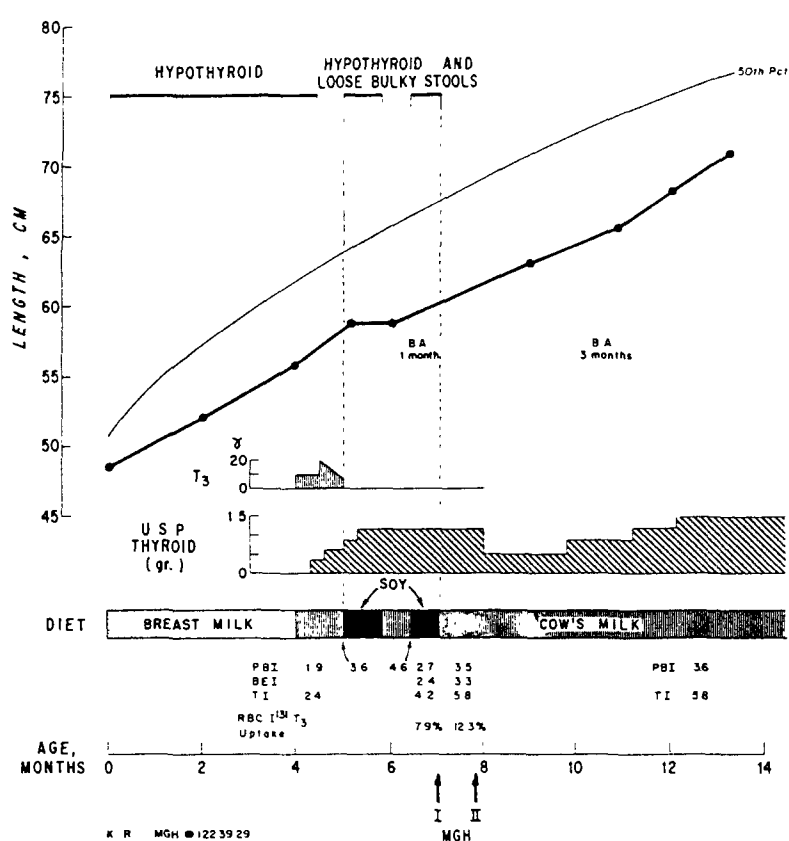


FIGURE 1. Clinical Course in Relation to Diet and Thyroid Medication.

iodine 5.8 microgm. per 100 ml. Although increases in the PBI and BEI were less than anticipated the patient was clinically euthyroid, the erythrocyte uptake of I^{131} -labeled tri-iodothyronine was normal (12.3 per cent), and the growth rate in subsequent weeks was markedly improved (Fig. 1).

RESULTS

The data in Table 1 indicate that fecal excretion of I^{131} on cow's milk was much less (31.6 per cent) than that observed on soy feeding (51 per cent). Fecal bulk was also less on cow's milk. Improved thyroxine absorption was further suggested by the increased urine and serum radioactivity in Study 2. Since the relatively low fecal I^{131} content might have been attributed in part to a delayed excretion on cow's milk the stool collection was continued to forty-eight hours. Despite this precaution only an additional 3.3 per cent of I^{131} was eliminated during the interval of thirty to forty-eight hours. The change in diet from Study 1 to Study 2 did not produce significant changes in the butanol solubility of fecal content of I^{131} (Table 1).

The clinical course of the patient in the ten months since these investigations has been satisfactory. Growth, physical findings and stool consistence

have been all normal. Despite generous thyroid therapy PBI measurements remain in the low-normal range. The significance, if any, of this finding is unknown.

DISCUSSION

The main aim of this study was to ascertain whether the refractoriness to thyroid medication manifested by this infant while he was receiving a soy-bean diet could be ascribed to an impaired absorption of thyroid hormones.

The control study, performed while the patient was on a whole-milk diet, revealed that thirty hours after the oral administration of I^{131} -labeled L-thyroxine, approximately 32 per cent of the radioactive label was excreted in the feces. Data on the thyroxine excretion of normal subjects of this age group are not available, but the value obtained in this patient is similar to those observed by Van Middlesworth⁹ in adults. When the infant was fed soy formula the fecal I^{131} excretion was higher, and the levels of radioactivity in the urine and serum were lower than those of the control study. Thus, evidence has been obtained that soy feeding may interfere with thyroxine absorption in human beings as well as in animals. This finding calls for caution in the

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TABLE 1. *Gastrointestinal Study of Absorption of I^{131} -Labeled-L-Thyroxine*

PERIOD OF STUDY	SOY FORMULA			WHOLE-MILK DIET		
	QUAN- TITY	I^{131} % of dose	BEI ¹³¹ % of dose	QUAN- TITY	I^{131} % of dose	BEI ¹³¹ % of dose
Feces.						
0-24 hr.	130 gm.	48.8	95	62 gm.	31.3	95
24-30 hr.	100 gm.	2.2	70	4 gm.	0.3	100
30-48 hr.	*	—	—	88 gm.	3.3	1
Total, 0-30 hr.	230 gm.	51.0	94	66 gm.	31.6	95
Urine.						
0-24 hr.	31.5 ml.	1.1		214 ml.	5.0	
24-48 hr.	59.0 ml.	1.3		88 ml.	4.0	
Total, 0-48 hr.	90.5 ml.	2.4		302 ml.	9.0	
Serum:						
24 hr.	—	17.0†	‡§	—	42.0†	95§

*Not collected.

†% of dose/liter.

‡Radioactivity not sufficient to permit this determination.

§PBI.

use of soybean diets in patients requiring thyroid medication.

Increased fecal loss of hormonal iodine in rats fed soy-flour diets has previously been observed.⁷⁻⁹ Changes in the bulk of the feces seem to be of importance in these connections. Diets high in cellulose or bran also produce a large fecal volume, with enhanced excretion of hormonal iodine.⁷ The work of Triantaphyllidis¹² clearly documents this direct proportionality between fecal volume and thyroxine content. In patients with pancreatic steatorrhea Hiss and Dowling¹³ found that a decrease in the fecal weight, induced by appropriate treatment, was associated with a significant reduction in thyroxine loss, although fecal hormonal iodine content remained abnormally high. In line with these observations the patient described above was noted to have bulky, loose and foul-smelling stools when given soybean feeding, and the weight of stools was three or four times higher during the thirty-hour study on soy diet than during the control study.

"Intestinal hurry" due to increased fecal bulk may not completely explain the fecal thyroxine losses, and other mechanisms may be involved. Increased biliary excretion, addition of thyroxine to the intestinal tract by extrabiliary sources and alteration of the thyroxine chemical structure have been excluded by the experimental work of Beck.⁸ The absence of significant changes in the fecal BEI in our study also suggests that soybean does not alter the thyroxine molecule.

Changes in the intestinal bacterial flora may affect fecal thyroxine losses. This hypothesis seems supported by the recent observation of Salvatore et al.¹⁴ that *Escherichia coli*, a normal component of the intestinal flora, has specific binding properties for the thyroid hormones. No relevant data were obtained in the patient presented above, but it would

be of interest to ascertain whether soybean increase the *Esch. coli* content of the intestine.

With the present observations in mind the soybean-induced goiters previously reported in human patients can be considered. Most of these goiter showed an increased avidity for I^{131} .^{1,4} Available histologic descriptions are of changes similar to those found in iodine deficiency.¹⁵ Iodide administration led to the disappearance of the goiter.⁴ Thus, the effects of soybean products on thyroid function are similar to those found in iodine deficiency. In this context the observation of Van Wyk et al.² that soybean products may act as thyroid-blocking agent remains unexplained.

The goitrogenic effect of soybean diet in animals has long been recognized.^{15,16} Iodide supplementation has been shown to prevent the thyroid enlargement.¹⁶ In rats Van Middlesworth^{7,9} has recently observed that soy-flour diets produce high-uptake goiters and excessive fecal thyroxine excretion where as iodide absorption and excretion are apparently unaffected. He has postulated that the goiter is due to an increased iodide requirement after thyroxine depletion.

The present report suggests that the hypothesis of fecal thyroxine wastage proposed by Van Middlesworth may be responsible for the goiters previously reported in infants on soy diets. The documentation of decreased absorption of exogenous thyroxine when this congenitally hypothyroid patient was on a soy formula suggests that soy diets in euthyroid infants might interfere with the reabsorption of endogenous thyroid hormone reaching the gut through the bile. Although the enterohepatic circulation of thyroxine is lower in human beings than in rats clinical investigations have shown that 25 to 30 per cent of human thyroxine may be lost in the feces.^{9,17} Thus, prolonged interference with intestinal thyroxine reabsorption could significantly alter iodine balance and eventually cause a goiter.

SUMMARY

An athyreotic cretin became resistant to thyroid medication when placed on a soybean diet. Studies with I^{131} -labeled L-thyroxine revealed that soybean decreased the intestinal absorption of exogenous thyroxine. This finding supports the theory that the goiters previously described in infants on soy diets were caused by fecal wastage of endogenous thyroid hormones.

We are indebted to Dr. J. B. Stanbury for advice and helpful suggestions and to Dr. J. T. Dunn for reviewing this manuscript.

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MEDICAL PROGRESS

THE D-STATE (Concluded)

A Review and Discussion of Studies on the Physiologic State Concomitant with Dreaming

ERNEST L. HARTMANN, M.D.*

BOSTON

PHARMACOLOGY AND THE D-STATE

A great many data, mostly from small informal studies, are available concerning the effect of drugs on the D-state, but as yet no definite pattern has emerged. A number of substances decrease the amount of time spent in the D-state. This is true in man for phenobarbital and several other barbiturates studied so far.²⁰ Phenothiazines likewise tend to decrease D-time; trifluoperazine (Stelazine) has some confusing early effects²¹ but probably actually decreases D-time like the others.²² Alcohol has been shown to cause a decrease in D-time,²³ and in fact it has been suggested that delirium tremens may represent a state of acute "rebound" from dream deprivation — that is, the patient has had his D-time suppressed over a prolonged period while drinking, and the vivid hallucinatory state when he finally stops drinking represents an effort to "catch up" on D-time.^{27,28} The authors present evidence for increased D-time after withdrawal from alcohol, but this is, of course, during sleep; the delirium-tremens condition is certainly not a typical D-period but might be seen as the condition of a "waking dreamer" in whom somehow both the mechanisms underlying the D-state and those underlying waking are operating at once.

Since hypnogenic and tranquilizing drugs have been shown to decrease D-time, it was thought that stimulants such as caffeine or the amphetamines might have the opposite effect. However, this does

not turn out to be the case: caffeine apparently has little effect²⁶; dextroamphetamine sulfate definitely reduces D-time, when it allows the subject to sleep at all.²⁹

These studies have been done on man, but it appears that in general these groups of drugs have the same effect on cats: in addition it has been shown that large doses of atropine effectively suppress D-periods in cats¹⁸ and rats.⁶⁰

Thus, a variety of drugs appear to be able to depress D-time; it would certainly be useful to have a drug that would increase D-time or at least provide sound sleep with a normal amount of D-time, in view of the dangers of dream deprivation, but no such drug has yet been found. However, large doses of eserine, an acetylcholinesterase inhibitor, have a tendency to increase the length of D-periods in cats although total D-time is not definitely increased.¹⁸ Lysergic acid diethylamide (LSD) has been found to have little effect, or if anything to decrease D-time in cats,⁶¹ but a recent study shows that within a very narrow dosage range, it may lengthen at least one D-period during the night in man, without increasing total D-time for the night.⁶²

Although there are no drugs that definitely increase D-time, one may with reasonable ease achieve a night of increased D-time pharmacologically by administering for a time, and then withdrawing, either phenothiazines, alcohol or dextroamphetamine sulfate.

Altogether, the pharmacologic findings do not point to any clear-cut conclusions as yet, except perhaps the conclusion that the D-state is quite

*Director, Sleep and Dream Laboratory, Boston State Hospital; assistant clinical professor of psychiatry, Tufts University School of Medicine; career investigator, National Institute of Mental Health, National Institutes of Health, United States Public Health Service.

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Morning Session

Session No. 57

NEURO-EPIDEMIOLOGY:
DETERMINANTS OF DEMENTIA

Thursday, April 17

10:45 AM - 12:00 PM

Room 309

Co-chairs: Walter Rocca, Rochester, MN
Karen Marder, New York, NYS57.001
10:45 AMNotice This Material May Be Protected
By Copyright Law (Title 17 U.S. Code)Association Between Dementia,
Alzheimer's Disease and Wine Consumption
in Bordeaux Area

L. Letenneur, J.-M. Orgogozo, C. Lacoste, S. Lafont, D. Commenge, J.-F. Dartigues, Bordeaux, France.

OBJECTIVE: To study the association between incident dementia, Alzheimer's disease (AD), and wine consumption.**BACKGROUND:** Alcoholic beverages are well known to be toxic for the brain when used without moderation. But, mild to moderate alcohol consumption has been recognized as protective against cardiovascular disorders. We studied the association between dementia, AD and alcohol on the Paquid cohort, with particular attention on wine consumption, since this beverage represents more than 97% of regular alcohol consumption in the Bordeaux area.**DESIGN/METHODS:** Paquid is a prospective study of a representative random sample of elderly people living around Bordeaux. 3,675 initially non-demented subjects older than 65 and living at home were followed-up for five years. 2,865 (77.2%) had at least one complete follow-up screening, 890 (10.6%) died before follow-up and 450 (12.2%) refused to participate in the follow-up. During this follow-up, 190 subjects developed an incident dementia classified as probable or possible AD in 120 cases according to the NINCDS-ADRDA criteria. Association between baseline wine consumption and the risk of subsequent dementia or AD was computed with a Cox model.**RESULTS:** Among the 3,665 subjects with known wine consumption, 1,601 (43.7%) did not drink wine at baseline, 1,495 (40.8%) had a mild consumption, 462 (12.3%) a moderate consumption and 107 (2.9%) a high consumption. After adjustment for age, gender and education, the relative risk (RR) of dementia related to moderate wine consumption was 0.35 (95% Confidence interval (CI)=0.17-0.73, $p=0.005$), while mild and high consumption were not significantly related to the risk of dementia. Same results were obtained for AD (RR=0.35, 95% CI = 0.14-0.84, $p=0.02$).**CONCLUSIONS:** Even if these findings could be explained by a decrease of wine consumption in the preclinical phase of dementia, we cannot exclude a possible protective effect of moderate wine consumption.S57.002
11:00 AMRisk Factors for Vascular Dementia: The
Honolulu-Asia Aging Study

Web Ross, Helen Petrovitch, Lon White, Kamal Masaki, David Curb, Beatriz L. Rodriguez, Honolulu, HI, Daniel J. Foley, Richard J. Havlik, Bethesda, MD.

OBJECTIVE: To investigate risk factors for vascular dementia (VaD) in a cohort of Japanese-American men, aged 71-93, living in Hawaii and participating in the Honolulu Heart Program (HHP).**BACKGROUND:** The HHP is a prospective study of heart disease and stroke that has accumulated risk factor data on a cohort of 8006 Japanese-American men since the study began in 1965. A recent examination of the cohort identified all cases of VaD using the California Alzheimer's Disease Diagnostic and Treatment Center's criteria.**DESIGN/METHODS:** 63 subjects with VaD were compared to 3335 subjects without dementia or stroke (NDNS) in a nested case control design. Subjects with VaD were also compared to 106 subjects with stroke who were not demented (NDS).**RESULTS:** In a multivariate logistic regression model for VaD compared to NDNS containing variables measured prospectively, age (OR=1.21, 95% CI=1.14-1.28), 1 hour post prandial serum glucose (OR=1.42; 95% CI=1.07-1.89), coronary heart disease (OR=2.76; 95% CI=1.50-5.06), a more western as opposed to oriental diet (OR=0.53; 95% CI=0.29-0.96), and supplementary vitamin E (OR=0.32; 95% CI=0.12-0.82) were found to be independently related to VaD. A similar model for the comparison of VaD to NDS revealed age (OR=1.29; 95% CI=1.16-1.42) and a more western diet (OR=0.37; 95% CI=0.16-0.84) to be independently related to VaD. In a model containing variables measured cross sectionally, left ventricular hypertrophy (LVH) (OR=13.00; 95% CI=2.00-84.55), abnormal ankle arm blood pressure index <0.9 (OR=3.43; 95% CI=1.11-10.56), and orthostatic hypotension (OR=3.78; 95% CI=1.01-14.25) discriminated VaD from NDS.**CONCLUSIONS:** Age, coronary heart disease, and elevated glucose may be important contributors to the development of VaD in late life. Peripheral vascular disease, LVH, and orthostatic hypotension may also influence the development of VaD. The antioxidant, vitamin E, and presently unknown factors related to a western as opposed to oriental diet, may be protective against developing VaD.

Supported by: Dept. of Veterans Affairs, National Institute on Aging

S57.003
11:15 AM

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Sex Differences in the Risk for Dementing
Diseases: EURODEM Collaborative Analysis

L.J. Launer, Rotterdam, NL, K. Andersen, Odense, Dk, L. Letenneur, Bordeaux, Fr, A. Ott, Rotterdam, NL, J.-F. Dartigues, Bordeaux, Fr, F. Kragh-Sorensen, Odense, Dk, L.A. Araducci, Florence, It, A. Lobo, Zaragoza, ES, J.M. Martinez-Lage, Pamplona, ES, A. Hoffman, Rotterdam, NL, MRC-CFAS Study Group, the EURODEM Risk Factor Group.

OBJECTIVE: To examine difference between men and women in the incidence of, and individual risk for dementing diseases.**BACKGROUND:** There is some evidence that women may be at higher risk for Alzheimer's disease, but estimates have often been based on small studies.**DESIGN/METHODS:** Data from four population-based studies conducted in Europe (Odense Study, PAQUID Study, Rotterdam Study, MRC-Alpha Study) were pooled. Cases were identified with a two step procedure whereby all subjects were screened using short cognitive tests, and screen positives underwent more detailed assessment including a clinical exam, neuropsychological testing and an informant interview. Diagnoses were made according to internationally accepted guidelines. Average follow-up time was 2.25 (0.77) yrs. A total of 26,378 person-years and 485 cases were included in the analyses. Rates (95% CI) were estimated using Poisson regression.**RESULTS:** Alzheimer's disease (AD) accounted for 65% of the dementias. Proportionately more women than men were diagnosed with AD (72.2% vs 52.7% of dementia cases). The incidence of AD at age 90 was significantly higher in women than men (rate per 1000 pyrs in women: 73 (57.3-93.7) and in men 22.1 (9.5-51.5). Differences by sex in the rate of vascular demen-

Thursday, April 17

daily collection, dialysis and reinfusion of the lymph of our patient was continued until February 22, 1965. His remarkably good clinical improvement persisted until February 12, 1965, when the insidious onset of weakness, anorexia, lethargy and a rise in blood pressure to 180/118 developed. By February 20, nocturnal hallucinations and a coarse tremor had developed. A deficiency of magnesium was suspected, and he was given 2.0 gm. magnesium sulfate parenterally on February 20 and 21. His response to these small doses was judged to be excellent, but on February 22, 1965, he suddenly had a severe grand-mal seizure and died.

Post-mortem examination revealed no immediate cause of death; therefore, we concluded that an insidious magnesium depletion developed as a result of the continuous dialysis of lymph for 78 days. This led to a severe convulsive seizure, with an anoxic period, causing ventricular fibrillation. Serial lymph levels for magnesium were obtained retrospectively and revealed a fall from 3.8 milliequiv. per liter initially to 0.48 milliequiv. per liter at the time of death.

If lymph collection for dialysis are only performed for 6 hours a day or for 2 days a week, we believe that the depletion of trace elements can be avoided without seriously decreasing the efficiency of the procedure.

The dialysis coils and the artificial kidney used in this project were supplied by the Travenol Company, Morton Grove, Illinois. The Silastic bags and catheters were provided by Dow Corning Center for Aid to Medical Research, Midland, Michigan. The analyses of lymph and urine for mercury were performed by Dr. Joseph Nash, director, Toxicology Laboratory, Department of Pharmacology, University of Texas Medical Branch. The analyses of lymph and blood

coagulation factors were performed by Dr. D. Mason Guest, professor and chairman of physiology and director of the Blood Coagulation Research Laboratory, University of Texas Medical Branch.

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THYROID REFRACTORINESS IN AN ATHYREOTIC CRETIN FED SOYBEAN FORMULA*

ALDO PINCHERA, M.D.,† MARGARET H. MACGILLIVRAY, M.D.,‡ JOHN D. CRAWFORD, M.D.,§ AND ALLAN G. FREEMAN, M.D.¶

BOSTON

GOITER and hypothyroidism have occasionally been reported in infants fed soybean diets.¹⁻⁵ In these patients both goiter and hypothyroidism have disappeared when soybean was eliminated. Since 1959 a widely used commercial brand of soybean has been supplemented with iodide,⁶ and to our knowledge no further cases of soybean-induced goiters have been observed.

*From the departments of Medicine and Pediatrics, Harvard Medical School, and the Medical Service (Thyroid Unit) and the Children's Service, Massachusetts General Hospital.

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†Clinical and research fellow in medicine, Harvard Medical School and Massachusetts General Hospital (work was done during the tenure of an international postdoctoral research fellowship of the National Institutes of Health; permanent address, Istituto Patologia Medica, University of Rome, Italy).

‡Clinical and research fellow in pediatrics, Harvard Medical School and Massachusetts General Hospital; trainee fellow of the National Heart Institute, National Institutes of Health, United States Public Health Service (HTS-1539).

§Associate professor of pediatrics, Harvard Medical School; chief, Metabolic Endocrine Unit Children's Service, Massachusetts General Hospital; United States Public Health Service senior fellow (GSF-14-82).

¶Attending physician, Elliot Community Hospital and pediatrician, Keene Clinic, Keene, New Hampshire.

The athyreotic cretin described below showed persistent hypothyroidism while being fed a soy formula, in spite of receiving large doses of desiccated thyroid. Since laboratory animals on soy diet acquire goiter associated with fecal wastage of thyroxine⁷⁻⁹ we proposed that refractoriness of this infant to exogenous thyroid might have been caused by impaired absorption of the hormone. Accordingly, the intestinal absorption of I¹³¹-labeled L-thyroxine was studied in conjunction with other measures of thyroid function while the patient was receiving soy formula and after its withdrawal. We are not aware of previous studies on intestinal absorption or fecal excretion of thyroid hormones in human beings receiving soybean diets.

CASE REPORT

K R (M G H 122 39-29), the product of a full-term uncomplicated pregnancy and delivery, weighed 3062 gm. (6 pounds, 12 ounces) and was 48 cm. long at birth. The neonatal period was unremarkable. During the 1st 3½

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months weight gain was satisfactory, but linear growth progressively deviated from the 10th percentile at birth to less than the 3d percentile.

At 3½ months of age severe hypothyroidism was evident. Notable clinical features were cretinous facies, dry skin, poor head control and an umbilical hernia. The diagnosis was confirmed by a protein-bound iodine (PBI) of 1.9 microgm. per 100 ml. (total iodine, 2.4 microgm. per 100 ml.). The bone age was less than that of a full-term newborn infant. Tri-iodothyronine (T_3), 4 microgm. 3 times a day, was started. Over the next 3 weeks this was gradually increased to 20 microgm. per day. A prompt improvement was observed, with increased alertness and general activity. The electrocardiograph showed an increase in voltage. At this time T_3 was withdrawn and replaced by a preparation of desiccated thyroid* (30 mg. per day), which was maintained throughout the period of observation (Fig. 1).

From 3½ to 5 months of age the infant was fed whole milk. In this period he had an episode of otitis media on the right side and frequent bouts of wheezing and nasal congestion. At 5 months he was clinically euthyroid on thyroid (45 mg. per day), and the PBI was 3.6 microgm. per 100 ml. Symptomatology suggestive of milk allergy prompted a trial with soybean formula†. Stools, which had been normal before soy feedings, became loose, bulky and foul. He also became hypothyroid again, and the dosage of thyroid was increased to 45 mg. alternating with 60 mg. daily. As shown in Figure 1, he was maintained on the soy formula for the next 7 weeks with the exception of a 2-week period of cow's milk, during which the stools became formed and of normal size. Despite these unusually high doses of thyroid in the range of 240 mg. per square meter of body-surface area per day the PBI at the end of the trial with cow's milk was only 4.6 microgm. per 100 ml.; the total serum iodine was 13.8 microgm. per 100 ml., reflecting the frequent use of an iodide-containing cough suppressant. The most striking clinical features on the soy formula were the virtual cessation of linear growth and clinical hypothyroidism. He also had frequent minor bouts of gastroenteritis and otitis media, and appeared irritable and unhappy.

Ten days before admission the soy formula was reinstituted, and the loose, foul, bulky stools returned.

On admission at the age of 6½ months he was a pale, irritable, alert infant with features suggesting partially treated hypothyroidism. These included slight facial puffiness and depressed nasal bridge, pale, dry and slightly mottled skin and cool extremities. No thyroid tissue was palpable. Semilunar ridges were present on the fingernails indicating the onset of thyroid replacement. The previously noted umbilical hernia was no longer present. Developmentally, he was within normal limits. He could sit with little support, had good head and trunk control and could reach for and transfer objects.

The hemoglobin was 10.1 gm. per 100 ml., and the white-cell count 10,400, with 32 per cent neutrophils, 47 per cent lymphocytes, 5 per cent monocytes and 16 per cent eosinophils. Urinalysis was within normal limits. Microscopical examination of the stools gave no suggestion of fat or of protein malabsorption. A sweat sodium value of 27 milliequiv. per liter was further evidence against fibrocystic disease of the pancreas. The serum calcium was 9.6 mg., the serum total protein 4.7 gm., and the serum cholesterol 178 mg. per 100 ml. Skin testing revealed a marked reaction to egg antigen. Milk precipitins were not demonstrable in a serum sample.

Laboratory evaluation confirmed the clinical impression of persistent hypothyroidism with refractoriness to thyroid replacement. The PBI was 2.7 microgm., the butanol-extractable iodine (BEI) 2.4 microgm., and the total iodine 4.2 microgm. per 100 ml. The erythrocyte uptake of I^{131} -

labeled T_4 was 7.9 per cent (normal, 12 to 16 per cent). The bone age was 1 month. Although the growth curve suggested some thyroid function in the early months of infancy, there was no significant thyroid uptake of radioactive iodine. Radioactivity over the neck area 6, 24 and 48 hours after the oral administration of I^{131} (0.5 microcurie) was only 5, 3 and 2 per cent of the dose respectively after subtraction of the nonthyroidal-tissue background obtained by counting over the thigh. The intravenous injection of I^{131} (1 microcurie) similarly revealed no significant thyroid uptake at 3, 20, 60 and 120 minutes. Because the patient was clinically and chemically hypothyroid at the time these studies were performed they probably indicate an absence of thyroid tissue rather than suppression of iodine uptake by administered thyroid.

The saliva-serum ratio of I^{131} 4 hours after the oral administration of radioactive iodide was 29, reflecting normal ability of the salivary gland to concentrate iodide. This distinguishes the patient from a previously described cretin with a defect in the iodide-concentrating mechanism.¹⁰

The uptake study of orally administered I^{131} was repeated after TSH stimulation while the patient was on a whole-milk diet. Again, there was no evidence of uptake by the gland.

The reports of fecal hormonal iodine wastage in laboratory animals fed soy¹¹ prompted a study of thyroxine absorption in this infant. The investigation was performed while he was receiving a soy diet and repeated after its withdrawal.

METHODS

A solution of 10 microgm. of sodium L-thyroxine (T_4) in saline solution containing 3 microcuries of I^{131} -labeled L- T_4 ‡ was fed to the patient by mixing with the diet. The I^{131} -labeled thyroxine administered was assessed for purity by chromatography and found to contain 92 per cent of total activity as T_4 , the remainder being iodide. The patient was kept on a metabolic bed, and feces and urine collected and measured as indicated in Table 1. A serum sample was drawn at twenty-four hours. Feces were weighed, dissolved in distilled water and homogenized. Total I^{131} content of the urine, serum and feces was measured in duplicate in a well-type scintillation counter. Corrections were made for the residue in the diet container. The fecal thyroxine-like radioactivity — that is, BEI¹³¹ — was isolated by acid butanol extraction followed by alkaline washing, a modification of the method of Man et al.¹¹ being used. Serum PBI¹³¹ was measured when radioactivity was sufficient to permit this determination.

Study 1. The soy diet had been started ten days before and continued throughout this study. Thyroid replacement was maintained at a level of 60 mg. daily.

After Study 1 was completed, the infant was discharged on a cow's milk formula and unaltered thyroid replacement.

Study 2. The patient was re-evaluated after a two-week period of cow's milk feeding. This diet and thyroid (60 mg.) were maintained throughout this study. At the time of Study 2 the PBI was 3.5 microgm., the BEI 3.3 microgm., and the total

*In the form of Armour thyroid, Armour Pharmaceutical Company, Kankakee, Illinois.

†In the form of Mull-Soy, Borden Company, Pharmaceutical Division, New York City.

‡Abbott Laboratories, Oak Ridge, Tennessee.

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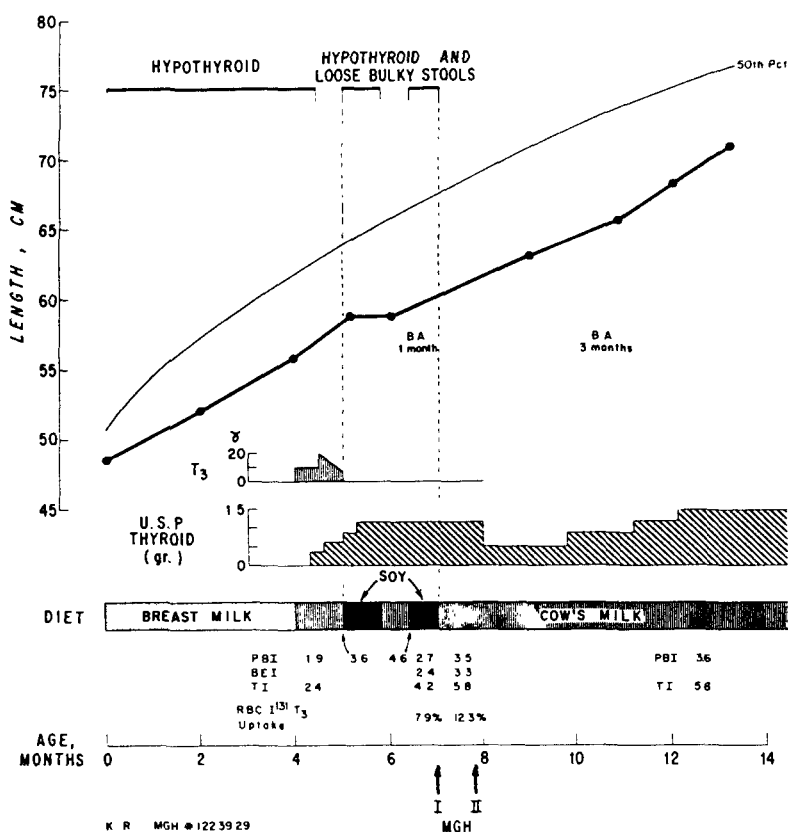


FIGURE 1. Clinical Course in Relation to Diet and Thyroid Medication.

iodine 5.8 microgm. per 100 ml. Although increases in the PBI and BEI were less than anticipated the patient was clinically euthyroid, the erythrocyte uptake of I^{131} -labeled tri-iodothyronine was normal (12.3 per cent), and the growth rate in subsequent weeks was markedly improved (Fig. 1).

RESULTS

The data in Table 1 indicate that fecal excretion of I^{131} on cow's milk was much less (31.6 per cent) than that observed on soy feeding (51 per cent). Fecal bulk was also less on cow's milk. Improved thyroxine absorption was further suggested by the increased urine and serum radioactivity in Study 2. Since the relatively low fecal I^{131} content might have been attributed in part to a delayed excretion on cow's milk the stool collection was continued to forty-eight hours. Despite this precaution only an additional 3.3 per cent of I^{131} was eliminated during the interval of thirty to forty-eight hours. The change in diet from Study 1 to Study 2 did not produce significant changes in the butanol solubility of the fecal content of I^{131} (Table 1).

The clinical course of the patient in the ten months since these investigations has been satisfactory. Growth, physical findings and stool consistence

have been all normal. Despite generous thyroid therapy PBI measurements remain in the low-normal range. The significance, if any, of this finding is unknown.

DISCUSSION

The main aim of this study was to ascertain whether the refractoriness to thyroid medication manifested by this infant while he was receiving a soy-bean diet could be ascribed to an impaired absorption of thyroid hormones.

The control study, performed while the patient was on a whole-milk diet, revealed that thirty hours after the oral administration of I^{131} -labeled L-thyroxine, approximately 32 per cent of the radioactive label was excreted in the feces. Data on the thyroxine excretion of normal subjects of this age group are not available, but the value obtained in this patient is similar to those observed by Van Middlesworth⁹ in adults. When the infant was fed soy formula the fecal I^{131} excretion was higher, and the levels of radioactivity in the urine and serum were lower than those of the control study. Thus, evidence has been obtained that soy feeding may interfere with thyroxine absorption in human beings as well as in animals. This finding calls for caution in the

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TABLE 1. *Gastrointestinal Study of Absorption of I¹³¹-Labeled-L-Thyroxine.*

PERIOD OF STUDY	SOY FORMULA			WHOLE-MILK DIET		
	QUAN- TITY	I ¹³¹ % of dose	BEI ¹³¹ % of I ¹³¹	QUAN- TITY	I ¹³¹ % of dose	BEI ¹³¹ % of I ¹³¹
Feces:						
0-24 hr.	130 gm.	48.8	95	62 gm.	31.3	95
24-30 hr.	100 gm.	2.2	70	4 gm.	0.3	100
30-48 hr.	*	—	—	88 gm.	3.3	1
Total, 0-30 hr.	230 gm.	51.0	94	66 gm.	31.6	95
Urine:						
0-24 hr.	31.5 ml.	1.1		214 ml.	5.0	
24-48 hr.	59.0 ml.	1.3		88 ml.	4.0	
Total, 0-48 hr.	90.5 ml.	2.4		302 ml.	9.0	
Serum:						
24 hr.	—	17.0†	‡§	—	42.0†	95§

*Not collected

†% of dose/liter.

‡Radioactivity not sufficient to permit this determination.

§PBI

use of soybean diets in patients requiring thyroid medication.

Increased fecal loss of hormonal iodine in rats fed soy-flour diets has previously been observed.⁷⁻⁹ Changes in the bulk of the feces seem to be of importance in these connections. Diets high in cellulose or bran also produce a large fecal volume, with enhanced excretion of hormonal iodine.⁷ The work of Triantaphyllidis¹² clearly documents this direct proportionality between fecal volume and thyroxine content. In patients with pancreatic steatorrhea Hiss and Dowling¹³ found that a decrease in the fecal weight, induced by appropriate treatment, was associated with a significant reduction in thyroxine loss, although fecal hormonal iodine content remained abnormally high. In line with these observations the patient described above was noted to have bulky, loose and foul-smelling stools when given soybean feeding, and the weight of stools was three or four times higher during the thirty-hour study on soy diet than during the control study.

"Intestinal hurry" due to increased fecal bulk may not completely explain the fecal thyroxine losses, and other mechanisms may be involved. Increased biliary excretion, addition of thyroxine to the intestinal tract by extrabiliary sources and alteration of the thyroxine chemical structure have been excluded by the experimental work of Beck.⁶ The absence of significant changes in the fecal BEI in our study also suggests that soybean does not alter the thyroxine molecule.

Changes in the intestinal bacterial flora may affect fecal thyroxine losses. This hypothesis seems supported by the recent observation of Salvatore et al.¹⁴ that *Escherichia coli*, a normal component of the intestinal flora, has specific binding properties for the thyroid hormones. No relevant data were obtained in the patient presented above, but it would

be of interest to ascertain whether soybean increase the *Esch. coli* content of the intestine.

With the present observations in mind the soybean-induced goiters previously reported in human patients can be considered. Most of these goiters showed an increased avidity for I¹³¹.^{3,4} Available histologic descriptions are of changes similar to those found in iodine deficiency.^{15,16} Iodide administration led to the disappearance of the goiter.⁴ Thus, the effects of soybean products on thyroid function are similar to those found in iodine deficiency. In this context the observation of Van Wyk et al.² that soybean products may act as thyroid-blocking agents remains unexplained.

The goitrogenic effect of soybean diet in animals has long been recognized.^{15,16} Iodide supplementation has been shown to prevent the thyroid enlargement.¹⁶ In rats Van Middlesworth⁷ has recently observed that soy-flour diets produce high-uptake goiters and excessive fecal thyroxine excretion whereas iodide absorption and excretion are apparently unaffected. He has postulated that the goiter is due to an increased iodide requirement after thyroxine depletion.

The present report suggests that the hypothesis of fecal thyroxine wastage proposed by Van Middlesworth may be responsible for the goiters previously reported in infants on soy diets. The documentation of decreased absorption of exogenous thyroxine when this congenitally hypothyroid patient was on a soy formula suggests that soy diets in euthyroid infants might interfere with the reabsorption of endogenous thyroid hormone reaching the gut through the bile. Although the enterohepatic circulation of thyroxine is lower in human beings than in rats clinical investigations have shown that 25 to 30 per cent of human thyroxine may be lost in the feces.^{9,17} Thus, prolonged interference with intestinal thyroxine reabsorption could significantly alter iodine balance and eventually cause a goiter.

SUMMARY

An athyreotic cretin became resistant to thyroid medication when placed on a soybean diet. Studies with I¹³¹-labeled L-thyroxine revealed that soybean decreased the intestinal absorption of exogenous thyroxine. This finding supports the theory that the goiters previously described in infants on soy diets were caused by fecal wastage of endogenous thyroid hormones.

We are indebted to Dr. J. B. Stanbury for advice and helpful suggestions and to Dr. J. T. Dunn for reviewing this manuscript.

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MEDICAL PROGRESS

THE D-STATE (Concluded)

A Review and Discussion of Studies on the Physiologic State Concomitant with Dreaming

ERNEST L. HARTMANN, M.D.*

BOSTON

PHARMACOLOGY AND THE D-STATE

A great many data, mostly from small informal studies, are available concerning the effect of drugs on the D-state, but as yet no definite pattern has emerged. A number of substances decrease the amount of time spent in the D-state. This is true in man for phenobarbital and several other barbiturates studied so far.²⁰ Phenothiazines likewise tend to decrease D-time; trifluoperazine (Stelazine) has some confusing early effects²¹ but probably actually decreases D-time like the others.²² Alcohol has been shown to cause a decrease in D-time,²³ and in fact it has been suggested that delirium tremens may represent a state of acute "rebound" from dream deprivation — that is, the patient has had his D-time suppressed over a prolonged period while drinking, and the vivid hallucinatory state when he finally stops drinking represents an effort to "catch up" on D-time.^{24,25} The authors present evidence for increased D-time after withdrawal from alcohol, but this is, of course, during sleep: the delirium-tremens condition is certainly not a typical D-period but might be seen as the condition of a "waking dreamer" in whom somehow both the mechanisms underlying the D-state and those underlying waking are operating at once.

Since hypnogenic and tranquilizing drugs have been shown to decrease D-time, it was thought that stimulants such as caffeine or the amphetamines might have the opposite effect. However, this does

not turn out to be the case: caffeine apparently has little effect²⁶; dextroamphetamine sulfate definitely reduces D-time, when it allows the subject to sleep at all.²⁷

These studies have been done on man, but it appears that in general these groups of drugs have the same effect on cats: in addition it has been shown that large doses of atropine effectively suppress D-periods in cats¹⁸ and rats.⁶⁰

Thus, a variety of drugs appear to be able to depress D-time; it would certainly be useful to have a drug that would increase D-time or at least provide sound sleep with a normal amount of D-time, in view of the dangers of dream deprivation, but no such drug has yet been found. However, large doses of eserine, an acetylcholinesterase inhibitor, have a tendency to increase the length of D-periods in cats although total D-time is not definitely increased.¹⁸ Lysergic acid diethylamide (LSD) has been found to have little effect, or if anything to decrease D-time in cats,⁶¹ but a recent study shows that within a very narrow dosage range, it may lengthen at least one D-period during the night in man, without increasing total D-time for the night.⁶²

Although there are no drugs that definitely increase D-time, one may with reasonable ease achieve a night of increased D-time pharmacologically by administering for a time, and then withdrawing, either phenothiazines, alcohol or dextroamphetamine sulfate.

Altogether, the pharmacologic findings do not point to any clear-cut conclusions as yet, except perhaps the conclusion that the D-state is quite

*Director, Sleep and Dream Laboratory, Boston State Hospital, assistant clinical professor of psychiatry, Tufts University School of Medicine, career investigator, National Institute of Mental Health, National Institutes of Health, United States Public Health Service.

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Morning Session**Session No. 57****NEURO-EPIDEMIOLOGY:
DETERMINANTS OF DEMENTIA****Thursday, April 17****10:45 AM - 12:00 PM****Room 309**Co-chairs: Walter Rocca, Rochester, MN
Karen Marder, New York, NY**S57.001**
10:45 AMNotice This Material May Be Protected
By Copyright Law (Title 17 U.S. Code)**Association Between Dementia,
Alzheimer's Disease and Wine Consumption
in Bordeaux Area**

L. Letenneur, J.-M. Orgogozo, C. Lacoste, S. Lafont, D. Com-menges, J.-F. Dartigues, Bordeaux, France.

OBJECTIVE: To study the association between incident dementia, Alzheimer's disease (AD), and wine consumption.**BACKGROUND:** Alcoholic beverages are well known to be toxic for the brain when used without moderation. But, mild to moderate alcohol consumption has been recognized as protective against cardiovascular disorders. We studied the association between dementia, AD and alcohol on the Paquid cohort, with particular attention on wine consumption, since this beverage represents more than 97% of regular alcohol consumption in the Bordeaux area.**DESIGN/METHODS:** Paquid is a prospective study of a representative random sample of elderly people living around Bordeaux. 3,675 initially non-demented subjects older than 65 and living at home were followed-up for five years. 2,865 (77.2%) had at least one complete follow-up screening, 390 (10.6%) died before follow-up and 450 (12.2%) refused to participate in the follow-up. During this follow-up, 190 subjects developed an incident dementia classified as probable or possible AD in 120 cases according to the NINCDS-ADRDA criteria. Association between baseline wine consumption and the risk of subsequent dementia or AD was computed with a Cox model.**RESULTS:** Among the 3,665 subjects with known wine consumption, 1,601 (43.7%) did not drink wine at baseline, 1,495 (40.8%) had a mild consumption, 462 (12.6%) a moderate consumption and 107 (2.9%) a high consumption. After adjustment for age, gender and education, the relative risk (RR) of dementia related to moderate wine consumption was 0.35 (95% Confidence interval (CI)=0.17-0.78, $p=0.005$), while mild and high consumption were not significantly related to the risk of dementia. Same results were obtained for AD (RR=0.35, 95% CI = 0.14-0.84, $p=0.02$).**CONCLUSIONS:** Even if these findings could be explained by a decrease of wine consumption in the preclinical phase of dementia, we cannot exclude a possible protective effect of moderate wine consumption.**S57.002**
11:00 AM**Risk Factors for Vascular Dementia: The
Honolulu-Asia Aging Study**

Web Ross, Helen Petrovitch, Lon White, Kamal Masaki, David Curb, Beatriz L. Rodriguez, Honolulu, HI, Daniel J. Foley, Richard J. Havlik, Bethesda, MD.

OBJECTIVE: To investigate risk factors for vascular dementia (VaD) in a cohort of Japanese-American men, aged 71-93, living in Hawaii and participating in the Honolulu Heart Program (HHP).**BACKGROUND:** The HHP is a prospective study of heart disease and stroke that has accumulated risk factor data on a cohort of 8006 Japanese-American men since the study began in 1965. A recent examination of the cohort identified all cases of VaD using the California Alzheimer's Disease Diagnostic and Treatment Center's criteria.**DESIGN/METHODS:** 68 subjects with VaD were compared to 3335 subjects without dementia or stroke (NDNS) in a nested case control design. Subjects with VaD were also compared to 106 subjects with stroke who were not demented (NDS).**RESULTS:** In a multivariate logistic regression model for VaD compared to NDNS containing variables measured prospectively, age (OR=1.21; 95% CI=1.14-1.28), 1 hour post prandial serum glucose (OR=1.42; 95% CI=1.07-1.89), coronary heart disease (OR=2.76; 95% CI=1.50-5.06), a more western as opposed to oriental diet (OR=0.53; 95% CI=0.29-0.96), and supplementary vitamin E (OR=0.32; 95% CI=0.12-0.82) were found to be independently related to VaD. A similar model for the comparison of VaD to NDS revealed age (OR=1.29; 95% CI=1.16-1.42) and a more western diet (OR=0.37; 95% CI=0.16-0.84) to be independently related to VaD. In a model containing variables measured cross sectionally, left ventricular hypertrophy (LVH) (OR=13.00; 95% CI=2.00-84.55), abnormal ankle arm blood pressure index <0.9 (OR=3.43; 95% CI=1.11-10.56), and orthostatic hypotension (OR=3.78; 95% CI=1.01-14.25) discriminated VaD from NDS.**CONCLUSIONS:** Age, coronary heart disease, and elevated glucose may be important contributors to the development of VaD in late life. Peripheral vascular disease, LVH, and orthostatic hypotension may also influence the development of VaD. The antioxidant, vitamin E, and presently unknown factors related to a western as opposed to oriental diet, may be protective against developing VaD.

Supported by: Dept. of Veterans Affairs, National Institute on Aging

S57.003
11:15 AM**Sex Differences in the Risk for Dementing
Diseases: EURODEM Collaborative Analysis**

L.J. Launer, Rotterdam, NL, K. Andersen, Odense, Dk, L. Letenneur, Bordeaux, Fr, A. Ott, Rotterdam, NL, J.-F. Dartigues, Bordeaux, Fr, P. Kragh-Sorensen, Odense, Dk, L.A. Araducci, Florence, It, A. Lobo, Zaragoza, ES, J.M. Martinez-Lage, Pamplona, ES, A. Hoffman, Rotterdam, NL, MRC-CFAS Study Group, the EURODEM Risk Factor Group.

OBJECTIVE: To examine difference between men and women in the incidence of, and individual risk for dementing diseases.**BACKGROUND:** There is some evidence that women may be at higher risk for Alzheimer's disease, but estimates have often been based on small studies.**DESIGN/METHODS:** Data from four population-based studies conducted in Europe (Odense Study, PAQUID Study, Rotterdam Study, MRC-Alpha Study) were pooled. Cases were identified with a two step procedure whereby all subjects were screened using short cognitive tests, and screen positives underwent more detailed assessment including a clinical exam, neuropsychological testing and an informant interview. Diagnoses were made according to internationally accepted guidelines. Average follow-up time was 2.25 (0.77) yrs. A total of 26,378 person-years and 485 cases were included in the analyses. Rates (95% CI) were estimated using Poisson regression.**RESULTS:** Alzheimer's disease (AD) accounted for 65% of the dementias. Proportionately more women than men were diagnosed with AD (72.2% vs 52.7% of dementia cases). The incidence of AD at age 90 was significantly higher in women than men (rate per 1000 pyrs in women: 73 (67.8-93.7) and in men 22.1 (9.5-51.5). Differences by sex in the rate of vascular demen-

Thursday, April 17

Early report

Exposure of infants to phyto-oestrogens from soy-based infant formula

Kenneth D R Setchell, Linda Zimmer-Nechemias, Jinnan Cai, James E Heubi

Summary

Background The isoflavones genistein, daidzein, and their glycosides, found in high concentrations in soybeans and soy-protein foods, may have beneficial effects in the prevention or treatment of many hormone-dependent diseases. Because these bioactive phyto-oestrogens possess a wide range of hormonal and non-hormonal activities, it has been suggested that adverse effects may occur in infants fed soy-based formulas.

Methods To evaluate the extent of infant exposure to phyto-oestrogens from soy formula, the isoflavone composition of 25 randomly selected samples from five major brands of commercially available soy-based infant formulas were analysed, and the plasma concentrations of genistein and daidzein, and the intestinally derived metabolite, equol, were compared in 4-month-old infants fed exclusively soy-based infant formula ($n=7$), cow-milk formula ($n=7$), or human breast-milk ($n=7$).

Findings All of the soy formulas contained mainly glycosides of genistein and daidzein, and the total isoflavone content was similar among the five formulas analysed and was related to the proportion of soy isolate used in their manufacture. From the concentrations of isoflavones in these formulas (means 32–47 $\mu\text{g/mL}$), the typical daily volume of milk consumed, and average body-weight, a 4-month-old infant fed soy formula would be exposed to 28–47 per day, or about 4.5–8.0 mg/kg body-weight per day, of total isoflavones. Mean (SD) plasma concentrations of genistein and daidzein in the seven infants fed soy-based formulas were 684 (443) ng/mL and 295 (60) ng/mL, respectively, which was significantly greater ($p<0.05$) than in the infants fed either cow-milk formulas (3.2 [0.7] and 2.1 [0.3] ng/mL), or human breast-milk (2.8 [0.7] and 1.4 [0.1] ng/mL), and an order of magnitude higher per bodyweight than typical plasma concentrations of adults consuming soy foods.

Interpretation The daily exposure of infants to isoflavones in soy infant-formulas is 6–11 fold higher on a bodyweight basis than the dose that has hormonal effects in adults consuming soy foods. Circulating concentrations of isoflavones in the seven infants fed soy-based formula were 13 000–22 000 times higher than plasma oestradiol concentrations in early life, and may be sufficient to exert biological effects, whereas the contribution of isoflavones from breast-milk and cow-milk is negligible.

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Clinical Mass Spectrometry Center (K D R Setchell PhD, L Zimmer-Nechemias MS, J Cai MS), and **Division of Gastroenterology and Nutrition** (J E Heubi MD), **Children's Hospital Medical Center**, 3333 Burnet Avenue, Cincinnati, Ohio 45229, USA

Correspondence to: Prof Kenneth D R Setchell

Introduction

More than a decade after attention was first drawn to the levels of phyto-oestrogens in soy infant-formulas,^{1,2} concerns are being expressed about the possibility of hormonal effects from exposure of infants to phyto-oestrogens from soy-based infant formulas.³ These concerns have prompted at least one government agency to issue statements and recommendations about the use of soy-based infant formulas in early life.⁴

The phyto-oestrogens in all soy-protein foods belong to the isoflavone class.¹ With few exceptions all soy-protein products and soybeans are rich in isoflavones.^{5,6} Variation in amount of isoflavones in different soy foods is accounted for mainly by the differences in industrial processing of the soybean, and the type and extent of incorporation of the soy protein into the food matrix.⁵ Isoflavones when ingested are metabolised extensively in the intestinal tract, absorbed, transported to the liver, and undergo enterohepatic recycling.^{1,7,8} Intestinal bacterial glucosidases cleave the sugar moieties and release the biologically active isoflavones, daidzein and genistein, and in the adult these can be further biotransformed by bacteria to the specific metabolites, equol,⁹ desmethylangolensin, and p-ethylphenol.¹⁰ All of these phyto-oestrogens are then eliminated, mainly by the kidney, and therefore share the physiological features and behaviour of endogenous oestrogens.⁷

In addition to acting as oestrogen mimics,^{7,8} isoflavones have important non-hormonal activities. Genistein, for example, is a potent inhibitor of tyrosine kinases¹¹ and interferes with cell signal-transduction pathways.¹² The ingestion of high concentrations of phyto-oestrogens has adversely affected reproduction in several animal species,^{13,14} and in premenopausal women daily ingestion of soy protein lengthens the menstrual cycle and suppresses the usual midcycle surge in pituitary gonadotropins,¹⁵ effects that epidemiological evidence suggests are beneficial in decreasing risk of breast cancer. The hypocholesterolaemic action of soy protein is well established and anticancer actions of soy isoflavones have been shown in in-vitro studies and in several classic animal models of chemically-induced breast cancer.^{7,8}

Although urinary analyses have indicated that isoflavones are absorbed by the infant fed soy-based infant formula, data on the composition of phyto-oestrogens in infant formulas are scant and the level of exposure of the infant fed soy-based formula to phyto-oestrogens is uncertain. We now describe a comprehensive analysis of the isoflavone composition of randomly selected samples of five different brands of commercially available soy-based infant formulas and a comparison of the plasma concentrations of isoflavones in 4-month-old infants fed soy infant-formula, cow-milk formula, and human breast-milk.

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Methods

Phyto-oestrogen analysis of soy-based infant formulas

Five samples each of five of the major commercial brands of soy-based infant formulas were purchased from three stores in the Cincinnati area. The brands were: Nursoy powdered formula (Wyeth Laboratories, Philadelphia, Pennsylvania), Isomil powdered formula (Ross Products Division Abbott Laboratories, Columbus, Ohio), Isomil "Ready to Feed" liquid formula (Ross Products Division Abbott Laboratories, Columbus, Ohio), Alsoy liquid formula concentrate (Carnation Nutritional Products Division, Nestlé Food Company, Glendale, California), and ProSobee liquid formula concentrate (Mead Johnson, Evansville, Indiana). Care was taken to obtain samples of different batch or lot numbers. Isoflavone concentrations were measured by reverse-phase high-pressure liquid chromatography (HPLC).¹

Accurately weighed portions of about 0.1 g of each of the powdered formulas and 1.0 mL volumes of the liquid formulas were taken for analysis, and equilenin (25 µg) was added as an internal standard for quantification. Isoflavones were extracted into 80% methanol (4 mL) by sonication for 15 min and shaking for 2 h at cold temperature. The sample was centrifuged and the supernatant was removed. Lipids were extracted by partitioning into hexane (2×4 vols). The sample was centrifuged and the hexane phase was discarded. The methanolic phase was taken for analysis by HPLC. Isoflavones and their conjugates were separated by reverse-phase HPLC on a 25×0.46 cm Aquapore (C8; particle size 7 µm) column under gradient elution conditions, essentially as previously described.⁵ The mobile phase consisted of an initial isocratic period of 2 min of 0.1% trifluoroacetic acid, followed by a gradient increasing over a 15-min period to 100% of a solution of 46.4% acetonitrile in 0.1% trifluoroacetic acid. Isoflavones were detected by the intense absorption at 260 nm, and quantified by their response factors relative to the internal standard. The within-batch precision of the method, as determined from replicate analyses (n=5) of the same soy formula, ranged from 1.6 to 3.8% (coefficient of variation, CV) for the major glycosidic conjugates of isoflavones in the concentration range 10–20 µg/mL, and from 14 to 20% (CV) for the aglycones, which are minor components (<2 µg/mL).

Measurement of plasma concentrations of isoflavones

Plasma samples were obtained from healthy 4-month-old white boys (n=21) who had been exclusively fed a typical soy-based infant formula (Isomil, n=7), a cow-milk formula (Similac, n=7, Ross Products Division Abbott Laboratories), or breast milk (n=7). These full-term infants had been randomised to these diets, and had been exclusively fed the formulas, from the first week of life. The blood sample (0.5–1 mL) was obtained between 0900 h and 1100 h. The infants were not fasted before collection. This observational study was approved by the hospital Investigational Review Board.

Plasma isoflavone concentrations were determined by gas-chromatography/mass-spectrometry, with liquid-solid extraction and liquid-gel chromatographic techniques to isolate the oestrogenic fractions.⁹ The samples were analysed in a blinded fashion. After addition and equilibration of an internal standard, dihydroflavone, to plasma (0.25–0.5 mL), the sample was diluted with four volumes of triethylamine sulphate and heated to 65°C to dissociate isoflavones and their conjugates from proteins. The warmed sample was then passed through a precharged solid-phase Bond Elut C18 cartridge. After washing of the cartridge with water (2×5 mL), the retained isoflavones were then recovered by elution with methanol (4 mL). Isoflavone conjugates were hydrolysed enzymatically overnight at 37°C with a β-glucuronidase and sulphatase preparation from *Helix pomatia* from which isoflavones had been pre-extracted. Isoflavones were then extracted from the hydrolysate by liquid-solid extraction and separated from neutral steroid-hormones on a lipophilic anion-exchange gel, triethylaminohydroxypropyl Sephadex LH-20. The phenolic/oestrogenic fraction containing

isoflavones was eluted from the gel with methanol saturated with CO₂ and evaporated to dryness. Isoflavones were converted to their tert-butyldimethylsilyl ether derivatives and analysed by mass spectrometry with selected ion-monitoring of the specific ions characteristic of daidzein and the internal standard (m/z 425), genistein (m/z 555), equol (m/z 470), dihydrodaidzein (m/z 472) and desmethyangolensin (m/z 543). Isoflavones were quantified by comparison of the ratio of the peak area response of the characteristic ion relative to the peak area response for the internal standard and interpolating this ratio against a calibration curve constructed from known amounts of the pure standards. Values are expressed as ng/mL and nmol/L. The between-batch precision of the method, as determined from replicate analysis over a 2-year period of a quality-control human serum sample having an isoflavone concentration of 72 ng/mL, was 8–11% (CV) for the individual isoflavones.

Statistical analysis

Isoflavone concentrations were expressed as mean (SD). An analysis of variance (ANOVA) was done to compare the log-transformed plasma concentrations of isoflavone constituents among infants fed the different regimens. The ANOVA was followed by the least significant difference test for multiple comparisons, and the pairwise differences in individual and total isoflavones were computed. The pairwise differences of the log-transformed isoflavones were computed, and then converted to their original scale.

Results

Isoflavone composition of soy-based infant formulas

A typical HPLC profile of the phenolic fraction of soy-based infant formula consists of a complex pattern of peaks with absorbance at 260 nm that were identified on the basis of retention index and mass spectrometry to be conjugated and unconjugated isoflavones.¹ The predominant isoflavones identified in all of the soy-based formulas were the β-glycosides, genistein and daidzein, and the 6-"O"-malonyglycosides and 6-"O"-acetylglycosides of genistein and daidzein. Glycitin was also found in significant amounts. When prepared for consumption according to the manufacturer's directions the isoflavone concentration in the powdered formulas was 46–47 µg/mL, similar to that in the Prosoabee or the Isomil formulas (44–47 µg/g formula, figure 1). The isoflavone

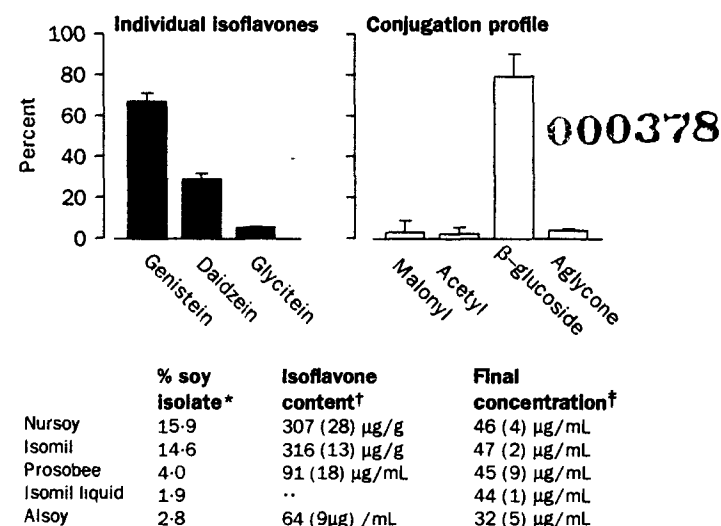


Figure 1: Summary of isoflavone composition of commercially available soy-based infant formulas

*Amount of soy isolate used in the manufacture of formula; †total isoflavone content in off-the-shelf preparation; ‡concentration of preparation for consumption according to the manufacturers' directions.

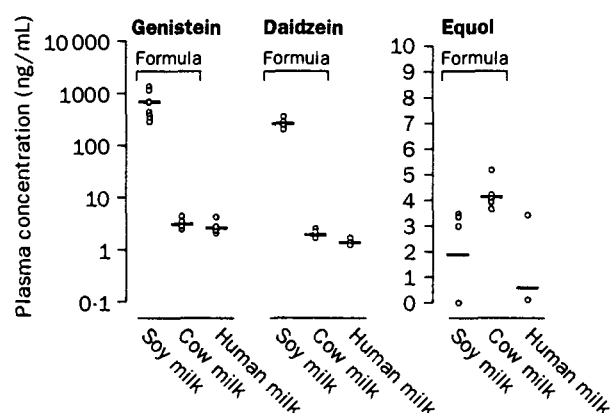


Figure 2: Plasma concentrations of genistein, daidzein, and equol measured in 4-month-old infants exclusively fed soy-based infant formula, cow-milk formula, or human milk. Plasma concentrations for daidzein and genistein plotted on a log scale. Normal plasma oestradiol concentrations in infants are typically <80 pg/mL²⁴. Horizontal bars=mean values.

concentration of the Alsoy liquid concentrate was lower than in the other formulas, but was similar when the proportion of soy isolate contained in this formula was taken into account. The proportion of the aglycones, daidzein, and genistein in all formulas was small and ranged from 3.2–5.8% of the total isoflavones present. The relative composition of the individual isoflavones and their conjugates in the formulas was essentially similar, although the proportions of malonyl glycosides tended to be lower in the liquid formula, presumably because of the instability of these conjugates to the heating procedure used in the preparation of the liquid formulas. The β -glycosides accounted for a mean 79.5% of the total isoflavones present. There was a higher proportion of genistein and its conjugates (mean 67.1% [4.5]) than of daidzein and its conjugates (mean 28.7% [3.2]) in all formulas.

Plasma concentrations of isoflavones

Data for daidzein and genistein are presented in figure 2 on a logarithmic (\log_{10}) scale because of the large difference in plasma concentrations found in infants fed soy-based formula compared with infants fed cow-milk formula or breast-milk. For soy-based formula feeding, mean plasma concentration was 684 (443) ng/mL, equivalent to 2.53 (1.64) $\mu\text{mol/L}$ for genistein, and 295.3 (59.9) ng/mL, equivalent to 1.16 (0.23) $\mu\text{mol/L}$ for daidzein. These values were significantly greater ($p<0.001$) than the mean values for plasma genistein and daidzein concentrations in infants fed either cow-milk formulas (3.16 [0.68] ng/mL, 11.6 [2.5] nmol/L, and 2.06 [0.29] ng/mL, 8.1 [1.1] nmol/L, respectively), or breast-milk (2.77 [0.73] ng/mL, 10.2 [2.70] nmol/L, and 1.49 [0.13] ng/mL, 5.86 [0.51] nmol/mL, respectively). Equol was consistently present in the plasma of all of the infants consuming cow-milk formula, the mean concentration being 4.11 (0.49) ng/mL or 16.9 (2.0) nmol/L. However, equol was detected only in the plasma of four of the seven infants fed soy-based formula and in one of the seven infants fed human breast-milk (figure 2). Desmethyldaidzein and dihydrodaidzein were not detected in the plasma.

There were large variations in plasma isoflavone concentrations in the infants fed soy-based formula. Wilk and Shapiro tests for normality indicated non-normal

distributions of these concentrations by formulas ($p<0.05$; $p<0.1$). ANOVA indicated significant differences in each of the plasma isoflavone concentrations among the three different groups of infants ($p<0.05$), while the pairwise comparisons indicated significantly higher plasma isoflavone concentrations in soy-based formula-fed infants than in infants on cow-milk formula and breast-milk ($p<0.05$). There was, however, very little difference in the plasma concentrations of isoflavones between the infants on cow-milk formula and breast-milk, except for the total isoflavone concentration, which was higher in the infants on cow-milk.*

Discussion

Since most brands of soy infant-formulas are prepared from soy isolates, the total isoflavone content should be similar among formulas, and related to the proportion of soy isolate incorporated. All five soy-based formulas analysed contained considerable amounts of isoflavones, and these were mostly glycosidic conjugates of daidzein and especially of genistein. Unconjugated isoflavones accounted for only 3–6% of the total. This composition is consistent with the reported findings for soy isolates.^{5,17} When the actual proportion of soy isolate used in their manufacture is taken into consideration, the total isoflavones per gram soy-protein was almost identical among the individual brands of formulas. These values are similar to, or slightly higher than, earlier reported values that used a technique that did not discriminate among the various conjugates². From our analyses of these five soy formulas, which contained 32–47 $\mu\text{g/mL}$ of total isoflavones, and on the basis of a typical daily intake of 900–1000 mL of milk at 4 months of age, the total isoflavone exposure for a 4-month-old infant is 28–47 mg per day, or 6–9 mg/kg bodyweight per day (figure 3). The total daily intake of isoflavones derived from soy-based infant formula is thus comparable to that of adults consuming modest amounts of soy-protein foods, and

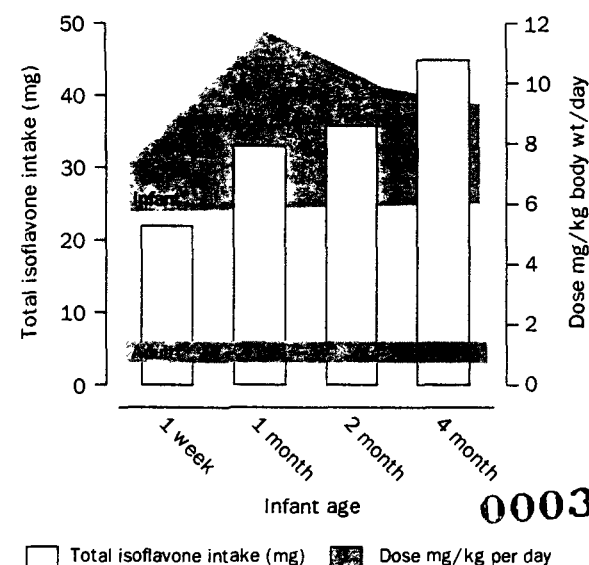


Figure 3: Total isoflavone intake (mg) in early life (solid vertical bars), and the exposure dosage (mg/kg bodyweight per day; shaded region)

Exposure dosage calculated from concentrations of isoflavones measured in soy-based infant formula, typical daily volumes of milk consumed by the infant, and the average body weights taken from standard paediatric growth charts.

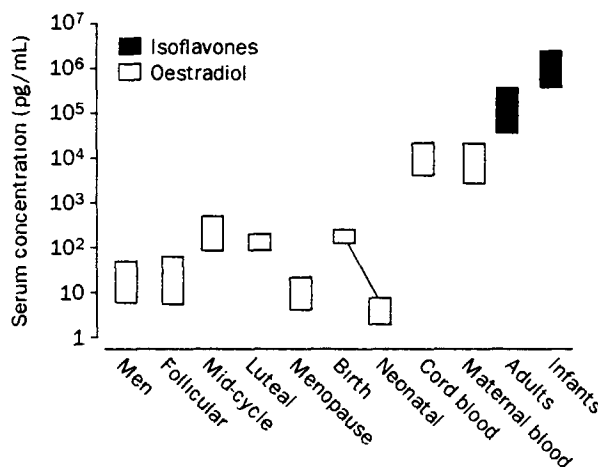


Figure 4: Comparison of plasma isoflavone concentrations in adults consuming soy foods and in infants fed soy-milk formulas with plasma oestradiol concentrations in adults and infants

Note logarithmic scale.

also of Japanese adults consuming a traditional diet.¹⁸ When bodyweight and milk intake are taken into account, an infant exclusively fed soy-based formulas would be exposed to a fairly constant dose throughout early life (figure 3). This dose represents a 6–11 fold higher level of intake of isoflavones than the bodyweight-adjusted intake (0.7 mg/kg per day) found to cause significant modifications to the hormonal regulation of the menstrual cycle of western women.¹⁵

The issue of bioavailability of isoflavones in the infant has been contentious. It was uncertain whether phyto-oestrogens circulated at concentrations sufficiently high to exert physiological effects. Our observational study provides information on plasma isoflavones in response to different infant-feeding regimens. Our previous studies indicated that infants fed soy infant-formulas readily absorb isoflavones, since daidzein and genistein were found in the urine.¹⁶ Urine concentrations of daidzein and genistein were variable and lower for infants¹⁶ than for adults consuming similar quantities of isoflavones,^{6,11,19,20} which suggested that either renal clearance or intestinal absorption was lower in infants than in adults. Absorption, however, seems to be relatively efficient as suggested by the extremely high plasma concentrations of daidzein and genistein in infants fed exclusively on soy-based formula. Plasma total isoflavone concentrations in these seven infants (range 552–1775 ng/mL, mean 980 ng/mL) is 2–5 fold higher than the peak plasma concentrations observed in adults after a single oral dose of 50 mg of the pure compounds (300 ng/mL),²¹ and significantly greater than reported concentrations (50–200 ng/mL) for adults consuming diets of soy-based foods containing similar amounts of isoflavones.^{22,23} These values are also much higher than plasma isoflavone concentrations of Japanese adults, which range from 40 to 240 ng/mL.²⁴ The high plasma concentrations may be accounted for in part by the steady-state condition arising from frequent and regular feeds through the day. Furthermore, because of developmental immaturity in the bacterial enzymes required for metabolic biotransformation of isoflavones,¹⁶ a greater proportion of daidzein and genistein may be available for absorption by the infant than in the adult.

Equol was detected in the plasma of only four of the

seven infants on soy-based formula and one of seven of the breast-milk-fed infants, and was generally low in all of the infants. Equol was found in highest concentrations in all of the infants fed cow-milk formula, which is explained by the presence of isoflavones in cow's milk.²⁵ These findings are in accord with data for the urinary excretion of equol in infants,¹⁶ and are explained by the ontogenic lack of a fully developed microflora in early life, or inactivity of the metabolic enzymes.

Our data do not support claims about human milk as a source of phyto-oestrogens.²⁶ Mean total plasma isoflavone concentration of breast-milk-fed infants was 4.7 (1.3) ng/mL, which is less than 1/200th the level attained by feeding on soy-based formula. Isoflavone concentrations of human breast-milk are also very low (5–15 ng/mL) and although they can increase up to 10-fold when the lactating mother consumes soy foods,²⁷ the daily intake by the infant of phyto-oestrogens from human milk is only 0.005–0.01 mg. Our data therefore provide little reason for concern about phyto-oestrogens from human breast-milk, even when mothers consume soy during lactation. Because of the weak oestrogenic activity of isoflavones (10^{-2} to 10^{-3} that of oestradiol),⁷ the dietary intake of phyto-oestrogens from human milk is unlikely to exert biological effects.

Phyto-oestrogens have hormonal and non-hormonal actions^{21,28} that can explain how a diet containing bioactive oestrogens may prevent hormone-dependent diseases, including cancer, osteoporosis, and cardiovascular disease.^{1,7,8,19,28} Furthermore, epidemiological data show that the incidence of hormone-dependent diseases is low in China and Japan, where soy is a staple.^{29–31} Although soy infant-formula is little used in Japan and China, infants there are exposed to soyfoods early in childhood, so it is perhaps the lifetime exposure to soy foods that confers these health benefits. However, diets are rapidly becoming westernised in these countries, which have also seen an increase in the incidence of western diseases.³¹

The reproductive dysfunction in Clover disease in sheep¹³ and veno-occlusive disease in captive cheetah¹⁴ were both attributed to the ingestion of isoflavones. The relation between prenatal exposure to diethylstilboestrol and genetic imprinting and predisposition of the infant to reproductive dysfunction and adenocarcinoma later in life is well established.³² These findings and the concerns over the possible effects of other environmental oestrogens³² have led to questions about the safety of phyto-oestrogens in soy-based infant formulas.³⁴ There is no evidence to suggest that ingestion of isoflavones, at levels consistent with amounts present in soy-protein foods, has adverse effects in human beings, but the potential effect that these bioactive compounds may produce by creating steroid-hormone imbalance, by competition with enzymes that metabolise steroids, drugs, and xenobiotics, or by influencing gonadal function, is unknown. There have been no specific examples to support these concepts for human beings, even in countries where soy has been consumed for millennia, nor have deleterious effects of soy-based infant formulas become apparent despite having been in use for more than 30 years. It would be difficult for human beings to consume sufficient amounts of isoflavones from natural soy-foods to reach the toxicological levels that induce the pathological effects recorded in animals. However, with the recent trend toward extracting

isoflavones from soy for commercialised over-the-counter soy isoflavone supplements, and because such products are not closely regulated, the potential dangerous effects from self-induced mega-dosing are a concern.

Since many major diseases of western populations are associated with oestrogen-deficient states, the beneficial effects of diets rich in phyto-oestrogen seem to outweigh any hypothetical disadvantages, at least for the adult. There may also be long-term benefits from exposing infants to soy-infant formulas containing isoflavones, because this may confer some protection later in life against the predisposition to hormone-dependent diseases. Prepubertal exposure of rat pups to genistein provides resistance to chemically induced breast cancer later in life,³³ which may be inferred to represent late-onset programming effects.

Our observational studies indicate that phyto-oestrogens circulate in soy-formula-fed infants at concentrations that are 13 000–22 000 times higher than plasma oestradiol concentrations, which range from 40 to 80 pg/mL in early life (figure 4).³⁴ Even allowing for their weak oestrogenic activity, dietary isoflavones must have some biological activity in the infant. The safety of soy-based infant formulas remains controversial, partly because of the paucity of data on the physiological behaviour and metabolic and molecular effects of phyto-oestrogens in human beings, and the difficulty of defining appropriate endpoints. To allay increasing concerns about soy-based formulas, long-term follow-up studies are needed to assess the potential beneficial or adverse effects of phyto-oestrogen exposure in early life.

Contributors

Kenneth D R Setchell carried out and supervised the research, Linda Zimmer-Nechemias did the laboratory work, Jinnan Cai operated the mass spectrometer, and James E Heubi oversaw the clinical studies.

Acknowledgments

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*Table summarising descriptive statistics for log-transformed values, pairwise differences of log-transformed plasma isoflavone concentrations between feeding regimens, and resultant anti-log transformed values with 95% CI available from *The Lancet*.

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Herbal Medicines, Phytoestrogens and Toxicity: Risk:Benefit Considerations¹ (44248)

DANIEL M. SHEEHAN²

Division of Reproductive and Developmental Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, DHHS, Jefferson, Arkansas 72079

Abstract. There are several suggested health benefits of phytoestrogens, particularly those found in soy products. Herbal medicines are also widely thought to confer health benefits. Additionally, drugs are prescribed to improve human health, but unlike phytoestrogens and herbal medicines, toxicities are defined in experimental animals and monitored in humans before and after marketing. Knowledge of toxicity is crucial to decrease the risk:benefit ratio; this knowledge defines appropriate conditions for use and strategies for development of safer products. However, our awareness of the toxicity of herbal medicines and phytoestrogen-containing foods is dramatically limited compared to drugs. Some aspects of the toxicity of herbal medicines are briefly reviewed; it is concluded that virtually all of our knowledge is derived from human exposures leading to acute toxicities. Importantly, detection of toxicity is sporadic, and little information is available from prior animal experimentation. Additionally, well-organized monitoring of human populations (as occurs for drugs) is virtually nonexistent. Important toxicities with long latencies are particularly difficult to associate with a causative agent during or even after large scale exposures, as exemplified by tobacco smoking and lung cancer; estrogen replacement therapy and endometrial cancer; diethylstilbestrol and reproductive tract cancers; and fetal alcohol exposure and birth defects. These considerations suggest that much closer study in experimental animals and human populations exposed to phytoestrogen-containing products, and particularly soy-based foods, is necessary. Among human exposures, infant soy formula exposure appears to provide the highest of all phytoestrogen doses, and this occurs during development, often the most sensitive life-stage for induction of toxicity. Large, carefully controlled studies in this exposed infant population are a high priority.

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Several lines of evidence suggest significant health benefits of phytoestrogens, plant chemicals possessing estrogenic activity. This evidence is reviewed in a number of papers presented in this volume (1-5), and while not the subject of this paper, clearly needs to be considered as part of an overall evaluation of potential health benefits. However, here I wish to discuss certain characteristics of herbal medicines, long used for health purposes, and to explore some broad cultural and scientific relationships that exist between herbal medicines and phytoestrogens. Specifically, both herbal medicines and phytoestrogens are

widely believed to be beneficial but can display toxic effects, and these, like the health claims, also need consideration in order to evaluate overall health effects properly.

Toxicologists can be perceived as having a negative impact on the development of a wide range of marketable products that may benefit society. This naive perspective is challenged by one of the important goals of the toxicologist: to provide information on risks to be included as part of a risk:benefit evaluation. The appropriate decision is directly dependent on the proposed use of a product. For example, drugs useful in diseases with high mortality can display serious toxicity but still be appropriate for use, whereas lower toxicity may not be accepted in a product for common minor ailments, such as colds. An important additional consideration is voluntary versus involuntary exposure; risks associated with involuntary exposures are less acceptable than risks from voluntary exposures (6). Thus toxicologists contribute to decision-making regarding whether a product should be on the market, and if so, under what specific

¹ The views expressed in this paper do not necessarily reflect the views of the FDA.
² To whom requests for reprints should be addressed at Division of Reproductive and Developmental Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, DHHS, Jefferson, AR 72079

conditions of use. These decisions are important to protect human health, which can be improved by having knowledge of toxicity. An example is provided by tamoxifen, a drug that is widely used for its beneficial effects as an antiestrogen in preventing recurrence of breast cancer. However, recent findings demonstrate that tamoxifen acts as an estrogen and increases endometrial cancer incidence in breast cancer patients (7). This knowledge has not resulted in the removal of tamoxifen for intended beneficial effects; rather, clinicians now know to monitor patients closely for clinical signs of endometrial abnormalities and to take appropriate medical action when these are found. This knowledge decreases the risk:benefit ratio by lowering the population risk.

This, then, is the context within which the information presented herein should be considered: How can we improve human health by understanding the toxicity of both herbal medicines and phytoestrogens?

Plants are chemical factories that directly provide about 25% of currently used drugs; another 25% of drugs are chemically altered natural products (8). Likewise, phytochemicals with known or potential health benefits are found in plants or plant products marketed either as herbal medicines (9, 10) or foods (11). The latter group includes soybeans, which have a high phytoestrogen content and are a growing component of the human diet. There is, however, a fundamental difference in the safety evaluation of drugs compared to herbal medicines or foods (excluding, in the latter case, chemicals added to foods). Marketing approval for drugs requires careful preclinical, clinical and post-market evaluation of both safety and efficacy; this is not a general requirement for herbal medicines or foods.

Preclinical safety testing in animals follows well-defined protocols involving short- and long-term dosing for evaluation of organ toxicity and death, and tests for mutagenic and carcinogenic activity, reproductive toxicity, and adverse effects on development, among others. Additional specific investigations may be necessary depending on the drug class or the nature of toxicity found in standard preclinical tests. A significant proportion of potential drugs never get to the market because toxicity data suggest a poor risk:benefit ratio.

Clinical testing, which follows animal testing, involves drug administration to volunteers with close monitoring of both efficacy and safety. Again, a number of drugs fail these evaluations. Once marketed, drugs continue to be scrutinized through post-market surveillance; for example, physicians report adverse effects possibly associated with drug treatment, and reports must be maintained by the drug sponsor. Drugs are occasionally found to have toxicities in post-market surveillance that were not detected in the rigorous preclinical and clinical testing. These findings can influence marketability, conditions of use, and patient monitoring as appropriate. Most drugs are available only by prescription. Patients are informed of possible adverse effects both by their physician and the drug label, and are monitored by

their physician. By these measures, most people are aware that drugs may have toxic effects. This knowledge can lead a patient to associate their drug ingestion with adverse outcomes.

Another difference between many drugs compared to herbal medicines and foods is the purity of the chemical of interest. Many drugs are pure chemicals (with fillers, excipients, etc., added for pharmacological purposes); others, however, are complex mixtures that may be partially purified, such as alcoholic extracts (tinctures). Foods and herbal medicines generally are complex mixtures.

Unlike drugs, herbal or folk medicines and food products directly derived from plants are not generally required to be tested for safety or efficacy. Food safety laws are complex and not the subject of this discussion, but it should be appreciated that chemicals added to foods during processing (e.g., antioxidants, emulsifiers, etc.) do require safety testing.

Herbal products have a long history of use based on religious and cultural traditions in which plants are viewed as sources of health remedies (12). This is clearly shown by the prevalence of plant products among prescription drugs. However, it is also true that plants have evolved defense mechanisms against animal and pest predation. These include thorns and other types of physical protection, as well as chemicals that either make plants unpalatable or that sicken or kill their predators and are widely distributed among plants. Such toxicity occurs in humans. The coexistence of beneficial and adverse effects is as true for plant products as for drugs. An important distinction is that our knowledge of drug safety is much superior to our knowledge of herbal product or food safety; we depend on the mostly random accumulation of reports of adverse effects in humans for the latter products (12), and this reporting system is poorly organized and greatly dependent both on luck and consumer as well as physician alertness.

What do we know about the use of herbal medicines and the attitudes of consumers? In a survey of HIV-positive patients, 22% reported regular use of an average of 4.5 herbal tablets per day. Over one-quarter of these reported adverse effects that could be caused by the herbal products (13). Approximately one in two Hong Kong residents use herbal medicines (14). Brown and Marcey (15) report that over 90% of 100 surveyed adults used at least one botanical remedy or another, with a median number of seven (range, 0-33). Of those with chronic conditions, more (58%) used home remedies than physician-prescribed treatments (21%). These findings demonstrate a strong belief in, and highly prevalent use of, herbal products, a phenomenon that cuts across cultures and economic classes. Additionally, in part due to the lack of adequate safety data, toxicities may not be expected; in fact, the possibility may be vigorously denied. Adverse outcomes, therefore, may not be recognized as being associated with herbal products.

However, numerous herbal products demonstrate toxicity; this relationship between known toxicity and the per-

ception of consumers stands in direct contrast to the more widespread knowledge of drug toxicity detailed earlier. The toxicity of herbal products may be classified as due to misidentification of plants or to toxicity of properly identified plants (16).

Misidentification has been documented for both self-collected plants and in commercial products. Foxglove is the original source of digitalis compounds and some leaf preparations remain available. Death due to arrhythmias and hyperkalemia are well described (17). An elderly man consumed tea prepared from leaves of a foxglove plant found in his back yard; acute cardiotoxicity resulted (17). A couple died following ingestion of tea prepared from foxglove mistaken for comfrey; other cases of foxglove poisoning are known (18). A red variety of common vetch was misidentified and sold as red lentil; vetch contains neurotoxins (19). Two infants were poisoned with a tea prepared with the herb *Senecio longilobus* mistakenly substituted for *Gordolobo yerba* (12, 18). One infant died and the other suffered chronic liver toxicity. Both of the latter examples were due to commercial products. Likewise, a large American marketer of herbs misidentified deadly nightshade as comfrey, resulting in atropine poisoning (20, 21). In any case, comfrey itself is hepatotoxic and, although widely marketed, should be completely avoided (12, 22, 23).

Misidentification also results from nomenclature problems. Ginseng (English common name) is also called renshen (transliteration), radux ginseng (latinized pharmaceutical name) and *Panax ginseng* (scientific name) (14). Additionally the same common name may be applied to different plants (14). "Cohosh" is used in New England for baneberry, which is toxic, while in areas of Appalachia it refers to black or blue cohosh; all three are different genera that show different patterns of toxicity (24). Huxtable (16) provides more detailed examples of nomenclature problems as described above; he as well describes the common confusion that one plant may have many different names (e.g., *Heliotropium angiospermum* has 31 common synonyms). Additionally, it is common to be unable to identify components of herbal medicines or teas when consumers present with clear signs of toxicity associated with their consumption (14). For example, an unidentified herbal tea was consumed by four women; one developed a skin rash, whereas the other three had veno-occlusive disease of the liver, from which one died. The tea contained pyrrolizidine alkaloids at a high concentration (25, 26).

However, proper identification alone cannot provide assurance of safety. Ridker (23, 27) lists 26 herbs with known toxicities; all are used to prepare teas, and most are available commercially. Almost 400 different herbs and spices are used for teas, and while more than 10% contain psychoactive ingredients, it is unclear if they induce responses (28). One chemical class of toxicants found in a number of herbal preparations is the pyrrolizidine alkaloids; over 8,000 cases of veno-occlusive disease of the liver have

been reported to be caused by this class of chemicals (16, 29) including probable human embryotoxicity (12, 30).

In addition to teas, herbs are also consumed by smoking. Some 20 years ago, 192 different herbs were available for such use (28). Of the mixtures used in 18 different products, almost half contained psychoactive ingredients.

Sassafras, long consumed as a tea in the southeastern United States, causes diarrhea and is hepatotoxic and hepatocarcinogenic (23). It contains the experimental animal carcinogen, safrole. Licorice (*Glycyrrhiza glabra*) can induce a syndrome of toxicities that appears clinically similar to primary aldosteronism (23). Alexander the Great's army used licorice during desert crossings, probably to conserve water by reducing urine output. Licorice contains glycyrrhizic acid, a metabolic precursor of an 11β -steroid dehydrogenase inhibitor which is almost certainly the cause of the primary aldosteronism of licorice (9). Ginseng is consumed by 5-6 million people in the United States. Frequent consumption can produce a syndrome of toxicities (hypertension, confusion, depression, insomnia) and severe hypotension upon withdrawal which together mimic corticosteroid poisoning (8).

Several plants in the southwestern United States contain adrenergic chemicals. One of these, ephedrine, has been consumed as a "natural amphetamine." About 500 reports of adverse effects, including eight deaths, were received in less than two years by the state of Texas (31, 32). Natives of Curacao consume teas prepared from *Croton flavens* and suffer a high incidence of esophageal cancer (33). This plant contains a family of diterpene esters that increase the risk of malignancies when given with a chemical carcinogen (co-carcinogen) or after a carcinogen (tumor promoter). Their potency is comparable to the phorbol-12,13-diester, such as TPA, which are widely used in experimental carcinogenesis studies. This is the first example that an herbal tea containing co-carcinogens and tumor promoters likely represents the primary carcinogenic risk in epidemiologically identified human malignancy (33).

The examples provided here, as well as numerous others (see Refs. 16, 23, 24, 26) demonstrate that a long history of accepted use of herbal medicines cannot provide great confidence in their safety. In fact, it has been asserted that plants injure or kill more people than animals (12).

Estrogen toxicity is well-known to be associated with plant exposures; phytoestrogens induce infertility (34, 35) and developmental toxicity (36-38) in animals. However, we have little evidence of the adverse effects of herbal preparations that contain phytoestrogens, although attention to phytoestrogens in herbal medicines is increasing (39). Chaparral (*Larrea tridentata*), a desert plant found in the Southwestern United States and Mexico, has long been used as an infusion (tea) for a number of diseases. The high content of nordihydroguaiaretic acid in chaparral appears responsible for hepatitis in users of the tea (40, 41). It has also been used as a contraceptive preparation (42), consistent with experimental data showing estrogenic activity and

actions as a reproductive toxicant. It has been marketed as an herbal medication. Obermeyer *et al.*, (40) have shown that the phenolic content, which is 80%–90% of the dry weight, is more effectively extracted in methanol than water. The major phenolic chemicals extracted are flavonoid aglycones and glycosides (quercetin, kaempferol, and luteolin) and lignans (43). Because chaparral is marketed as capsules or tablets, the bioavailability of these estrogenic chemicals is expected to be higher than in teas (40). One of the chemicals that may be responsible for the reproductive toxicity (anti-implantation activity) is 3'-demethoxy-6-O-demethyl-isoguaiacin, which is estrogenic in rats (44). Given that one traditional use is as a contraceptive agent (consumed as a tea), increased phytoestrogen bioavailability from capsules or tablets may induce involuntary infertility in unsuspecting consumers. Other herbal products contain phytoestrogens that have been detected in bioassays using either extracts of the herbal medicines or saliva from individuals consuming them (39). An herbal medicine derived from *Vitex agras casta* may increase follicular phase estradiol concentrations and induce an ovarian hyperstimulation condition (45). The phytoestrogen content is unknown.

Despite the fact that numerous herbal medicines are traditionally recommended for various disorders and conditions of female reproduction and pregnancy, and that numerous plants contain estrogenic chemicals, no information unambiguously links the phytoestrogen content of herbal medicines to estrogenic effects in humans. Given the poor monitoring of exposure and effects in humans, it cannot be considered that such a relationship does not exist.

In addition to the high phytoestrogen content in soy products, which are estrogenic and developmentally toxic in animals (38), there are other well-described examples of phytoestrogen-containing plants inhibiting fertility *via* estrogenic activity. These include "sheep clover disease" due primarily to the phytoestrogen coumestrol (34, 46) and "moldy corn syndrome" in pig and cattle fed corn contaminated by *Fusarium sp.*, which produce the estrogenic β -resorcylic acid lactone, zearalenone (47). Both of these chemicals display typical estrogen effects during reproduction and development. Another example is inhibition of reproduction of California quail by phytoestrogens produced by plants growing in dry conditions (34).

These examples in animals suggest that the phytoestrogen content of herbal medicines and soy products may induce unintended adverse effects on reproduction and development in humans. Herbal product use is prevalent and perceived as safe. Some herbal medicines induce toxicity, and these outcomes are not usually detected by an organized and systematic monitoring of the exposed population. How can we apply these findings to a consideration of the health effects of soy products? First, soy product use is prevalent and perceived as safe; it demonstrates toxicity in livestock and experimental animals; and exposed populations are not systematically monitored for adverse effects. Based on this

comparison with herbal medicines, confidence that soy products are safe is clearly based more on belief than on hard data.

A general argument can be made that the long history of apparent safe use of soy argues that it is not toxic, similar to assertions made for herbal medicines. It is important to point out that almost all known human toxicities of herbal medicines are acute; toxicities with long latencies to appearance are infrequently described and are usually associated with long-standing use of a product. Adverse outcomes with long latencies following discontinuation of herbal medicine use have rarely been demonstrated. Does this mean that such toxicities do not exist or that our abilities to detect them are sharply limited? Without numerous well-designed studies, we simply cannot answer the first question, but there are clear examples that demonstrate the difficulty in associating long latency toxicities with a specific chemical exposure. Four such examples are provided.

Since its introduction to Europe 5 centuries ago, tobacco use has increased. However, heavy smoking was relatively infrequent soon after use in Europe began and was not suggested to be associated with lung cancer until 1761 (48). Not until the middle of this century were convincing studies presented linking tobacco use to malignancies, primarily lung cancer (48). To this day, most tobacco companies and some consumers deny the clear and compelling evidence that smoking causes lung cancer, which shows a latency from initiation of smoking to disease detection of several decades. Thus does belief trump data.

Likewise, the use of unopposed estrogen replacement therapy (i.e., lacking a cyclical progestin component) for menopausal symptoms is now well known to increase the risk of endometrial adenocarcinoma (49). The relative risk increases about one unit for every year of use (e.g., 5 years of exposure results in a 5-fold higher risk of disease occurrence). Prescription drugs were causative. Physicians, drug companies, consumers aware of possible drug toxicities, and the Food and Drug Administration were all involved in defining and advising against unopposed estrogen replacement therapy. Yet even under much more favorable conditions than for detection of adverse effects caused by tobacco, almost 3 decades elapsed before a high level of human exposure to unopposed estrogen therapy was unambiguously associated with this serious toxicity.

In both of these examples, most of the individual exposures were continuing at the time of detection of the malignancies. Thus while there was a long latency to disease appearance, exposure was generally concurrent with disease detection, allowing the association of cause and effect to be made more easily.

Two other examples suggest that when exposure is brief, a long latency to disease appearance may be an even more difficult obstacle to finding the causation. Diethylstilbestrol (DES) exposure of 3–5 million women occurred during pregnancy; a majority of female offspring and a

smaller portion of male offspring showed various developmental abnormalities and malignancies of the reproductive tract that were diagnosed some 12–25 years following the exposure (50). Treatment with this estrogenic drug continued for over 20 years before this association was made by alert physicians who saw, in a short space of time, a handful of young women with clear-cell carcinoma of the vagina or cervix (51). This malignancy is extremely rare, particularly in young females, and it was this unusual feature that led to the demonstration of DES causation. If this had been a more prevalent disease in young women, or if it had not occurred at all or more rarely following DES exposure, it is questionable that the much higher prevalence (50) of benign abnormalities of the female reproductive tract would have been associated with DES exposure. This is due to the fact that similar benign abnormalities occur at a lower prevalence in young women not exposed to DES, and thus an increased incidence might have gone undetected. Thus, simple luck and alertness appears to have played a large role in understanding the role of DES in inducing human malignancies and malformations.

Finally, a very informative example is provided by Fetal Alcohol Syndrome (FAS). FAS occurs in some infants of mothers who consumed alcohol during a critical stage of fetal development. It is characterized by a distinctly recognizable pattern of facial abnormalities and other significant problems, primarily but not exclusively, in the central nervous system (52). This syndrome and its association with maternal alcohol consumption was first described in the 1970s (53, 54). Yet alcohol consumption during pregnancy has certainly occurred over several thousands of years. There is no reason to assume FAS is a recent occurrence; it has been described in every species examined, including nonhuman primates, and in all ethnic groups examined (52). Humankind has looked closely at the faces of its newborn infants over the entire period that maternal alcohol consumption has occurred, yet FAS was only described from a cause and effect perspective three-quarters of the way through this century. The latency between exposure and detection is less than nine months, and the features of FAS are clearly visible at birth. This is an extraordinary demonstration of our inability to associate a clear externally displayed manifestation of toxicity with a well-defined exposure over thousands of years in an untold number of cases.

To my knowledge, there are no long-term studies in humans in which a possible association between soy exposure and toxicity has been systematically and rigorously explored. Given the prevalence of soy exposure and the possible health benefits, it is appropriate to include adverse effects in any future large-scale, long-term epidemiological studies. Because reproductive and developmental toxicity have been demonstrated in animals and humans with a wide variety of estrogens, and phytoestrogen exposure has been shown to induce reproductive and developmental toxicity in experimental animals and livestock, these endpoints should

receive particular attention. Given the parallels with herbal medicines with respect to attitudes, monitoring deficiencies, and the general difficulty of detecting toxicities with long latencies, I am unconvinced that the long history of apparent safe use of soy products can provide confidence that they are indeed without risk.

One use of soy, in infant formulas, results in a high phytoestrogen exposure during development (55). Consumption of phytoestrogen-containing soy products by women produces demonstrable estrogenic responses at phytoestrogen doses about 5-fold lower than in soy infant formula exposed infants (55–57). Unfortunately we know very little regarding the toxicity of estrogens generally in human infants. Premature breast development, (gynecomastia in males and premature thelarche in females), can be caused by infant exposure to oral contraceptives *via* mothers milk (58). Rhesus monkeys are used as an animal model for human reproduction and development. A low dose of DES (500 ng/Kg) administered daily to infant rhesus monkeys altered the normal postnatal gonadotrophin pattern (59). Such findings raise concerns for the potential adverse effects from an infant diet exclusively composed, in many cases, of only soy infant formula.

Additionally, goiter has been described in soy formula fed infants (60), although iodine supplementation of the formula was thought to reverse this problem (61). However, a recent study shows an increased risk of autoimmune thyroid disease in infants consuming soy formula (62). Some isoflavones found in soy formula inhibit thyroid peroxidase, the key enzyme in thyroid hormone synthesis. Inhibition can be reversible or irreversible depending on whether iodine is present (63). Inhibition of thyroid peroxidase would lower thyroid hormone (T3 and T4) serum levels and thus increase Thyroid Stimulating Hormone (TSH) levels in a homeostatic attempt to increase thyroid hormone production. The increased TSH also increases thyroid growth, potentially leading to goiter and malignancy (63). These findings, taken together, suggest that careful studies of the soy infant formula-exposed population should be undertaken, as it is a well-identified group, and phytoestrogen doses can be estimated with some accuracy. Such studies should include not only the infants currently consuming soy infant formulas, but older children, adolescents, and adults previously exposed. They should incorporate estrogenic and thyroid hormone related endpoints, as well as a wide variety of other endpoints of toxicity, as history has shown us that the specific type of toxicity encountered is not always obvious *a priori*. Additionally, given the potential health benefits of soy (1–4) and particularly the finding of inhibition of chemically-induced breast cancer by developmental treatment of rats with genestein (5), measures of possible beneficial effects should be included. Only by such studies can risk: benefit data be collected in order for health professionals to provide appropriate advice to the public. At present, we are conducting a . . . large, uncontrolled, and basically

unmonitored human infant experiment . . . with uncertain risks and benefits (64–65).

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Isoflavone Content of Breast Milk and Soy Formulas: Benefits and Risks

To the Editor:

A recent editorial [1] properly credited Franke and Custer [2] for an important study of urinary and breast milk concentrations of isoflavones in women consuming soybeans. This editorial cited literature concerning potential health benefits of phytoestrogens and, to a much lesser extent, potential toxicity. In the accompanying letter below, Franke points out that infants consuming soy-based formula are exposed to high concentrations of phytoestrogens.

Estrogens are two-edged swords in humans; both risks and benefits can be demonstrated in the same person. Two examples are oral contraceptives (benefit: fertility control; risk: increased incidence of breast cancer [3]) and unopposed estrogen replacement therapy (benefit: reduction in mortality due to heart disease and osteoporosis and relief of menopause symptoms; risk: increased incidence of endometrial cancer [4]). Given this characteristic of estrogens generally, what do we know of risks from phytoestrogens?

Adverse effects of phytoestrogens on reproduction and development in wildlife [5], livestock [6], and experimental animals [7] have been documented. Developmental exposure to phytoestrogens results in toxicities similar or identical to those of other estrogens. Neonatal rodents have long been used as a model of human prenatal diethylstilbestrol (DES) exposure on the basis of developmental staging and similar outcomes from exposure [8]. However, the neonatal rodent and postnatal human are not at equivalent morphological stages of development [9] and the neonatal rodent does not model the infant human. In addition to lacking a rodent estrogen model of the human infant, we also have little clinical experience with human infant exposure to estrogens generally. Although the data are limited for developmental effects of phytoestrogens, the similarity of DES and

phytoestrogen effects in newborn rodents should be considered a cautionary note for the developmentally later exposure that occurs with soy infant formula. As the editorial points out, the beneficial effects of soy-based formulas or of milk from mothers consuming phytoestrogens is speculative. The same is true for potential risks.

Phytoestrogen exposure is quite high; ~20% of American infants receive an isoflavonoid dose from soy formula (expressed as mg/kg) that is about five times higher than the dose that lengthened the follicular phase of the menstrual cycle and lowered lutropin and follitropin concentrations in adult women [10]. To my knowledge, only one study is underway in soy formula-exposed infants, despite our great uncertainty concerning benefits and risks of isoflavone exposure.

While metabolism and disposition data are important in both animals and humans, another crucial need is to define appropriate animal models and to explore phytoestrogen effects in these models; to characterize biological effects of phytoestrogens in infants, particularly those consuming soy-based infant formulas; and to be able to compare results across animals and humans. These studies need to define effects as either beneficial or adverse, and to explore a large variety of effects. They should also consider dose response, age at exposure, and length of exposure. Only after completion of such studies can we know the benefits and risks of infant phytoestrogen exposure and thus be able to provide the best advice to parents concerning infant exposures from breast milk and soy-based formulas.

In the meantime, this large, uncontrolled, and basically unmonitored human infant experiment continues unabated.

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Daniel M. Sheehan

National Center for Toxicol. Res.

USFDA

3900 NCTR Rd., HFT-130

Jefferson, AR 72079

To the Editor:

The editorial by Slavin [1] summarizes the effects of isoflavones and recent research on human isoflavone exposure, including our studies on breast milk concentrations after soy consumption [2]. Some brief comments may be helpful to add to the current knowledge in the area of isoflavonoid research.

Extensive analyses of isoflavone concentrations in legumes showed that exclusively soy foods contained considerable amounts of these agents, whereas other legumes such as lentils, beans, and chickpeas contained trace or nondetectable amounts [3]. A recent study confirmed these results by reporting isoflavone concentrations in lentils, beans, and chickpeas to be lower by a factor of 50 to 5000 relative to soybeans [4].

The higher urinary isoflavone recovery after consumption of fermented vs nonfermented soy foods [5] needs to be verified because the protocol applied resulted in differen-

Current issues regarding phytoestrogens

Daniel M. SHEEHAN and Kevin MEDLOCK

National Center for Toxicological Research, 3900 NCTR Road
Jefferson, AR 72079-9502, USA

Abstract: Estrogens produced by plants are found in diets of wildlife, domestic animals and humans. Both beneficial and adverse effects of phytoestrogens are known. To make good risk/benefit decisions concerning phytoestrogens much more information needs to be acquired. We have identified some major research needs to fill knowledge gaps.

Phytoestrogens are plant chemicals which possess estrogenic activity. Also frequently included in this group are the mycoestrogens produced by various species of fungi. The prototypical estrogen is 17β -estradiol (Fig.), which plays critical roles in the reproduction of vertebrates and is produced primarily by the ovaries, but also by the adrenal glands and adipose tissue.

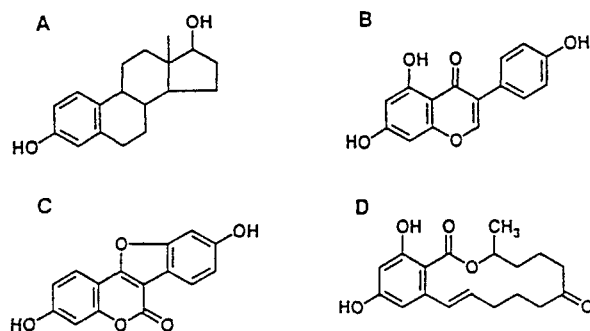


Figure. The structures of some estrogens. A, steroid (17β -estradiol) B, isoflavonoid (genistein) C, coumestan (coumestrol) D, β -resorcylic acid lactone

Numerous plant chemicals possess estrogenic activity and their chemical structures vary widely. While several hundred plants are now known to produce phytoestrogens (Farnsworth *et al.*, 1975), little data exist for many phytoestrogens beyond the characterization of their plant source, biological activity and chemical structure. The most well studied of the phytoestrogens are representatives of three chemical classes, the coumestans, the isoflavonoids and the β -resorcylic acid lactones (Fig.). The biological activity of estradiol is greatly dependent on the presence of hydroxyl groups, particularly on the A ring of the

steroid nucleus. Unlike 17β -estradiol, phytoestrogens are not steroids, but they possess a hydroxyl group (or can be hydroxylated) that can be aligned in a stereochemical position analogous to that of estradiol (Fig.).

The biological activity of phytoestrogens can be measured in several ways, the most common being the increase in the weight of the uterus (a classic estrogen target organ) of rodents. Using this measure, the potencies of phytoestrogens are generally several orders of magnitude lower than for estradiol, although potency of different phytoestrogens can vary widely. Some chemicals that are estrogenic also possess antiestrogenic characteristics. It is important to characterize individual phytoestrogens with respect to both estrogenic and antiestrogenic activities, as opposed to generalizing that all phytoestrogens have these properties.

Interference with the complex biological activity of endogenous estrogens can lead to infertility. Indeed, infertility in livestock was one clue that led ultimately to the discovery of phytoestrogens. Sheep feeding on Australian pastures containing subterranean clover (*Trifolium subterraneum* L.) failed to reproduce, and displayed abnormalities of the genital tract (Adams, 1995). The major phytoestrogens in clover are isoflavones (Fig.). Soybeans are also a rich source of phytoestrogens, the most important of which is genistein. Alfalfa is rich in coumestrol (Fig.). Mycoestrogens were discovered following the observation of infertility in swine herds; the cause was determined to be contamination of feed corn by *Fusarium* sp. which primarily produced zearalenone (Fig.) (Sheehan, *et al.* 1984).

A recent resurgence of interest in phytoestrogens has been driven by a number of different considerations including the potential for both

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beneficial and adverse effects. The new interest is reflected in the number of meetings dealing with phytoestrogens. The National Center for Toxicological Research (NCTR) sponsored the first two meetings devoted entirely to phytoestrogens (see *Proc. Soc. Exp. Biol. Med.* 208, 1-138 for the presentations from the Second International Conference on Phytoestrogens). We will hold another meeting in December, 1995. The National Cancer Institute subsequently held a meeting on this topic. Phytoestrogens were also discussed at a recent soybean conference, at a meeting sponsored by the NIEHS on Estrogens in the Environment and at a US EPA workshop on Endocrine Disruptors.

Phytoestrogens have been increasingly postulated to be anticarcinogenic or antitumor agents. One report in the literature suggested that ingestion of soy products and lignans lowered the incidence of breast cancer in Japanese and Finnish women, respectively (Adlercreutz, 1990). Soybeans have also been implicated in the hormonal regulation of normal as well as neoplastic prostatic growth (Makela *et al.*, 1995). Currently, the phytoestrogen receiving the most research attention appears to be genistein. In cell culture and in rodent tumorigenesis models, genistein has been shown to prolong latency to tumor appearance and to decrease tumor multiplicity (Wei *et al.*, 1995; Lamartiniere *et al.*, 1995). Recently, there has been a marked increase in research dealing with the mechanisms of action by which genistein and other phytoestrogens elicit their effects. Although the term phytoestrogen implies that these compounds work through the estrogen receptor (ER), in studies with T47D cells with and without expressed ER, genistein inhibited the growth of both cell lines equally well perhaps via signal transduction pathways (Barnes and Peterson, 1995). This suggestion is supported by the findings that genistein inhibits both topoisomerase II and tyrosine kinases. Such activities may be the mechanism(s) by which it inhibits cell multiplication while inducing cell differentiation (Constantinou and Huberman, 1995). The impairment of the signal transduction pathway which appears to inhibit the growth of cancer cell lines is thought to block critical points of cell cycle control leading to apoptosis (Pagliacci *et al.*, 1994). Not all of the effects of phytoestrogens may be beneficial. Most of the detrimental effects described involve the developing organism, particularly in the reproductive tract and the neuroendocrine system. In mice, very low doses of coumestrol caused premature vaginal opening while higher doses elicited persistent vaginal cornification (PVC) (Burroughs, 1995). In rats, a 0.01% dose of coumestrol in the diet of nursing dams had no

effect on vaginal opening but did result in PVC (Whitten *et al.*, 1995). All of the adverse effects listed above are characteristic of estrogens in general. Gestational exposure of rats to high levels of genistein appears to delay vaginal opening but subsequently does not disrupt estrous cyclicity (Levy *et al.*, 1995). Early neonatal exposure of rats to coumestrol caused a reduction in the ER level and uterine weight along with inhibition of uterine gland genesis (Medlock *et al.*, 1995).

There are a number of major challenges in this area that must be addressed. The best analytical methods currently depend on gas chromatography/mass spectrometry techniques, which while accurate, are slow and expensive (Adlercreutz *et al.*, 1986). We need rapid, inexpensive, sensitive and accurate methods for detection and quantitation of specific phytoestrogens. Current techniques greatly limit the analysis of phytoestrogens in plants, in plant-derived foods and in serum and tissues of experimental animals, livestock, wildlife and humans. The health effects of phytoestrogens should be studied in experimental animals and humans, with particular attention paid to reproductive, developmental and anticarcinogenic/carcinogenic outcomes. Also important is distinguishing those effects which are mediated via the ER (*i.e.*, estrogenic or antiestrogenic activities) versus those mediated by non-ER mechanisms (*i.e.*, tyrosine kinase or topoisomerase inhibition).

We must recognize that estrogens of all sorts are two-edged swords; the same estrogen that is beneficial in one circumstance or organ may be a hazard in another. Estrogen replacement therapy protects against osteoporosis, hot flashes and other menopausal symptoms, but increases the incidence of endometrial adenocarcinoma (Grady *et al.*, 1995). Oral contraceptives have introduced a revolution in family planning and population control, yet continued use during pregnancy may increase the risk for birth defects (Li *et al.*, 1995). We should expect that phytoestrogens, either individually or as mixtures (as found in animal and human diets), will also demonstrate both benefits and risks as a function of the type of phytoestrogen(s), target organs, species-specificity, dose-dependence, developmental-stage specificity and mechanism. When we obtain such information, we will be able to make more informed risk/benefit decisions concerning exposure to phytoestrogens in animals and humans.

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The role of polyphenols as chemopreventers

Bozidar STAVRIC

Food Research Division, Food Directorate, Health Canada, Tunney's Pasture, Banting Bldg
Postal Locator No. 2203D, Ottawa, On. K1A 0L2, Canada

Abstract: Certain polyphenols from vegetables, fruit and tea, described either as functional foods, nutraceuticals, chemopreventers or phytochemicals, have recently received considerable attention because of their apparent beneficial effect for human health. Although the precise mechanism of action in preventing chronic diseases is still unclear, they exhibit various modes of action, both extra- or intra-cellularly. More research is required to establish their value in health protection.

It has been well established that dietary factors play a major role in the development of human chronic diseases, such as cardiovascular disorders and cancer (Diet Nutrition and Cancer, 1982; Hertog *et al.*, 1993). Human diet, in addition to the essential nutrients, contains a number of natural non-nutritional components, some of which may provide protection against these chronic diseases (Wattenberg, 1992; Stavric, 1994). These components, frequently referred to as *chemopreventers*, (and also as *phytochemicals*, or *nutraceuticals*) can be found in all types of food, with polyphenols from vegetables, fruits and tea providing the most apparent beneficial effects (Wattenberg, 1992; Graham, 1992).

The precise mechanism of action for the beneficial effects of polyphenols is still unclear. It seems that individual polyphenols in different situations may exhibit various modes of action (Stavric and Matula, 1992). Generally, most chemopreventers act either extra- or intra-cellularly (Stavric *et al.*, 1992), and the antioxidant properties of polyphenols (*e.g.* protecting against the degradation of vitamins, scavenging free radicals), appear to play the most significant role in their overall beneficial effect (Tanaka, 1994).

Animal experiments have shown that polyphenols acting extra-cellularly could hinder the uptake of xenobiotics from the gastrointestinal tract,

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SOYBEAN GOITER*

Report of Three Cases

THOMAS H. SHEPARD, M.D.,† GORDON E. PYNE, M.D.,‡ JOSEPH F. KIRSCHVINK, M.D.,‡ AND
CAPTAIN MALCOM McLEAN, M.C., USAR§

SEATTLE, BUCKLEY AND TACOMA, WASHINGTON

ALTHOUGH goiters associated with soybean feeding have been produced experimentally in laboratory animals¹⁻³ we have been able to find only 2 similar reports in human beings.^{4,7} In view of the widespread use of soybean milks in infancy, it is pertinent to record the appearance of goiters in 3 additional infants fed soybean formulas.

CASE REPORTS

CASE 1|| A goiter developed when this female infant was placed on a soybean formula at 3 months of age. At 11½ months when cow's milk was substituted, the goiter subsided. She was admitted to Children's Orthopedic Hospital at 10 months of age because of a mass in the neck.

The pregnancy and delivery were uneventful, and she was well until 2 months of age, when there was the onset of moderately severe eczema. At 3 months of age she was placed on a soybean formula|| and rice cereal. She was placed in a foster home 1 month later, when a slight swelling was noted in the neck by the foster mother. No additional solids were added to the diet.

There was no family history of thyroid disease. The mother had not ingested goitrogenic drugs or foods during the pregnancy. There was no consanguinity.

At 10 months of age, because of continued and increasing thyroid enlargement, the isthmus of the gland was biopsied, at operation the gland was found to be large and firm. The microscopical picture was that of hyperplastic thyroid tissue (Fig. 1 and 2). The specimen was composed of pale-staining, tall columnar cells in folds. Minimal numbers of acini and only small amounts of colloid were present.

The patient was discharged from the hospital on a soybean formula and was seen in the Endocrine Clinic at Children's Orthopedic Hospital at 11 months of age. At this time she was active and healthy appearing, weighing 8.1 kg and being 72 cm. tall. Further measurements are recorded in Table 1. The flush blood pressure was 95.

From the Department of Pediatrics, University of Washington School of Medicine and the Children's Orthopedic Hospital, Seattle, Ramer State Training School, Buckley and Madigan Army Hospital, Tacoma, Washington.

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†Assistant professor of pediatrics, University of Washington School of Medicine, director, Endocrine Clinic, Children's Orthopedic Hospital.

‡Clinical instructor, Department of Pediatrics, University of Washington School of Medicine.

§Pediatric assistant resident, Madigan Army Hospital.

||Revised by Dr. Samuel Tarica.

In the form of Mull-Soy, Borden Company, Pharmaceutical Division, New York City.

There was diffuse enlargement of the thyroid gland, the right lobe measuring 5.5 cm. and the left 4.5 cm. in length (Fig. 3). A bruit was not present. The eyes were normal, and there was no exophthalmos or lid lag. The skin was warm and of normal texture. The remainder of the physical examination was within normal limits.

At 10 months of age, the hemoglobin was 10.8 gm. per 100 ml., and urinalysis was negative; the serum cholesterol was 100 mg., and the serum protein-bound iodine 3.4 microgm per 100 ml. X-ray study of the hand and foot at 12 months of age revealed calcification in the epiphyses of the hamate, capitate, 1st cuneiform and cuboid bones.

At 11 months of age the alkaline phosphatase was 12 Bodansky units, and the serum cholesterol 94 mg., and the protein-bound iodine 4.9 microgm. per 100 ml. ¹³¹I uptake was 88 per cent in 24 hours.

After preliminary laboratory tests the soybean-milk formula was discontinued at 11½ months of age, and at 15 months of age the thyroid gland had become barely palpable (Fig. 4). The ¹³¹I uptake had dropped to 38 per cent in 24 hours, and growth and development were progressing normally. The thyroid gland was not detectably enlarged at 17 months of age.

CASE 2. In this female infant a goiter developed after she had been placed on a soybean formula at birth. The goiter diminished after the patient was started on cow's milk at 3 months of age. She was admitted to Madigan Army Hospital at 3 months of age because of a swelling in the neck. Pregnancy and delivery had been normal, and she had appeared healthy at birth, when she was placed on the same soybean formula as Case 1 because of a strong family history of allergy. The formula was continued without addition of solid foods until admission. She had done well except for excessive mucus in the nasopharynx.

There was no family history of goiter, and the mother had not received goitrogenic substances during pregnancy.

Physical examination revealed a healthy-appearing infant except for mucopurulent material in the nares. She weighed 5.2 kg. and was 61 cm. tall. The pulse was 140; the blood pressure was not recorded. The eyes were normal. The skin was of normal texture and warmth. A symmetrically enlarged thyroid gland without nodules was palpable. The remainder of the examination was within normal limits.

The hemoglobin was 10.8 gm. per 100 ml. Urinalysis was negative. The serum cholesterol was 115 mg., and the protein-bound iodine 3.4 microgm. per 100 ml. ¹³¹I uptake was 86 per cent in 4 and 42 per cent in 24 hours.

The patient was discharged from the hospital on a diet of cow's milk, fruit, vegetables and meats, and 1½ months later the thyroid gland was found to be normal in size. A repeat determination of the ¹³¹I uptake at this time revealed a 4-hour value of 29.5 per cent and a 24-hour value of 45 per cent.

CASE 3. This mentally retarded girl was placed on a soybean formula at 3 months of age because of eczema.

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Thyroid enlargement was first noted after 3 years and 3 months of a soybean formula. Fifteen days after the addition of iodine to the diet the goiter could no longer be palpated.

The patient was admitted to the Rainier State Training School for mentally retarded children at the age of 2 and 9 months with problems including mental and growth retardation, congenital laryngeal stridor requiring tracheostomy, multiple hemivertebras, eczema and febrile convulsions.



FIGURE 1. *Histologic Appearance of the Thyroid Gland in Case 1, Showing Increased Cellularity and Absence of Colloid.*

Gestation and birth were considered normal. She weighed 3.1 kg. and was 45 cm. long at birth. She was first admitted to the hospital at 3 months of age because of respiratory stridor and eczema, and at that time was placed on the soybean formula. After this, she was given solid foods in restricted amounts, but their exact nature was not recorded.

She was subsequently admitted many times for respiratory infections and eczema. Physical examination at 2 years and 9 months of age revealed a small, well proportioned child without speech development who weighed 13 kg. and was 86.3 cm. tall. The blood pressure was 108/65, the pulse 88, and the respirations 20.

Skin changes compatible with eczema behind the ears, over the face and in the popliteal and antecubital spaces were present. The head circumference was 44 cm., and the supraorbital areas were puffy because of the eczema. Twenty teeth were present. The thyroid gland was enlarged, the right lobe measuring 3 cm., and the left lobe 2 centimeters in length. A tracheostomy tube was in place. The heart and lungs were normal. There was a small umbilical hernia, with a 3-cm. muscular defect.

Neurologic examination was negative. The extremities were warm. Laboratory studies after admission revealed normal blood counts and a negative urinalysis. The serum cholesterol was 121 mg., and the protein-bound iodine 3.7 microgm. per 100 ml. I^{131} uptake at 24 hours was 75 per cent. The bone age was 3 years.

At the age of 3 years and 7 months 500 microgm. of iodide in the form of Lugol's solution was added to the

daily diet. Fifteen days after the start of iodine the goiter could no longer be palpated. After 8 weeks of iodide administration the I^{131} uptake was 36.5 per cent after 24 hours. During this time the patient was kept on soybean milk; however, the brand was changed inadvertently to another preparation.* (Table 1 summarizes the laboratory and clinical data.)

DISCUSSION

Clinical Features and Pathological Findings

The cases of soybean goiters in human beings reported above are similar to the 2 previously reported. The first involved a one-year-old girl who had been on soybean milk since birth.⁶ The goiter caused respiratory obstruction, necessitating a tracheostomy. After the soybean milk was discontinued, the goiter



FIGURE 2. *Lower-Power Magnification of Tissue Shown in Figure 1.*

A few small follicles can be seen.

disappeared. Although detailed clinical studies were not reported by Rawson and Rall⁶ the biopsy deserves further comment (as discussed below).

The second was a ten-month-old girl who had been on soybean milk since birth and in whom a goiter with clinical evidence of hypothyroidism developed.⁷ Van Wyk et al.⁷ studied her by means of I^{131} -uptake kinetics both while she was continuing on soybean milk and after she had changed to cow's milk. The conversion of I^{131} to protein-bound I^{131} was found to be inhibited on the soybean formula.

*In the form of Soyloc, Loma Linda Foods, Loma Linda, California.

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In the cases presented above, the exact elapsed time after the patient was placed on the soybean milk before the appearance of the goiter is unknown. In Case 1 the goiter was detected by the

Values for serum protein-bound iodine (Table 1) were slightly low (Normal mean and 1 S.D. = 6.3 ± 1.0^8). The serum cholesterol was normal in all these patients.

TABLE 1 Clinical Features

CASE NO.	AGE AT RECOGNITION OF THYROID ENLARGEMENT	WEIGHT	HEIGHT	BONE AGE	PROTEIN-BOUND IODINE	CHOLESTEROL	I^{131} UPTAKE ON SOYBEAN	I^{131} UPTAKE ON COW'S MILK
	mo.	kg.	cm.	yr.	microgm./100 ml.	mg./100 ml.	% in 24 hr.	% in 24 hr.
1	10	8.1	72.0	--	4.9	94	88	38
2	3	5.3	57.3	--	3.4	115	42	45
3	39	13.3	86.3	3	3.7	121	75	37

mother after one month. In Case 2 the physician noted the goiter after three months. In Case 3 the thyroid enlargement was not noted until after thirty-

The patterns of I^{131} uptake were of considerable interest. In Cases 1 and 3 there was a marked increase over normal in twenty-four hours while the patients were on soybean milk. In Case 2, also while she was on soybean milk, there was a marked in-



FIGURE 3. Thyroid Enlargement in Case 1 at the Age of Seven Months, while the Patient Was on Soybean Milk.

months. These goiters may have been present for a variable time before detection. This applies particularly to Case 3 because the thyroid area was distorted by scarring from several tracheostomies and by the presence of a tracheostomy tube.

The 3 patients described above were euthyroid as judged by physical findings and normal growth



FIGURE 4. Reduction in Size of the Thyroid Gland in Case 1 at the Age of Fifteen Months, Three Months after the Soybean Milk Was Discontinued.

crease in the I^{131} uptake at four hours, with a return to a high-normal value at twenty-four hours. The first 2 patients had normal I^{131} uptakes after soybean milk was discontinued. Case 3 was kept on

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the soybean formula while iodine was added to the diet. The I^{131} uptake also returned to normal in this patient. Conversion ratios to serum protein-bound iodine were not measured.

In the first 2 patients the goiters greatly diminished in size when soybean was discontinued, as did the goiter in Case 3 when iodide was added to the diet. Unfortunately, this protective influence of iodide is not strictly comparable to animal studies because the brand of soybean milk was changed at the same time the iodide was started.

The histologic picture of the thyroid gland in Case 1 (Fig. 1) is identical to that shown by Rawson and Rall.⁶ Both glands were hyperplastic and devoid of colloid and showed virtually no acinar structures. A similar picture in the thyroid glands of soybean-fed rats has been described.^{2,3,5} These changes are similar to the hypertrophy and hyperplasia found in thyroid glands early in iodine deficiency.⁹

Since soybean diets are commonly employed in infancy for allergic states it is surprising that soybean goiters have not been more frequently encountered. This may be related to the relative difficulty in detecting thyroid enlargement in the infant because of the shortness of the neck. The brief duration of employment of soybean milk may also be a factor in this regard. However, in Case 1 the goiter was noted after only one month of soybean milk.

All these cases occurred during the first six months of 1959, and since then, no more have been reported to us.

Pathogenesis

Soybean goiter²⁻⁵ appears to differ from the thyroid enlargement caused by thiourealike compounds, which interfere with the incorporation of iodide and the enzymatic synthesis of thyroxine,¹⁰⁻¹³ and that caused by the thiocyanate-type compounds, which prevent normal iodide trapping in the gland.⁹ A specific goitrogen has not been isolated from soybean.³ The following four points offer some evidence that soybean goiters are due to iodide deficiency:

Iodide content of soybean milks. Relatively small amounts of iodide will prevent soya goiter production in animals.^{2,3} Thiourea goiters do not respond to the administration of even large amounts of iodide,¹¹ but thiocyanate goiters do respond. Another widely used commercial source of soybean milk,* which has 18.7 microgm. of iodide added to each can,¹⁴ has not been associated with goiter production. The recommended minimal daily requirement for iodide during infancy is 2 to 4 microgm. per kilogram of body weight.¹⁵ The commercial product given in the 3 cases reported above, as in the case presented by Van

Wyk et al.,⁷ has not in the past contained any added iodide.†

Thyroidal avidity for I^{131} . Another observation suggesting iodide lack is the thyroidal avidity for I^{131} that has been demonstrated in animals fed soybean⁴ and in 2 of the patients described above. This is similar to the uptakes observed by Stanbury¹⁷ and Roche et al.¹⁸ in persons living in iodine-deficient areas, and is unlike the decreased uptakes usually observed when thiourea and cyanatelike compounds are administered.

The histologic picture. The histologic picture in Case 1 resembles the microfollicular colloid-deficient picture reported in newborn infants in iodine-deficient areas.¹⁹

Stool loss of iodide. Another point that supports the theory advanced above and offers a hypothesis to explain the iodide lack is experimental work carried out by Van Middlesworth.⁴ His study suggests that certain changes in content of the gastrointestinal tract cause abnormal losses of iodide. By injecting soya-fed rats intraperitoneally with I^{131} -labeled thyroxine, he demonstrated excessive losses of I^{131} in the stool. He produced the same loss by feeding rats high amounts of cellulose. The conclusion suggested was that the thyroxine excreted through the biliary system into the bowel could not be normally reabsorbed owing to the soybean diet. Although this type of interhepatic circulation of thyroxine is known to be of little importance in man,²⁰ pertinent measurements have not been made in infants. On this point Triantaphyllidis²¹ has offered evidence that the amount of stool thyroxine loss in various animals is proportional to the grams of stool per kilogram of body weight per day. It is also conceivable that infants in whom soybean goiters develop have a defect in the mechanism that conserves iodine or thyroxine.

Several observations may not fit with the iodine-deficient pathogenesis proposed above. Both Wilgus et al.³ and Sharpless, Pearsons and Prato² have shown that heating of soya diets decreases their goitrogenicity. This could be due to resulting changes in the property of the soya protein rather than to destruction of a specific goitrogen. Van Wyk and his associates⁷ have demonstrated that the feeding of soybean to volunteers will in a small percentage of cases (2 out of 14) reduce the conversion of administered I^{131} to protein-bound iodine. The presence of reduced amounts of protein-bound I^{131} in the serum does not necessarily indicate reduced production; it is equally possible that an increased rate of loss accounts for the low value.

Van Wyk et al.⁷ have also shown a decreased uptake of intravenously administered I^{131} and an absence of I^{131} conversion to protein-bound I^{131}

*In the form of Sobee, Mead Johnson and Company, Evansville, Indiana.

†This product has recently been fortified with potassium iodide so that the final iodide content is 300 microgm. per reconstituted quart.¹⁴

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when their patient was briefly put back on soybean milk. This observation of a decreased uptake is difficult to explain on the basis of an iodide lack, and this discrepancy from the observed uptakes in the patients described above may suggest a different mode of goitrogenesis.

Differences in calcium content and in methods of commercial preparation of soybean milk may also be of importance in their goitrogenicity but are not considered within the scope of this communication.

SUMMARY AND CONCLUSIONS

Three children in whom goiters developed on soybean milk are described. Although they were clinically euthyroid their protein-bound iodines were of v-normal levels. The I^{131} uptakes were high, and came normal when the soybean milk was discontinued. The goiters became much smaller or disappeared in 2 cases when the soybean milk was discontinued and in the other when iodine was added to the diet.

The pathogenesis is discussed. The major amount evidence seems to support an iodide-lack type of ter.

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EFFECT OF HEPARIN ON THE BLOOD AMMONIA DETERMINATION*

HAROLD O. CONN, M.D.†

NEW HAVEN, CONNECTICUT

To present the blood ammonia determination is widely used in clinical medicine and research. Various authors have reported enormous differences in normal range of blood ammonia concentrations in both the Conway¹ and the Seligson² method. Values for blood ammonia nitrogen in normal subjects with the Conway technic have ranged from 5³ to 20⁴ microgm. per 100 ml.⁴ Similarly, with Seligson method, wide variations have been reported,^{2,5-10} normal limits ranging from 30 to 90⁷ to 200 microgm. per 100 ml.^{6,†}

From the Medical Service, Veterans Administration Hospital, West Haven, and the Department of Internal Medicine, Yale University School of Medicine.

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Assistant professor of medicine, Department of Internal Medicine, University School of Medicine; clinical investigator, Veterans Administration Hospital, West Haven.

Should be emphasized that two "Seligson" methods are currently for the determination of blood ammonia. One of them, the method of Seligson and Seligson,¹¹ is a general microdiffusion technic for the determination of nitrogen liberated as ammonia that was designed for measurement of ammonia in liquids low in protein content. This procedure, which has frequently been employed for the measurement of ammonia in blood,⁷⁻¹⁰ is not ideally suited for this purpose.² The second method, that of Seligson and Hirahara, was designed specifically for analysis of ammonia in blood, and its advantages have previously been discussed.

The determination of blood ammonia is affected by a number of factors, including temperature,^{1,3} the concentration of carbon dioxide,^{1,12} the degree of alkalization,^{1,2,13} the interval between the shedding of blood and its analysis^{1,14} and the duration of diffusion.^{1,13} Undoubtedly, modifications of the original methods account for some of these variations in results.^{4,15,16}

Another major source of error that has not been emphasized is the effect of various heparin preparations on the blood ammonia determination. At present most investigators who use the Seligson method, and some of those who use the Conway technic,¹⁵⁻¹⁷ employ heparin as an anticoagulant. Several have mentioned that heparin may liberate small amounts of ammonia.^{3,14} Gross variations in the ammonia content of different commercial brands of heparin have been reported to interfere with the accurate estimation of the blood ammonia concentration.¹⁷

Our own observations have suggested that different commercial preparations of heparin and different

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THE EFFECTS OF A SOYBEAN PRODUCT ON THYROID FUNCTION IN HUMANS

Judson J. Van Wyk, M.D., Mary B. Arnold, M.D., James Wynn, M.D., and Francis Pepper, M.D.

Departments of Pediatrics and Radiology, University of North Carolina School of Medicine, and Departments of Medicine, Veterans Administration Hospital, and Duke Medical Center, Durham, North Carolina

OVER 25 years ago Sir Robert McCarrison¹ demonstrated marked thyroid enlargement in rats fed a diet of raw soybeans. The goitrogenic property of soybeans has been confirmed by other investigators²⁻⁴ in the rat and in other species. Rats are protected from this effect if the diet is supplemented with iodine in amounts which are greater than the normal daily requirement.⁴ This goitrogenic effect of soybean flour in rats can be diminished by extracting it with organic solvents or by heating.^{4,5} Although these observations suggest that there is a specific goitrogenic agent in soybeans, such a substance has not been isolated nor has the mechanism of goitrogenesis been defined.

Soybeans constitute a major source of nutrition for certain segments of the world's population and in recent years soybean products have been widely employed as a substitute for cow's milk in the feeding of infants with an allergic background. Except for a single case in an infant reported by Rawson,⁶ a causative role of soybeans in the production of goiter in humans has not been demonstrated.

We had the opportunity to study a 10-month-old infant who developed a goiter and hypothyroidism while receiving a soybean product (Mull-Soy®). These studies were later extended to healthy, euthyroid, adult volunteers in an effort to define further the effects of this soybean product on human thyroid function. The results of these studies are presented.

CASE REPORT

A 10-month-old white female (NCMH 07-14-74) was referred to the Pediatric Service of the North Carolina Memorial Hospital in June, 1958, with the diagnosis of cretinism. She was the fourth child born to highly intelligent parents and was considered normal at birth. Her birth weight was 2,950 gm. One paternal aunt had had surgery for goiter at the age of 48 years. Other siblings did not have a goiter and no other history of thyroid disturbance on either side of the family could be elicited. The patient's mother and siblings had a strong allergic history and in accordance with a current pediatric practice,⁷ this infant was fed a soybean product (Mull-Soy®) beginning shortly after birth.

Solid foods were added to the diet at the age of 3½ months. Mull-Soy® was continued until 10 months of age, 2 weeks prior to hospital admission. At that time whole cow's milk was started.

The local pediatrician observed the child at frequent intervals and reported normal growth and development until the age of 8 months when he first noted a thick tongue. In the next 2 months, the child's voice became hoarse and the tongue progressively thicker. Two determinations of serum protein bound iodine were reported as 2.9 and 2.7 µg/100 ml. respectively.

Physical examination revealed an alert, well-nourished female infant with puffy face, marked pallor, peripheral mottling and a carotenoid complexion (Fig. 1). Her height was 68.6 cm (height-age, 8 months) and her weight was 7.96 kg (weight-age, 8 months). The pulse was 120/min. Her tongue was enlarged and

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Dr. Van Wyk was a John and Mary R. Markle Scholar in Medical Science.

Dr. Wynn was a Clinical Investigator, Veterans Administration.

ADDRESS (J.J.VanW.): University of North Carolina School of Medicine, Chapel Hill, North Carolina.
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protruding from the mouth. An unmistakable goiter was present by inspection, and by palpation the thyroid was soft and diffusely enlarged to approximately three times the normal size. No nodules, irregularities, or areas of tenderness were present. The heart was not enlarged by percussion. Deep-tendon reflexes were active and bilaterally symmetrical with a quick relaxation phase. The remainder of the physical examination was unremarkable.

Laboratory studies revealed the hemoglobin to be 11.2 gm/100 ml, leukocyte count, 400 mm³ with a normal differential count;

appeared, and the serum protein bound iodine was 7.2 µg/100 ml.

SPECIAL STUDIES

Procedures

Carrier-free sodium iodide¹³¹ was administered intravenously to the infant and to 12 of 14 adult subjects. Serial measurements of thyroidal iodine uptake were made with a scintillation counter using formula #2 as described by the Medical Division of the Oak Ridge Institute of Nuclear Studies.⁸ Where indicated, residual radioactivity in the thyroid



FIG. 1. Patient at 10 months of age

adequate platelets. Urinalysis disclosed no abnormalities. A roentgenogram of the chest revealed a cardiac shadow which was normal in size and configuration. Skeletal roentgenograms revealed a bone age of 7 months with evidence of epiphyseal dysgenesis.

Although unequivocal signs of hypothyroidism were present, the child was alert, responsive, and did not appear to be mentally retarded. She could pull to a standing position and responded appropriately. The unusual association of goiter and hypothyroidism in a previously healthy infant stimulated us to undertake studies of the effect of this soybean product on the infant's thyroid function.

At the conclusion of these studies the infant was treated with Lugol's solution, 1 drop for 4 days, and the soybean product was replaced by whole cow's milk. With no further therapy the deficits in height and bone age were progressively restored (Fig. 2).

In May 1959, at the age of 22 months, she was euthyroid, the goiter had completely dis-

appeared, and the thyroid gland was determined prior to the administration of I¹³¹.

In two of the adults I¹³¹ was given orally after the following preparation: 75 µc of I¹³¹ and 465 ml of the liquid diet were equilibrated for 24 hours by constant stirring at 5°C. All of this mixture was then administered over a 15-minute period.

Blood was collected in heparinized syringes. Plasma was separated and frozen immediately for further studies.

Methods

DETERMINATIONS OF TOTAL I¹³¹ IN PLASMA:

Radioactivity of 3-ml aliquots of plasma was counted in a scintillation well at the 360 kev peak of I¹³¹. Results were expressed in counts/min/ml. The sensitivity of the well counter was determined daily and it averaged 390,000 counts/min/µc. The background averaged 30 counts/min. A total of 640 counts were determined on each sample.

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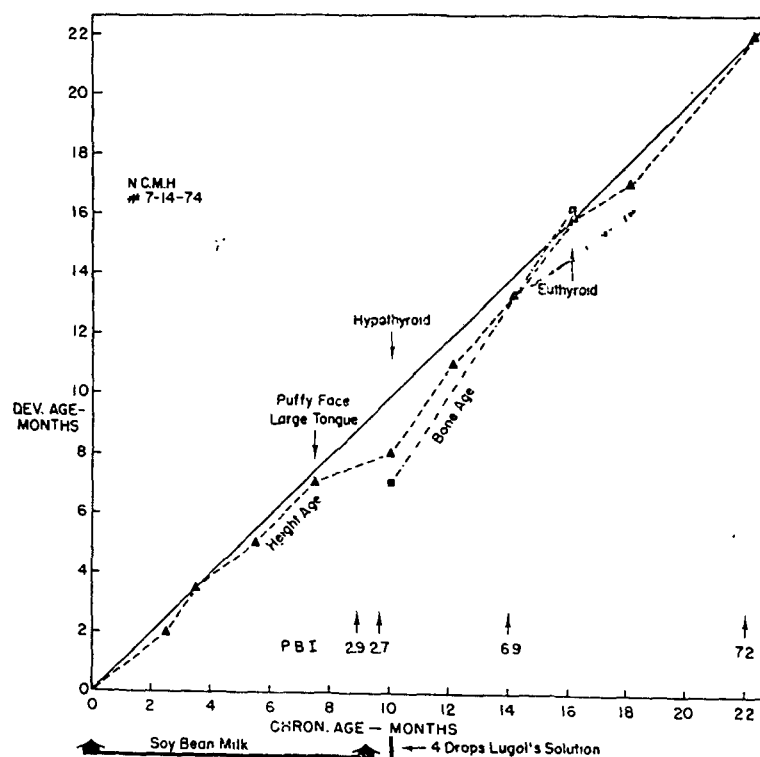


FIG. 2. Effect of diet and therapy on growth.

DETERMINATION OF PLASMA PROTEIN BOUND IODINE¹³¹ (PBI¹³¹): Aliquots of 3 ml of plasma were used. Protein was precipitated in 10% (w/v) trichloroacetic acid. The precipitate was washed three times with 7 ml of the 10% trichloroacetic acid and then dissolved in 2 ml of 5 normal sodium hydroxide. Precipitated I¹³¹ was counted as described in the determination of total I¹³¹. All counts were corrected for physical decay to the time of injection. When indicated, counts of residual PBI¹³¹ were determined prior to the injection of I¹³¹. The results were expressed in counts/min/ml. The studies on the infant were also expressed as percent of the injected dose per liter of plasma.

DETERMINATION OF PLASMA PROTEIN BOUND IODINE¹²⁷: The plasma protein bound iodine¹²⁷ was determined by the Moran modification of the Chaney method.⁹

CHROMATOGRAPHY OF IODINATED FRACTIONS: The iodinated amino acids in the plasma of the hypothyroid infant were extracted, separated and characterized by methods previously described.¹⁰ The I¹³¹ amino acids in plasma were adsorbed on Dowex #1 and eluted with a gradient concentration of formic acid (5 to

88% (w/v) in water). Radioactive fractions were characterized by their position on elution from resin and by comparison with the positions of I¹²⁷ standard iodoamino acids eluted simultaneously and identified by paper chromatography.

STUDIES OF HYPOTHYROID INFANT

Four studies were designed to determine the effect of the soybean product (Mull-Soy®) on thyroidal uptake and discharge of I¹³¹.

STUDY 1: This study was performed after the patient had been receiving a normal diet for 2 weeks. During this study she received only cow's milk at 4-hourly intervals. 17.5 µc of I¹³¹ was administered and thyroid uptake was measured at 1, 3, 6, 8 and 24 hours. Eighteen hours after the administration of the I¹³¹, 1 gm of potassium perchlorate was given by gastric tube and the thyroid uptake was thereafter measured at 15-minute intervals for 90 minutes. The blood sample for the determination of total and protein bound I¹³¹ was obtained 96 hours after the I¹³¹ was administered.

STUDY 2: Twenty-four hours after the administration of I¹³¹ in Study 1, feedings of

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Mull-Soy® iso-caloric with whole cow's milk were started; 72 hours later the patient was given 25 μC of I^{131} . Thyroid uptake was determined as in Study 1. No potassium perchlorate was administered during this study. Blood samples were taken at 6 and 24 hours. Feedings of the soybean product were continued throughout the 24-hour period of this study.

STUDY 3: Feedings of whole cow's milk were started at the completion of Study 2; 48 hours later, 12.5 μC of I^{131} were administered. Thyroid uptake and blood studies were obtained at the same time intervals as in Study 2.

STUDY 4: This study was carried out at the age of 14 months when the child was euthyroid and the goiter had disappeared. The child received only whole cow's milk during this study; 10 μC of I^{131} were administered.

RESULTS OF STUDY OF HYPO- THYROID INFANT

Thyroidal Uptake of I^{131}

The determination of thyroid uptake in Study 1 was abnormal in that there was a rapid uptake during the first few hours fol-

lowed by a rapid decrease (Fig. 3). The administration of potassium perchlorate caused only a small discharge of I^{131} from the gland. This discharge of I^{131} is similar to that described by Roche *et al.*¹¹ in patients with iodine deficiency.

The discharge of I^{131} from the thyroid during Study 2 was rapid and similar to that of Study 1, although a smaller percentage of the administered dose was accumulated by the thyroid. The uptake in Study 3 was almost identical to that obtained during Study 1. Four months later the uptake curve was entirely normal.

Plasma PBI^{131}

Plasma PBI^{131} studies were more striking than the thyroid uptake studies (Table I). Ninety-six hours after the first dose of I^{131} the plasma PBI^{131} was abnormally high, as judged by the normal values for infants given by Oliner *et al.*¹² After the second dose of I^{131} no additional PBI^{131} appeared in the blood. After the third dose of I^{131} a large in-

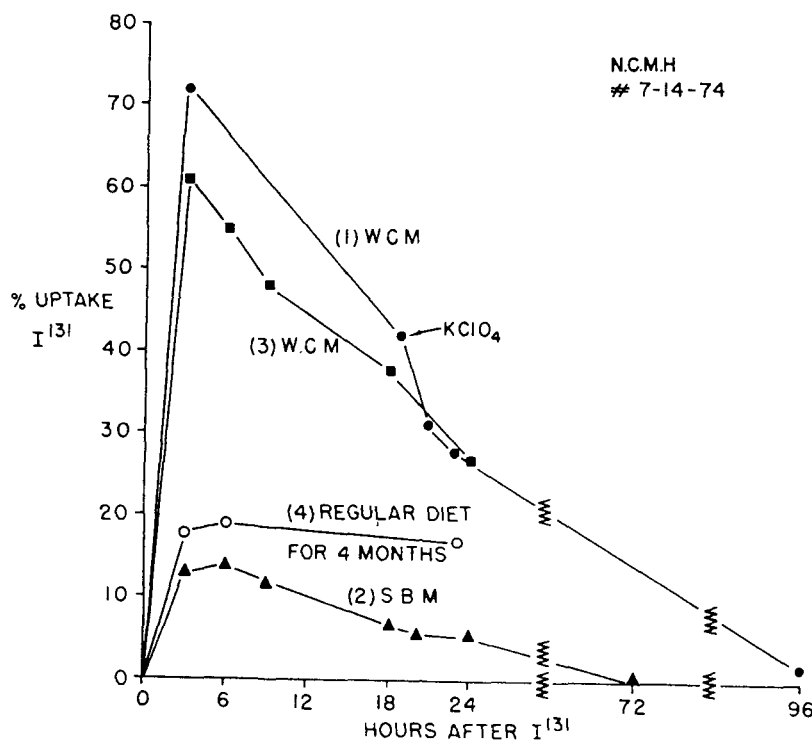


FIG. 3. Uptake of I^{131} by thyroid gland in the infant. Diets. 1) whole cow's milk, 2) soybean product; 3) whole cow's milk, 4) regular diet.

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TABLE I
STUDIES OF I^{131} IN BLOOD AFTER ADMINISTRATION OF I^{131} TO THE INFANT*

Study	Hours After I^{131} Admin.	Total I^{131} (c/m/ml)**	PBI I^{131} (c/m/ml)	PBI I^{131} (% dose/liter)***	Chromatography
1. (6/12/58) Whole milk 17.5 μ c I^{131} , i.v.	96	148	121	1.7	
2. (6/16/58) Soybean product 25 μ c I^{131} , i.v.	6 24	323 47	0 0	0 0	
3. (6/19/58) Whole milk 12.5 μ c I^{131} , i.v.	6 24	565 815	295 744	6.0 15.2	79% thyroxine 17% triiodothyronine
4. (10/3/58) Regular diet for 4 months 10 μ c I^{131} , i.v.	6 24	255 40	14 6	0.36 0.15	

* All counts corrected for radioactive decay to time of injection. Counts in studies II and III corrected for residual radioactivity in blood at beginning of study.

** Counts/min/ml.

*** Percent of injected dose per liter of plasma.

crease in PBI I^{131} again occurred. After chromatography, 79% of the protein bound I^{131} was recovered in the thyroxine fraction and 17% in the triiodothyronine fraction. Four months later, after the fourth dose of I^{131} , the plasma PBI I^{131} was lower and well within the normal range.

STUDIES WITH ADULTS

Procedures

Ten male and four female adults were selected for this part of the study. All subjects were euthyroid, had no goiter, and gave no history of thyroid disease. Preliminary determinations of serum protein bound iodine were within the normal range.

All studies were carried out in an identical manner with two distinct dietary regimens. In nine subjects the diet in the first 5-day period consisted of a soybean product and in the second 5-day period consisted of whole cow's milk, as the only sources of food. In five subjects the dietary sequence was reversed. Feedings were spaced at 4-hour intervals and totalled 2,600 calories in each 24-hour period. When expressed on a per kilogram basis the feedings received by the adults in each 24-hour period were less than half those received by the infant. After the ad-

ministration of I^{131} on the fourth day of each dietary period, thyroidal uptake of I^{131} was measured at 1, 3, 6, 8 and 24 hours. Fifty microcuries of I^{131} was administered intravenously to 12 of the subjects and 75 μ c was given with milk to two others. In two subjects 1,396 gm of sodium thiocyanate was administered intravenously 3 hours after the administration of I^{131} . Total plasma I^{131} and protein bound I^{131} were determined in 11 of the subjects 24 hours after the administration of I^{131} .

RESULTS OF STUDIES WITH ADULTS

In Table II it may be seen that all values for 24-hour I^{131} uptake were in the normal range, except those which were obtained after the administration of sodium thiocyanate. In these two subjects, although sodium thiocyanate prevented the further accumulation of I^{131} by the thyroid gland, it caused no discharge of I^{131} from the gland. In addition, the thyroid uptake of I^{131} in the two subjects who received I^{131} orally was not significantly different from the thyroidal uptake of subjects given I^{131} intravenously. There was no tendency for the 24-hour thyroid uptake to be higher with one regimen than with the other under the conditions of

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TABLE II
EFFECT OF DIET ON UPTAKE OF I^{131} BY THYROID
GLAND: NORMAL ADULTS, AT 24 HOURS

Subject	Age (yr)	Sex	WCM (%)†	SBM (%)
T.D.	23	M	23	25
J.W.	23	F	16	17
M.H.†	23	F	20	23
W.H.†	24	M	22	18
C.D.	24	F	22	14
R.D.	25	M	16	15
K.B.	22	M	17	21
M.G.	21	M	16	15
J.F.	25	F	21	23
T.F.	26	M	14	18
B.C.	21	M	10*	6*
W.P.	22	M	22	26
T.G.	21	M	9*	15*
G.P.	21	M	13	20

* Received 1 gm sodium thiocyanate intravenously at 3 hours.

† Received 75 μ c I^{131} orally.

‡ % test dose.

WCM = whole cow's milk.

SBM = soybean product.

this study. The greatest difference observed was not statistically significant.¹³

The results of the determinations of plasma protein bound I^{131} and protein bound I^{127} are presented in Table III and Figure

4. In most of the subjects no significant difference in the values for plasma protein bound I^{131} was observed with either of the two regimens. However, the two subjects (T.D., C.D.) with exceptionally high values for plasma protein bound I^{131} fractions with the whole cow's milk diet had a highly significant decrease in this fraction when given the soybean product. This change is similar to that seen in the hypothyroid infant in Study 2. In these two subjects there was no decrease in PBI^{127} with the soybean product in the dietary regimen. Subjects W.H. and G.P. similarly had a 50% reduction in the PBI^{131} fraction with the soybean-product regimen. However, since these changes reflect only a small difference in the total number of counts, the decrease in these two individuals is not significant.

DISCUSSION

The results of these studies support the observation that the soybean product used may cause goiter and, on occasion, hypothyroidism in humans. The occurrence of goiter in infants reared on soybean products is apparently rare. Since the report of Rawson⁶ and the recognition of the present case, we have learned of eight additional infants ob-

TABLE III
PLASMA PROTEIN BOUND I^{131} AND PLASMA PROTEIN BOUND I^{127} IN NORMAL ADULTS 24 HOURS AFTER
ADMINISTRATION OF I^{131} IN RELATION TO DIET

Subject	WCM			SBM		
	Total I^{131} (c/m/ml)*	PBI^{131} (c/m/ml)	PBI^{127} (μ g/100 ml)	Total I^{131} (c/m/ml)	PBI^{131} (c/m/ml)	PBI^{127} (μ g/100 ml)
T.D.	82	52	6.3	133*	1*	6.5
M.H.	96**	11**	7.0	86	12	—
W.H.	54**	4**	7.6	106	2	—
C.D.	173**	89**	5.1	146	43	5.1
R.D.	170**	14**	6.6	152	17	—
K.B.	89**	12**	—	127	14	—
M.G.	114**	20**	7.9	171	14	—
B.C.	88	9	5.1	119*	13*	—
W.P.	100	11	5.2	149*	8*	—
T.G.	97	7	6.8	187*	13*	—
G.P.	69	16	7.9	73*	8*	—

* Counts/min/ml. All counts corrected for physical decay to time of injection.

** Corrected for residual counts in plasma prior to injection.

WCM = whole cow's milk. SBM = soybean product.

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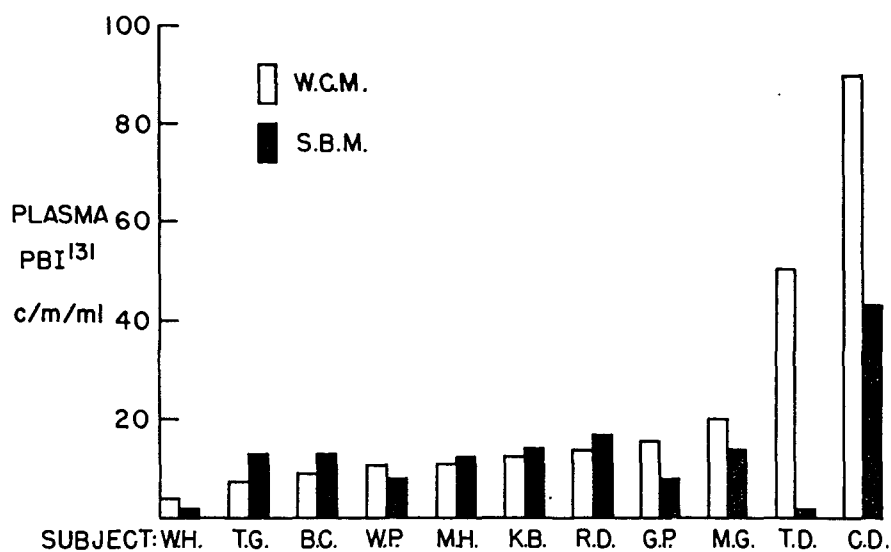


FIG. 4. Effect of whole cow's milk (WCM) and soybean product (SBM) on serum PBI¹³¹ in normal adults. All determinations obtained 24 hours after administration of I¹³¹.

served in other clinics who have developed goiter while receiving this soybean product (Mull-Soy®).*

The mechanism by which a soybean derivative may interfere with iodine metabolism is defined in part by these studies. Two features of the studies of the hypothyroid child stand out. A rapid, high uptake of I¹³¹ by the thyroid followed by rapid discharge into the blood as hormonal I¹³¹ is characteristic of either iodine depletion or the rebound phenomena which follow the withdrawal of any goitrogenic agent. These findings were noted when the infant was still hypothyroid from the prior soybean diet, although the soybean product had been withdrawn. When soybean product was reinstituted, the thyroidal uptake of I¹³¹ and the blood level of PBI¹³¹ were severely depressed. It thus appears that this soybean

product acted as a thyroid blocking agent. When these studies were repeated after 4 months of a normal diet, the uptake curve was normal and normal amounts of thyroid hormone were discharged into the blood.

It is possible in this infant that soybean decreased intestinal absorption of I¹³¹ and/or increased the fecal excretion of hormonal I¹³¹ in addition to its direct effect on the thyroid. Increased fecal excretion of hormonal iodine and a rapid rate of disappearance of thyroxine from the blood have been observed in the rat with a soybean diet.^{14, 15} Albert and Keating¹⁶ demonstrated in the rat that 100% of circulating thyroxine is re-circulated through the enterohepatic circulation per hour. Under these circumstances only a slightly impaired reabsorption would soon lead to significant thyroxine wastage and ultimately to iodine depletion. Limited studies which have been carried out in adults with biliary fistulae indicate that the biliary excretion of thyroxine labeled with I¹³¹ is considerably less than that reported in rats.¹⁷ Neither the intestinal absorption nor fecal excretion of I¹³¹ were examined in the present infant.

Extension of these studies to normal subjects who were not depleted of iodine further revealed that this soybean product did

* We are indebted to the following for permission to cite their patients: Dr. Melvin Grumbach, Babies Hospital, Columbia-Presbyterian Medical Center, New York, 2 patients; Dr. John A. Ripp, Old Westbury, N.Y., 1 patient; Dr. Alvin Hayles, Mayo Clinic, Rochester, Minnesota, 1 patient; Dr. Thomas Shepard, University of Washington School of Medicine, Seattle, Washington, 4 patients. Dr. Ripp and Dr. Shepard will report their patients separately (See Letter to the Editor, this issue of PEDIATRICS).

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not interfere with the absorption of iodine, that it did not impair iodine uptake, that it did not interfere with the oxidation of iodine in the thyroid gland, and that in most of the subjects it did not interfere with the release of PBI^{131} into the blood. However, two subjects, who had a high level of PBI^{131} in the plasma while receiving whole cow's milk, had a significant suppression in the PBI^{131} while receiving this soybean product.

The fact that only two adults showed any effect of the soybean product would indicate that only a particular kind of individual is affected by this soybean product under the circumstances of these investigations. Neither of these adults showed significant depression of thyroid uptake, but both showed a marked depression in the blood level of hormonal I^{131} while receiving the soybean product. If the low blood levels of PBI^{131} had been due to fecal loss, then the plasma protein bound I^{127} should have been depressed concomitantly. This was not the case, however. The studies on the normal adults therefore also suggest that this soybean product did impair thyroid hormone synthesis in susceptible individuals. Investigations are now in progress to delineate further the biochemical defect in the thyroid gland which may be induced by such a soybean product.

The fact that a disturbance in thyroid function has been only occasionally manifested among infants who presumably have had equivalent exposure to soybeans, is characteristic of a number of environmental goitrogens, such as cobalt, para-aminosalicylic acid and thiocyanate. Whether the factor determining the development of goiter is an inborn susceptibility, or a modifying action of other environmental factors is not known. It was considered possible that the preparations used in infant feeding are less goitrogenic than the soybean flour diets used to induce goiters in laboratory animals. Anderson^{*} has provided relevant

information on this point by demonstrating that different lots of soy vary widely in their goitrogenic properties and that the heat treatment used in processing infant foods lessens, but does not abolish the goitrogenic effects. In all cases he showed that it was possible to overcome the goitrogenic activity of the soy ration and increase the rate of growth in rats fed a soybean product by fortifying it with additional iodide.

Since these studies were completed the soybean product (Mull-Soy[®]) responsible for all of the cases cited in this paper (footnote p. 758) has been fortified with potassium iodide to provide 300 μg iodine/15 $\frac{1}{2}$ oz.[°]

SUMMARY

A 10-month-old infant reared on a soybean product (Mull-Soy[®]) from birth developed a goiter and hypothyroidism, which was cured by the administration of 4 drops of Lugol's solution and the replacement of the soybean product by whole cow's milk.

After this soybean product was withdrawn, but while the patient was still hypothyroid, the thyroid had a high uptake of I^{131} with rapid discharge into the blood as PBI^{131} . Reinstitution of this soybean product suppressed the thyroidal uptake of I^{131} and completely inhibited the appearance of PBI^{131} in the plasma.

Studies in normal adult subjects revealed that this soybean product did not interfere with the absorption of iodine, iodine uptake by the thyroid, oxidation of iodide to iodine or the release of PBI^{131} in most subjects. Two subjects, however, who had a high plasma level of PBI^{131} while receiving whole cow's milk had a significant suppression in the PBI^{131} while receiving this soybean product. This was not associated with a concomitant reduction in the plasma PBI^{127} , thereby indicating that excessive loss of hormonal iodine had not taken place.

These studies suggested that a goitrogenic studies. The full report will be published elsewhere. (See Letter to Editor this issue of PEDIATRICS).

[°] There is no evidence that this is necessary for all soybean infant foods (see Letter to Editor this issue of PEDIATRICS, p. 855)—EDITOR.

^{*} We are indebted to Dr. David Anderson of the Borden Company for permission to cite these

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genic agent was present in this particular soybean product, which interfered with thyroid hormone synthesis in susceptible individuals, and which raised the daily requirement for iodine.

Acknowledgment

The authors are indebted to Dr. William H. Patton, Morganton, North Carolina, for providing us with his careful and complete observations of this infant.

We gratefully acknowledge the help of Miss Dorothy Tate, Head Therapeutic Dietician of the North Carolina Memorial Hospital, for the preparation of the diets used in this study.

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Apolipoprotein E allelic variation and receptor interaction in Alzheimer disease.
O. Heinonen*, S. Yli-Herttuala, P. Riekkinen Sr and H. Soininen.
Department of Neurology and MIV-Institute, University and University Hospital, Kuopio, POB 1627, 70211 Kuopio, Finland.

The aim of the present study was to investigate whether apolipoprotein E (apoE) genotype affects immunoreactivity for α_2 -macroglobulin/LDL-related protein (α_2 MR/LRP), apolipoprotein E, amyloid- β -protein (A β), LDL-receptor and apolipoprotein B (apoB) in the AD brain. We used brain tissue from 6 definite AD patients (3 ϵ 4 ϵ , 2 ϵ 3 ϵ , 1 ϵ 4 ϵ), 1 possible AD (ϵ 4 ϵ) case and 2 nondemented controls (both ϵ 3 ϵ). The immunoreactivity for scavenger receptor peptides and malonylaldehyde modified LDL (oxidized LDL); two important factors in the formation of atherosclerotic lesions, was also studied. Immunopositivity for LDL-receptor, apoB and atherosclerotic markers was weak or not found. Instead, the study demonstrates the increase in the immunopositivity for apoE and α_2 MR/LRP in the frontal cortex and the hippocampal formation of AD patients with ϵ 4 ϵ . The increased immunopositivity may be a consequence of the impaired function and turnover of apoE associated with A β . All these components serve as substrates for the lysosomal systems that may be impaired in a very early stage of AD. Impaired substrate digestion or removal of digested products could lead to the accumulation of apoE and A β as well as to increased α_2 MR/LRP expression.

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Hippocampal cholinergic neurostimulating peptide (HCNP) - related components specifically accumulate in Hirano bodies

E. Katada*, S. Mitate, and K. Ojika

Second Department of Internal Medicine, Nagoya City University Medical School, Mizuho-ku, Nagoya 467, Japan

Hippocampal cholinergic neurostimulating peptide (HCNP), a novel undecapeptide isolated from the hippocampus of young rats, specifically enhances the cholinergic activity of the septo-hippocampal system in vitro. Cloning and base sequence analysis of HCNP-specific cDNA from rat and human cDNA libraries revealed that HCNP aligns at the N-terminal region of its 21 kDa precursor protein. To investigate the immunohistochemical distribution of HCNP in the human brain of the elderly individuals, the affinity-purified rabbit antibodies against rat as well as human HCNP were prepared. Both antibodies react with HCNP-related components in the soluble cytosol fraction of human brain tissue. The immunohistochemical examination revealed that almost all Hirano bodies (HBs) in the hippocampus were specifically stained, but not normal tissues or other neuronal inclusions. The number of HCNP-positive HBs in Sommer's sector was greater in patients with Alzheimer's disease than in age-matched non-demented individuals. HBs occur preferentially in the neuronal processes of the stratum pyramidale of the hippocampus. Occasionally, they can be seen as small inclusions intermingled with neurofibrillary tangles (NFTs) and in association with senile plaques (SPs). The anti-HCNP antibody also recognized the HBs within NFTs and with SPs. HCNP immunoreactivity was identified by immunoelectron microscopy in the paracrystalline structure of HBs. These findings suggest that HCNP-related components are involved in HB formation.

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Fibroblast Growth Factor (FGF)-9 Immunoreactivity in Senile Plaques

S. Nakamura, T. Todo, M. Mizuguchi, K. Arima, S. Haga, T. Aizawa, Y. Namba, N. Otsuka, Y. Motoi, Y. Otsuka, A. Ueki, K. Ikeda

Department of Ultrastructure and Histochemistry, Tokyo Institute of Psychiatry, 2-1-8 Kamikiazawa, Setagaya, Tokyo 156, Japan and Jichi Medical School, Omiya Medical Center, Omiya, Japan

FGF-9, initially referred to as a gliotrophic factor (GLA), is a newly identified member of FGF family including acidic FGF (FGF-1) and basic FGF (FGF-2). Recently we found by immunohistochemistry that FGF-9 was present in neurons and astrocytes in human and rat brains. Since senile plaques and neurofibrillary tangles of Alzheimer's disease (AD) brain are associated with FGF-2 immunoreactivity (Siopka et al, *Biochem Biophys Res Commun*, 171: 690-696, 1990) and both FGF-1 and FGF-2 are implicated in the AD pathogenesis, we performed immunohistochemical studies of AD autopsy brain tissues by using antibodies to FGF-9.

Hippocampal sections including occipitotemporal neocortex from 10 AD cases and 10 age-matched controls were cut from formalin-fixed, paraffin-embedded blocks in our departmental archives, treated with formic acid and subjected to immunohistochemistry. Antibodies to FGF-9 were raised in rabbits against human recombinant FGF-9 or synthetic peptides of FGF-9 (87-102 and 193-208, respectively). Specificity was verified by Western blotting and ELISA analyses. Our previous studies showed that three antibodies gave similar results in human and rat brain tissues with respect to neuronal and glial staining.

In AD brain sections antibodies to FGF-9 strongly immunostained pyramidal and granule cell neurons of hippocampus and less intensely neocortical neurons. Neuropil staining was conspicuous in hippocampal subfields and neocortex. Glial cells in the white matter were stained. FGF-9 antibodies strongly stained dystrophic neurites of senile plaques. Comparison of the adjacent sections stained with either tau antibody or FGF-9 antibodies revealed that the senile plaques with FGF-9 positive dystrophic neurites were almost equal in number to the plaques with tau positive dystrophic neurites. Neurofibrillary tangles were not stained. In control sections, both neuronal and neuropil stainings were weak compared to AD sections. Dystrophic neurites of senile plaques, when present, were also stained.

Epidemiology & Risk Factors III

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Association of mid-life consumption of tofu with late life cognitive impairment and dementia: the Honolulu-Asia Aging Study

L. White*, H. Petrovitch, GW Ross, K. Masaki
National Institute on Aging, NIH, Bethesda, MD; Kurekuni Medical Center and U Hawaii; Honolulu Department of Veterans Affairs

Tofu and other soybean foods contain isoflavones - three-ringed molecules bearing structural resemblance to steroidal hormones and having significant estrogen agonistic or antagonistic activities apparently related to their interactions with estrogen receptors and/or with enzymes involved in estrogen metabolism.

There is evidence suggesting that estrogens modulate neural and synaptic plasticity during aging. Male neurons have both estrogen and androgen receptors. Further, an enzyme (aromatase) that converts androgens to estrogens has been demonstrated in the medial forebrain, limbic system, hippocampus, and hypothalamus.

It was hypothesized that men who had consistently high dietary intakes of tofu during middle life would experience different patterns of cognitive decline and dementia in late life, compared with men reporting little or no tofu consumption.

The Honolulu-Asia Aging Study is a longitudinal study of aging and dementia conducted in Japanese-American men who are members of the Honolulu Heart Program cohort. Mid-life patterns of consumption of tofu and several other foods were defined on the basis of food frequency interviews conducted in 1965 and 1972. The Cognitive Abilities Screening Instrument was administered to more than three thousand surviving participants aged 71-93 years during the 1991-93 examination cycle. DSM-III-R, NINCDS-ADRDA, and California criteria were used for the diagnosis of dementia (all cause), AD, and VaD.

We found an association of consistently high levels of tofu consumption in mid-life with low cognitive test scores ($p=0.02$) and (independently) with Alzheimer's disease in late life, controlling for all other relevant variables. The odds ratio for AD in persons who reported eating tofu at least twice weekly was 2.4 (95% CI 1.14-5.09), compared with persons reporting tofu consumption rarely or never.

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Prevalence of Dementia in Older Japanese-American Men in Hawaii

The Honolulu-Asia Aging Study

Lon White, MD; Helen Petrovitch, MD; G. Webster Ross, MD; Kamal H. Masaki, MD; Robert D. Abbott, PhD; Evelyn L. Teng, PhD; Beatriz L. Rodriguez, MD, PhD; Patricia L. Blanchette, MD, MPH; Richard J. Havlik, MD, MPH; Gilbert Wergowske, MD; Darryl Chiu; Daniel J. Foley, MS; Carolyn Murdaugh, PhD; J. David Curb, MD, MPH

Objective.—To determine prevalence of dementia and its subtypes in Japanese-American men and compare these findings with rates reported for populations in Japan and elsewhere.

Design and Setting.—The Honolulu Heart Program is a prospective population-based study of cardiovascular disease established in 1965. Prevalence estimates were computed from cases identified at the 1991 to 1993 examination. Cognitive performance was assessed using standardized methods, instruments, and diagnostic criteria.

Participants.—Subjects were 3734 Japanese-American men (80% of surviving cohort) aged 71 through 93 years, living in the community or in institutions.

Main Outcome Measures.—Age-specific, age-standardized, and cohort prevalence estimates were computed for dementia (all cause) defined by 2 sets of diagnostic criteria and 4 levels of severity. Prevalence levels for Alzheimer disease and vascular dementia were also estimated.

Results.—Dementia prevalence by *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised* ranged from 2.1% in men aged 71 through 74 years to 33.4% in men aged 85 through 93 years. Age-standardized prevalence was 7.6%. Prevalence estimates for the cohort were 9.3% for dementia (all cause), 5.4% for Alzheimer disease (primary or contributing), and 4.2% for vascular dementia (primary or contributing). More than 1 possible cause was found in 26% of cases. The Alzheimer disease/vascular dementia ratio was 1.5 for cases attributed primarily to Alzheimer disease or vascular dementia.

Conclusions.—Prevalence of Alzheimer disease in older Japanese-American men in Hawaii appears to be higher than in Japan but similar to European-ancestry populations. Prevalence of vascular dementia appears to be only slightly lower than in Japan, but higher than in European-ancestry populations. Further cross-national research with emphasis on standardized diagnostic methods is needed.

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PAST PREVALENCE surveys indicate that 4% to 11% of persons over the age of 65 years have some form of de-

menting illness.¹ The national economic impact of the dementing diseases is staggering; the human costs to patients and their families are devastating.

Research on differences in rates of dementia in diverse populations represents one approach to the identification of modifiable risk factors and, ultimately, to the prevention of dementing diseases. While overall dementia rates seem to be generally similar among nations, relative frequencies of the 2 major subtypes of dementia, Alzheimer disease (AD) and vascular dementia (VsD), vary. Alzheimer disease is the major subtype in most Western nations. In contrast, VsD has usually been reported to be the domi-

nant subtype in Japan and possibly in other Asian nations.^{1,2} However, because case finding and classification methods have not been well standardized, these reported differences may reflect methodologic differences. Alternatively, true genetic, constitutional, social and/or environmental differences among people from distinct cultures may account for these disparate rates.

For editorial comment see p 993.

The Honolulu-Asia Aging Study (HAAS) is a member of an informal consortium of epidemiologic studies on dementia in the United States, Japan, and Taiwan that were designed collaboratively with special attention to the use of standardized methods, instruments, and diagnostic criteria.² The HAAS is conducted in cooperation with the Honolulu Heart Program (HHP), an established, longitudinal, long-term study of heart disease and stroke in Japanese-American men. In this article, we present age-specific and age-standardized prevalence estimates for total dementia, AD, and VsD in the HHP cohort.

METHODS

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The HHP is a longitudinal study of heart disease and stroke in Japanese-American men born 1900 through 1919 and living on Oahu when the study began in 1965. The World War II Selective Service Registration file was used to identify 12 417 possibly eligible men and 8006 participated in the first examination. Eighty-eight percent of the men were born in Hawaii, 12% in Japan. Continuous surveillance for mortality and hospitalizations has been carried out since the study's inception.³ Research on dementia began at the fourth examination of the cohort in 1991 with the establishment of the HAAS.

A total of 4678 surviving men were

From the National Institute on Aging (Drs White and Havlik and Mr Foley), National Institute for Nursing Research (Dr Murdaugh), National Institutes of Health, Bethesda, Md; Honolulu-Asia Aging Study, Kuakini Medical Center, Honolulu, Hawaii (Drs Petrovitch, Masaki, Rodriguez, and Curb and Mr Chiu), Department of Veterans Affairs, Honolulu (Dr Ross), Department of Medicine, University of Hawaii, John A. Burns School of Medicine, Honolulu (Drs Petrovitch, Ross, Masaki, Rodriguez, Blanchette, Wergowske, and Curb), Division of Biostatistics, University of Virginia, Charlottesville (Dr Abbott), and University of Southern California, Los Angeles (Dr Teng).

Reprints: Lon White, MD, Honolulu Heart Program, Honolulu-Asia Aging Study, 347 N Kuakini St, Honolulu, HI 96817.

eligible to participate in the fourth examination. All but 5 were found and invited to participate. Of 3741 men who were seen at the fourth examination, 3734 participated in phase 1 of the dementia case-finding effort. Ages ranged from 71 through 93 years (average, 78 years). Average years of education were 10.5. Thirteen percent had fewer than 8 years, and 45% had completed 12 or more years of education.

Interviews and testing were conducted in the participant's preferred language, English or Japanese. Subjects were fully informed regarding study participation and signed informed consent forms.

Dementia Case-Finding Methods

Dementia case-finding occurred in 3 phases (Figure 1). A total of 3734 participants (80% of the eligible cohort) were administered the Cognitive Abilities Screening Instrument (CASI) at phase 1. Eighty-five percent were seen at the clinic, 13% at home, and 2% in nursing homes. The CASI has been validated as a screening instrument for dementia in the United States and Japan, in both English and Japanese languages.⁴ Designed for use in comparative cross-national studies of dementia in the United States and Japan, it is a composite of the Hasegawa Dementia Screening Scale (widely used in epidemiologic studies in Japan),⁶ the Folstein Mini-Mental State Examination,⁶ and the Modified Mini-Mental State Test.⁷ The CASI includes tasks assessing attention, concentration, orientation, short- and long-term memory, language ability, visual construction, word list generation, abstraction, and judgment. The score range is 0 to 100.

Phase 1 CASI score was used to stratify the cohort into low scorers (CASI <74), intermediate scorers (CASI=74-81.9), and high scorers (CASI ≥82). All low scorers and men aged 85 years or older were invited back for the phase 2 examination. Participants were sampled from intermediate- and high-scoring groups by a probability sampling system. Of 1063 subjects invited to return, 948 (89%) completed phase 2. Most were examined within 12 weeks of their phase 1 appointment.

The phase 2 examination included a second CASI, neurologic examination, and testing of the participant's hearing and vision. Examiners were shielded from information obtained at phase 1. An informant (usually the wife) was given a standardized interview that included the Informant Questionnaire on Cognitive Decline in the Elderly (IQCODE), to assess changes in cognitive function and behavior over the prior 10 years. An IQCODE score of 3.6 or higher is a strong indicator of dementia.^{8,9}

Participants invited for phase 3 assessment included individuals with persistently low CASI scores (better of 2 scores <75) or IQCODE score greater than 3.6, plus a probability sample of remaining participants. Of the 507 subjects selected, 426 (84%) received the full phase 3 dementia evaluation.

Phase 3 examination included a standardized interview and neurologic examination by a neurologist with advanced training in behavioral neurology and dementia research, as well as the neuropsychological test battery from the Consortium to Establish a Registry for Alzheimer's Disease (CERAD).^{10,11} The neurologist obtained a structured history from the informant based on CERAD clinical evaluation protocol.¹⁰ Phase 3 examiners were shielded from information obtained at phases 1 and 2.

Those participants judged by the study neurologist to meet *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised*¹² (DSM-III-R) criteria for dementia had brain computed tomographic (CT) scans and blood tests (complete blood cell count, chemistry profile, vitamin B₁₂ level, folate level, rapid plasma reagin, and thyroid function tests).

Diagnostic Methods and Criteria

Dementia was defined using 2 independent sets of diagnostic criteria: those of Cummings and Benson¹³ and the DSM-III-R.¹² The Cummings and Benson criteria define dementia as acquired impairment in at least 3 of 5 neuropsychological domains (memory, speech/language, visuospatial function, higher cognition, and mood/personality). Impairment in a specific domain was a clinical decision based on the neurologist's evaluation and neuropsychological test scores. The DSM-III-R criteria require impairment in both long- and short-term memory and 1 other domain, with the additional requirement that the impairment be severe enough to interfere with social or occupational functioning. Semistructured guidelines were used to define functional impairment that required: (a) an informant's assessment of social/occupational expectations and capabilities of the participant prior to and after the onset of cognitive decline and (b) the decline in functioning be related to cognitive impairment and not physical disability.

Final diagnosis and clinical dementia rating (CDR)¹⁴ index were assigned by a panel consisting of the study neurologist and at least 2 other physicians with expertise in geriatric medicine and dementia. The panel was provided with all information accrued at phase 3, including CT scans, laboratory results, and the neurologist's diagnostic impression.

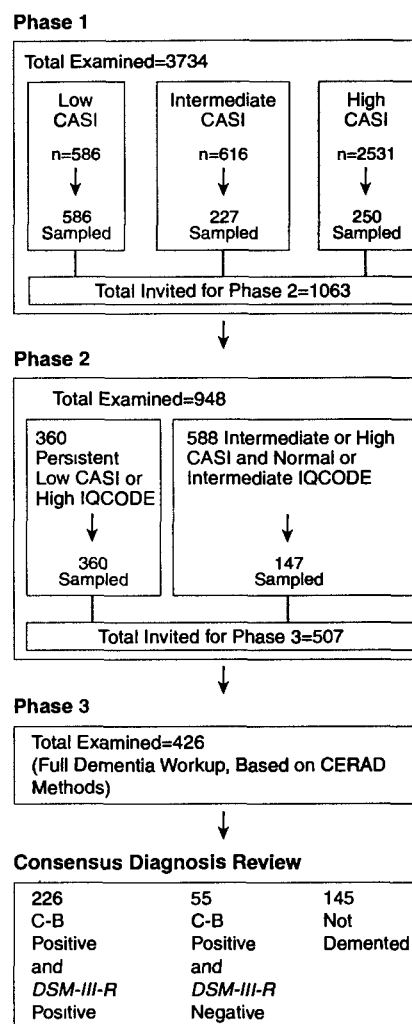


Figure 1.—Outline of study design. CASI indicates Cognitive Abilities Screening Instrument; IQCODE, Informant Questionnaire on Cognitive Decline; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; DSM-III-R positive, meeting criteria for dementia of the *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised*; DSM-III-R negative, not meeting criteria for dementia of the *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised*; and C-B positive, meeting Cummings and Benson criteria for dementia.

The panel was shielded from information gathered during phases 1 and 2. For those participants meeting DSM-III-R criteria for dementia, diagnostic subtypes were also determined. Alzheimer disease was diagnosed according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA).¹⁵ Criteria for VsD were based on those proposed by the California Alzheimer's Disease Diagnostic and Treatment Centers (AD-DTC).¹⁶ Dementia due to a degenerative parkinsonian disorder was diag-

Table 1.—Estimated Age-Specific and Age-Standardized Prevalence for Dementia (All Cause), Alzheimer Disease, and Vascular Dementia*

	Age Group, y				Overall (426/3734)	Age-Standardized‡ ≥65 y
	71-74 (34/1084)†	75-79 (122/1528)	80-84 (107/705)	85-93 (163/417)		
Dementia (All Cause) by Diagnostic Criteria						
Cummings-Benson						
%	3.0	9.2	17.2	46.2	13.0	10.3
95% CI	0.0-19.7	4.5-16.6	10.2-26.4	38.1-54.6	9.8-16.8	7.4-13.7
Cases identified at phase 3 evaluation	18	66	74	123	281	...
DSM-III-R						
%	2.1	6.2	12.9	33.4	9.3	7.6
95% CI	0.0-15.4	2.3-12.6	6.6-21.0	25.9-41.6	6.5-12.6	5.1-10.8
Cases identified at phase 3 evaluation	17	51	60	98	226	...
DSM-III-R Positive Dementia by Cause						
Alzheimer disease§						
%	0.9	3.7	7.0	20.6	5.4	4.7
95% CI	0.0-15.4	0.9-9.4	2.6-14.3	14.3-28.0	3.4-8.3	2.8-7.5
Cases identified at phase 3 evaluation	8	25	28	61	122	...
Vascular dementia						
%	1.1	2.3	6.4	15.9	4.2	3.8
95% CI	0.0-15.4	0.1-7.1	2.0-13.1	10.1-22.5	2.3-6.6	2.1-6.4
Cases identified at phase 3 evaluation	8	25	35	47	115	...

*CI indicates confidence interval; and DSM-III-R, Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised.¹²

†Participants at phase 3/participants at phase 1.

‡Standardized to the 1990 US population age distribution.

§Includes all cases with Alzheimer disease as the sole or contributory cause.

||Includes all cases with vascular dementia as the sole or contributory cause.

nosed if the subject had a clearly defined parkinsonian syndrome (any 2 of rest tremor, bradykinesia, rigidity, or postural instability) not related to medication or cerebrovascular disease, that preceded or occurred simultaneously with onset of cognitive impairment. No separate category of cortical Lewy body disease was made because of the difficulty in making this diagnosis without benefit of autopsy findings.

Mixed dementia involving AD was defined as possible AD (NINCDS-ADRDA criteria) with a second disorder sufficient to cause dementia.¹⁵ Mixed dementia involving VsD was defined according to ADDTC criteria.¹⁶ Other mixed cases were defined using the best clinical judgment of the consensus panel. An effort was made to designate the condition that was primary (most important).

Statistical Methods

Prevalence estimate computations used standard methods for a 2-stage stratified probability sampling strategy.¹⁷ At the first stage, a stratified random sample of men who had participated in phase 1 were invited to return for the phase 2 examination (Figure 1). A second stratified random sample of men who participated in phase 2 were invited to return for the phase 3 examination (Figure 1). Preliminary prevalence estimates for each phase 2 sampling stratum were computed using data from subjects seen at phase 3. Estimates were then computed for the full cohort of phase 1 participants. Ninety-five per-

cent confidence limits for the final prevalence estimates were derived from exact methods for a binomial parameter.¹⁸ Logistic regression models were also fit to the estimated prevalence rates across age groups using the method of maximum likelihood.¹⁹ Age-specific prevalence rates derived from these models were applied to the United States population age structure for men only and for both sexes combined (all ethnicities) age 65 years and older based on the 1990 census.

RESULTS

As illustrated in Figure 1, 3734 men received an evaluation of cognitive functioning at the phase 1 examination, 948 participated in the phase 2 examination, and 426 received a full dementia evaluation at phase 3. Subjects who declined to return for phase 2 or 3 examination tended to be somewhat older and had slightly poorer CASI scores than those who did participate. We requested supplementary information about these men from their personal physicians. Responses were received from 120 of 149 physicians queried. Eighty-four physicians stated that their patient was not demented, 19 stated that the patient was demented, and 17 were uncertain. These data indicate that dementia was probably more prevalent among nonresponders compared with fully participating cohort members. Thus, prevalence estimates given below may slightly underestimate true prevalence levels in the full study population.

We identified 281 men who met Cum-

mings and Benson criteria for dementia; 55 of these failed to meet DSM-III-R criteria. All 226 men who met DSM-III-R criteria also met Cummings and Benson criteria. Twelve participants could not be evaluated because of severe aphasia or were judged to be severely impaired consequent to a single catastrophic event such as brainstem stroke. These individuals were not included as dementia cases.

Age-specific prevalence estimates for dementia defined by DSM-III-R and Cummings and Benson diagnostic criteria are presented in Table 1. Consistent with prior dementia surveys, change in prevalence estimates with age had an exponential appearance. For dementia defined using DSM-III-R criteria, prevalence increased from 2.1% at aged 71 through 74 years to 33.4% in participants older than 85 years. Overall prevalence in the study cohort was 9.3% and 13% for DSM-III-R and Cummings and Benson criteria respectively.

Participant ages ranged from 71 to 93 years (average, 78 years). Most other surveys have been conducted in populations aged 65 years and older. Logistic curves, extended from age 65 years, were fit to available data for participating cohort members to generate comparable prevalence estimates for Japanese-American men in Hawaii (Figure 2). Application of these curves to the United States population aged 65 years and older (1990 census) yielded age-standardized dementia prevalence estimates of 7.6% (DSM-III-R criteria) and 10.3% (Cummings and Benson criteria) (Table

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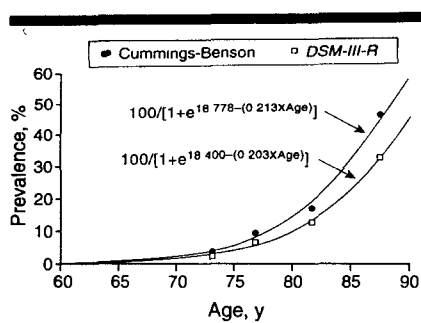


Figure 2.—Estimated age-specific prevalence curves for Japanese-American men in Hawaii aged 60 to 93 years. Dementia defined respectively by Cummings-Benson and *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised* (DSM-III-R) criteria. The upper and lower logistic equations were used to model the percent prevalence of dementia according to the Cummings-Benson and DSM-III-R criteria, respectively.

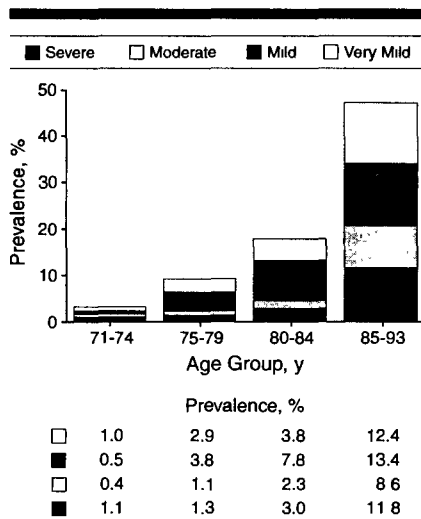


Figure 3.—Age-specific prevalence of dementia by severity among men aged 71 to 93 years old. Rates are estimated from 281 cases meeting Cummings-Benson criteria. Very mild is equivalent to a clinical dementia rating (CDR) index of 0.5; mild, CDR index of 1.0; moderate, CDR index of 2.0; and severe, CDR index of 3.0 or greater.

1). When the reference population included only males the standardized prevalence estimates fell to 6.1% and 8.3%, respectively, reflecting the lesser numbers of men in the oldest age strata of the general population.

To compare our results with those of other surveys in which prevalence values were reported according to dementia severity, we estimated age-specific prevalence at 4 levels of severity (defined by CDR index) using all the 281 cases identified by application of Cummings and Benson diagnostic criteria. The subset of very mild (CDR index=0.5) cases is represented by the top segment of each bar of Figure 3. This subset corresponds nearly perfectly with dementia cases who met Cummings and Benson

Table 2.—Specific Diagnosis Classification for 226 Cases of Dementia Meeting DSM-III-R Criteria*

Classification	No.
Probable Alzheimer disease	69
Possible Alzheimer disease (atypical course)	8
Probable vascular dementia	49
Possible vascular dementia	19
Mixed dementia: possible Alzheimer disease primary cause with	41
Vascular dementia	22
Vascular dementia + other†	8
Vitamin B ₁₂ deficiency	8
Subdural hematoma	1
Depression	1
Chronic alcohol use	1
Mixed dementia: probable vascular dementia primary cause with	7
Alzheimer disease	3
Vitamin B ₁₂ deficiency	1
Vitamin B ₁₂ deficiency plus Parkinson disease	1
Subdural hematoma	1
Neurosyphilis	1
Mixed dementia: possible vascular dementia primary cause with	5
Alzheimer disease	1
Vitamin B ₁₂ deficiency	2
Parkinson disease	1
Progressive supranuclear palsy	1
Other dementia	22
Parkinson disease	7
Parkinson disease + other‡	5
Progressive supranuclear palsy	4
Progressive supranuclear palsy + vascular dementia	1
Subdural hematoma	2
Trauma	2
Vitamin B ₁₂ deficiency	1
Dementia cause undetermined	6

*DSM-III-R indicates *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised*.¹²

†Subdural hematoma (n=2), vitamin B₁₂ deficiency (n=2), folate deficiency (n=1), depression plus Parkinson disease (n=1), chronic alcohol use (n=2).

‡Vascular dementia (n=3), vascular dementia plus subdural hematoma (n=1), chronic alcohol use (n=1).

criteria but failed to meet DSM-III-R criteria. Prevalence estimates that included mild, moderate, and more severe cases (CDR index ≥ 1) closely approximated estimates based on dementia cases defined by DSM-III-R diagnostic criteria. Approximately half of the DSM-III-R positive cases of dementia had CDR indexes of 1 (mild dementia).

Specific diagnoses for the 226 cases of dementia meeting DSM-III-R criteria are shown in Table 2. Alzheimer disease was identified as sole cause of dementia (ie, all probable AD plus possible AD without other contributing cause) in 77 cases (34%). Among mixed cases, AD was the primary cause in 41 cases and the secondary cause in 4 cases. Overall, AD was identified in 122 (54%) of the 226 dementia cases.

Cerebrovascular disease was the only apparent cause for dementia in 68 cases (30%). Among mixed cases, VsD was the primary cause in 12 cases, and the secondary cause in 35 cases. Overall, VsD was present in 115 cases (51%).

ADDC criteria specify that mixed dementia cases may be classified as either probable or possible VsD. Of the 226 dementia cases, 66 met the ADDTC

criteria for probable VsD while another 49 met criteria for possible VsD. Focal signs supporting prior stroke were noted for 67% of probable and possible cases. History of sudden onset or stepwise progression of cognitive problems was obtained for 49% of probable cases and 25% of possible cases. The CT scans showed multiple strokes for 64 (97%) of probable cases; the other 2 cases showed evidence of a single stroke judged to be of a size and location sufficient to explain the dementia. The most common strokes noted in the probable VsD group were lacunes in basal ganglia, thalamus, or frontal white matter. The CT scan and clinical picture were evidence for a diagnosis of Binswanger disease (as a type of VsD) for 1 person with probable VsD (who also had multiple strokes) and for 6 others with possible VsD.

Figure 4 shows age-specific prevalence estimates for VsD in the study cohort. The solid component and lowest cross-hatched component of the bars together represent cases meeting ADDTC criteria for probable or possible VsD with no other apparent cause. The top 2 components represent VsD with concurrent AD or some other disease possibly contributing to the dementia. The component for concurrent AD and cerebrovascular disease represents exactly the same cases as in the corresponding bar components of Figure 5. The cohort prevalence for VsD either alone or in conjunction with another possible cause (such as AD or Parkinson disease) was 4.2%.

Figure 5 shows age-specific prevalence estimates for probable or possible AD. Overall prevalence of probable or possible AD in the cohort (including cases in which AD was designated a contributing cause) was 5.4%.

Prevalence estimates for AD and VsD are summarized in the lower half of Table 1. There is substantial overlap between AD and VsD because individuals with both processes are counted in both categories. As shown, age-standardized prevalence estimates were 4.7% for AD and 3.8% for VsD. When the reference population was defined to include only men, estimates fell to 3.7% for AD and 2.9% for VsD.

COMMENT

Variations in the observed prevalence of dementia across populations may reflect differences in age, sex, education, and ethnicity composition. Our study cohort consists of Japanese-American men aged 71 through 93 years. Since the decision to exclude women from the HHP cohort was made more than 30 years ago, we are able to provide estimates only for men. Several but not all surveys have reported a slightly higher de-

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mentia prevalence in women.²⁰⁻²⁴ The distribution of education in the HHP cohort is roughly similar to that of other older American populations, with an average of 10.5 years of schooling completed. As in other populations, the oldest participants reported fewer years of schooling completed.²⁵⁻²⁷

Participants chosen to receive dementia evaluations were identified using stratified random sampling methods. This allowed estimation of the number of cases in all strata. Previous surveys that have used an initial screening step with less than 100% sensitivity and have not sampled subjects who scored above the cut-point are likely to have underestimated the true prevalence of dementia. The magnitude of underestimation can be substantial, as noted in a survey conducted in Stockholm, Sweden. When adjustment was made for cases missed because of false-negative screening test scores, prevalence among men aged 75 through 84 years doubled.²³

In our study, approximately 80% of dementia cases identified with Cummings and Benson criteria also met *DSM-III-R* criteria. Those who did not were nearly all very mildly demented (CDR index=0.5). The definition of dementia used in the East Boston Study appears to approximate that achieved with Cummings and Benson criteria.²⁸ Using Cummings and Benson criteria, our age-standardized prevalence estimate for dementia (10.3%) is still below the prevalence of dementia (approximately 12.2%) attributable to all causes among noninstitutionalized residents of East Boston.^{23,29} Using *DSM-III-R* dementia criteria, our age-standardized prevalence estimate is close to recent estimates from Stockholm^{23,30} and Japan.^{21,31-33}

Variability in prevalence estimates among studies may also be related to different severity thresholds used in the definition of a case. In the Framingham Dementia Study the reported prevalence of 3% among men was based on moderate to severely demented subjects, ie, with CDR indexes greater than 1.²⁰ Our age-standardized prevalence estimate for men (referenced to American males aged 65 years or older) is 6.1%. When only cases of moderate or greater severity (CDR index >1) are included (as in the Framingham study), our prevalence levels are lowered by about half at every age. Thus, once differences in population age distributions and severity criteria are taken into account, prevalence rates are similar in the 2 cohorts.

In contrast to most previous reports, we found a rather high proportion (26%) of cases having more than a single contributing cause. Of these, over half were classified as mixed AD and VsD. High

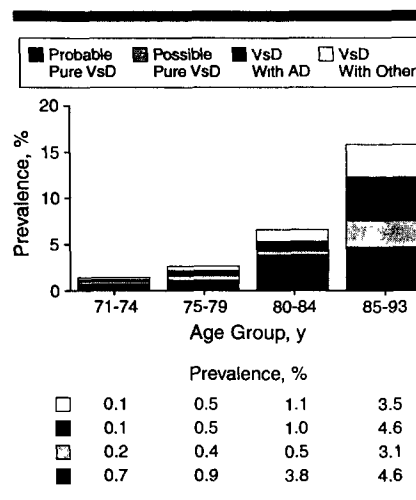


Figure 4.—Age-specific prevalence of vascular dementia (VsD) among men aged 71 to 93 years. Rates are estimated from 226 cases meeting *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised* criteria for dementia. Pure VsD defined by California Alzheimer's Disease Diagnostic and Treatment Centers criteria. In addition to meeting criteria for probable or possible VsD, cases defined as pure VsD required the absence of other systemic disorders that could account for the dementia. Mixed cases include probable or possible VsD with Alzheimer disease (AD) and other dementias.

proportions (12% and 13%) of mixed AD and VsD have recently been reported from community surveys conducted in the United States and Canada.^{20,22} Others have reported lower proportions of mixed dementia; from 0% to 7%.^{21,23,28} Because both AD and VsD become increasingly more prevalent with aging, the chance of having both is likely to be much greater in older populations. This may partially explain the high proportion of mixed cases in our study and in the Canadian study where subjects were over age 71 years and 85 years, respectively. In a study of 85-year-olds in Sweden, 69 of 118 demented subjects were thought to have VsD, including 12 of mixed cause.³⁰ A second factor that might contribute to the high rates of mixed AD/VsD in the present study is an enhanced recognition of cerebrovascular disease as a result of routine use of CT scans for diagnostic classification. A lack of neuroimaging data may have contributed to underrecognition of mixed AD and VsD in the Stockholm and East Boston studies.^{23,28}

Ratios of prevalence of AD to prevalence of VsD are useful as a means for identifying differences across populations. In a majority of reports from the United States and Europe, the AD/VsD ratio has been in the range of 2 or greater. In contrast, lower AD/VsD ratios (usually <1) have been reported in many surveys conducted in Japan during the past 2 decades.^{21,34-36} We found

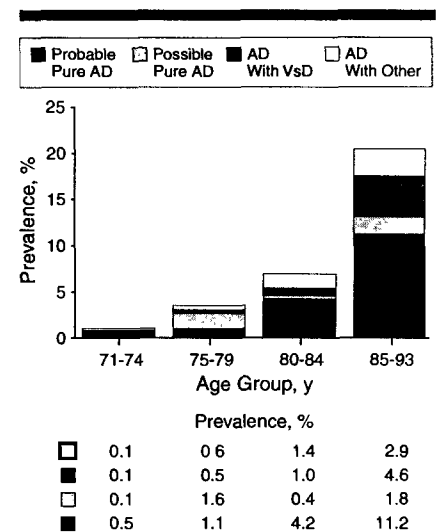


Figure 5.—Age-specific prevalence of Alzheimer disease (AD) among men aged 71 to 93 years. Rates are estimated from 226 cases meeting *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised (DSM-III-R)* criteria for dementia. Probable and possible AD defined by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer Disease and Related Disorders Association criteria. Possible AD subdivided into pure cases and mixed cases including vascular dementia (VsD) and other dementias.

118 men whose dementia was attributed solely or primarily to AD, and 80 whose dementia was attributed solely or primarily to VsD. In this group of cases, the AD/VsD ratio was 1.5, intermediate between most prior American and Japanese reports.

Age-specific and age-standardized prevalence values for AD in the HAAS population are quite close to estimates reported from several other surveys in North America and Europe.^{1,23,36,37} Reported prevalence of AD in East Boston is more than double the AD prevalence in Hawaii and in most other surveys.²⁸ The lower prevalence of AD in Framingham reflects the stringent severity criteria used to define cases in that study; when the reported prevalence in Framingham is compared with Honolulu prevalence estimates based on AD cases of at least moderate severity (CDR index >1), rates are similar.

In contrast, estimated prevalence of VsD in Honolulu is substantially higher than in Framingham and other populations in North America and Europe.^{1,20,23,37} Although prevalence of VsD was not specified in the East Boston Dementia Study, a possible vascular cause was noted in only about 5% of persons with moderate or severe dementia, much lower than found in Honolulu.²⁸

Although data from fully comparable studies in Japan are not yet available, there is no lack of published prevalence

reports from Japan using methods of variable comparability to those used in our study. Most Japanese surveys done in past years have reported dementia prevalence estimates between 4% and 6% with AD/VsD ratios less than 1.³⁴ In a recent study conducted in Hisayama City, overall prevalence of dementia was 6.7%.²¹ The Hisayama study is especially relevant and more comparable than most previous Japanese studies because of the thoroughness of dementia case finding, conduct of the survey in a stable community, use of *DSM-III-R* criteria for dementia, inclusion of both mild and severe cases, frequent use of neuroimaging, and validation of diagnoses by autopsy in a high proportion of cases. In the survey of 887 Hisayama residents aged 65 years or older, prevalence of AD was 1.5%, and the prevalence of clinically diagnosed VsD was 3.2% (overall AD/VsD ratio, 0.47). While diagnoses during life were based on clinical find-

ings, including a low Hachinski score, the relative infrequency of AD as a cause of dementia in this population was neuropathologically confirmed. A more recent follow-up study in the same population generated an AD/VsD ratio of 0.4 for men, 1.2 for women, and 0.8 for both sexes combined, based on new (incident) cases developing since the baseline survey.³⁵ Together, the 2 Hisayama reports strongly support the veracity of many prior surveys in Japanese populations that have found AD to be less prevalent than VsD.

Despite substantial problems related to comparability of studies conducted in Japan and the United States, it appears likely that older Japanese-American men in Hawaii experience a prevalence of AD approaching that of European-ancestry Americans and a prevalence of VsD only slightly below that observed in Japan. These observations lead us to speculate that environmental or cultural

exposures associated with migration from Japan to Hawaii may have influenced the development of AD in HAAS cohort members, while factors involved in the pathogenesis of VsD have remained relatively unaffected. These speculations underscore the need for further well-standardized studies specifically intended to allow comparisons of rates and risk factors for dementia in these diverse populations.

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1 INTRODUCTION

The Archer Daniels Midland Company (ADM) have provided the Food and Drug Administration (FDA) with notice that it has determined that the substance soy isoflavone is generally recognised as safe (GRAS). This notice was made in accordance with the FDA proposed rule 'Substances Generally Recognized as Safe' 21 CFR Parts 170, 184, 186 and 570.

In support of this notice, ADM have provided a document entitled 'An information document reviewing the safety of soy isoflavones used in specific dietary applications'.

In my opinion soy isoflavone (or more correctly, the soy isoflavones) should not be granted GRAS status. In fact given the current state of knowledge in the body of scientific literature it would make more sense, in terms of risk assessment, to prohibit the addition of soy isoflavones to foods. Further, manufacturers should act to minimise the exposure of the human and animal population to these compounds that appear to occur in all foods that contain soy protein. This opinion is based on my understanding of the scientific literature on soy isoflavones and some experience as a researcher in the field.

I have read the ADM supporting document and have found that, as a scientific document, it is seriously lacking. Firstly, the document contains several important factual errors. Some of these errors were so blatant that it caused me to reflect on ADM's moral and legal obligation to present an accurate case to the FDA. Secondly, I noted that the references cited in the ADM document frequently misrepresented, and sometimes bore no relation to, the conclusions made by the authors cited. Thirdly, the ADM document does not present the full body of current scientific evidence regarding soy isoflavones. Several notable omissions exist in relation to the potential for soy isoflavones to cause breast cancer and thyroid disease. Finally, many of the conclusions reached in the ADM document are not based on factual evidence and logic but rather assumption and belief. As such the ADM document does not represent good science.

These scientific deficiencies in the ADM document, and counter arguments, are detailed in the sections below.

2 SOY ISOFLAVONES: HISTORY OF USE

In order to prove the GRAS status of soy isoflavones it is critical for ADM to demonstrate that soy isoflavones have enjoyed a long and safe history of use. Hence ADM claim that 'these isoflavone components...have been consumed by millions of humans for over two thousand years'. However, their claim is not based on fact and neither is there any evidence provided to substantiate their claim.

The claim that isoflavones have been consumed for thousands of years has become quite common in isoflavone scientific literature, however it is no more than an assumption and appears based on the general perception that historical soybean consumption was widespread in Asia.

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Although soybean products have been consumed in some parts of Asia for many hundreds of years (1) they did not form a significant part of the diet (2). Also, the traditional soybean was quite different to the soybean as we know it today.

Glycine soja, the wild soybean, is found in northern, north-eastern and central China, adjacent areas of the former USSR, Korea, Taiwan and Japan. *Glycine soja* is the species of soybean that was consumed traditionally and is the ancestor of the modern cultivar, *Glycine max* (3).

The isoflavone content of *Glycine max* was first reported about 60 years ago (4) but it is impossible to know with certainty whether *Glycine soja* contained isoflavones. It is well established that *Glycine max* is, compositionally, quite different to *Glycine soja*. For example, *Glycine max* contains approximately 21.0% oil compared with 9.8% in *Glycine soja* and *Glycine max* also contains more protein (3). This is quite expected because *Glycine max* has been cultivated to have maximised economic potential.

It has also been shown that plants such as that as *Glycine max* produce phytoestrogens such as the soy isoflavones as a defence mechanism in response to pests (5). Increased disease resistance has been a consistent goal of soybean breeders and it is quite conceivable that this goal has served to increase the levels of isoflavones, and other naturally occurring toxins, in *Glycine max*.

It is also well established that different cultivars of *Glycine max* can contain widely variable levels of isoflavones (6). If this is so then it is not implausible that the traditional Asian soybean, *Glycine soja*, contained quite low levels of isoflavones, or perhaps none at all.

Therefore, a counter argument to the ADM claim of long and safe use could be that isoflavones have entered the human food chain only in relatively recent times. It has been the cultivation of *Glycine max* coupled with mass production technology and incorporation of soy protein into numerous foods that has resulted in these compounds being almost unavoidable in the human diet. This mass exposure has only occurred in the last 30 years and it is still undetermined whether isoflavones are safe or not.

In summary, ADM cannot show a long and safe history of use because there is no evidence to substantiate their claim 'that isoflavones have been consumed by millions of humans for over two thousand years'.

3 SOY ISOFLAVONES: SAFETY OF USE

ADM claim 'a long safe history of consumption for soy products and soy foods'. The issue of the safety of soy products in relation to isoflavone toxicity and risk/benefit considerations has been the subject of a recently published paper (7) by a senior scientist at the FDA National Center for Toxicological Research (NCTR), Dr Daniel Sheehan. Sheehan is 'unconvinced that the long history of apparent safe use of soy products can provide confidence that they are indeed without risk' and likens soy

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products to herbal medicines stating that the 'confidence that soy products are safe is clearly based more on belief than hard data'.

Even if ADM'S claims in relation to soy isoflavones, 'no toxic effects at normal dietary levels', were correct (which they are not, see Section 4) this does not provide evidence that soy products are safe. This is because the potential harmful effects of soy isoflavones have never been thoroughly investigated.

There have been several studies that attempt to define the acute toxicity of soy isoflavones in various experimental animals and these are cited in the ADM document. However, the prime concern in relation to estrogenic compounds such as the soy isoflavones is the potential for chronic endocrine system and reproductive toxicity and alterations to the immune system (8,9). As such the harmful effects of soy isoflavones would not have been obvious if they did exist. A compelling example is the estrogenic drug, diethylstilbestrol (DES). Treatment with DES continued for over 20 years before physicians fortuitously made the association between its use and the incidence of a rare type of malignancy in DES daughters (10). In the case of soy isoflavones, however, the fact that estrogenic compounds are present in soy foods has not been general knowledge to health professionals until quite recently. Therefore, any link between effect and cause is unlikely to have been made.

Until more extensive epidemiological studies are undertaken with clearly identified endpoints (such as breast cancer, thyroid disease or immune system dysfunction) it must be concluded that there is no certainty that soy isoflavones are safe at all.

4 SOY ISOFLAVONES: ADVERSE EFFECTS

ADM argue that 'these isoflavone components...have been consumed by millions of humans for over two thousand years with no recorded adverse effects'. Furthermore ADM claim that 'published epidemiology and feeding studies in both animals and humans indicate no toxic effects at normal dietary levels' and that 'soy isoflavones, as part of a soybean based diet, are not associated with reports of adverse health effects'.

It is difficult to reconcile these statements with published scientific literature which is replete with reports of adverse effects and toxicity of isoflavones at dietary levels. In fact it was the toxicity of dietary levels of isoflavones to animals that first raised the awareness of the scientific community to the fact that soy isoflavones were endocrine disruptors (11).

Reproductive effects, infertility, thyroid disease or liver disease due to dietary intake of isoflavones had been observed for several animals including cheetah (12), quail (13), mice (14), rats (15), sturgeon (16) and sheep (17).

With regard to sheep toxicity ADM claim that the 'adverse effects were attributed to feeding on subterranean clover and are associated with coumestrol and the isoflavone formononetin'. This is another example of misinformation in the ADM document. In fact it is generally accepted that sheep metabolise formononetin to the soy isoflavone

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daidzein. Daidzein is, in turn, metabolised to equol which is believed to be responsible for the type of infertility referred to as 'clover disease' (18). There can be no doubt that if sheep were fed a diet supplemented with soy isoflavones they would, depending on dose and duration, develop clover disease.

In another study it has also been reported that 9 out of 20 young calves died when fed a soybean milk replacer (19). The authors implicated 'phenolic compounds' as the reason of increased prostaglandin synthesis, gastrointestinal disorders, tachycardia, bronchoconstriction and death. Soy isoflavones have the potential to interfere with normal prostaglandin synthesis and are, therefore, a likely explanation for this toxicity in calves. It should be noted that in a control group of calves fed an ethanol extracted soybean milk replacer, only 4 out of 20 deaths occurred. Ethanol extraction reduced the amount of phenolics, which would have included isoflavones, in the soybean milk replacer 2.18% to 1.00%.

ADM claim that 'infertility effects are not general to all animals' citing work by Lundh (20). However, this author does not even investigate inter-species differences in reproductive toxicity due to isoflavones. Rather, his work shows how different species metabolise isoflavones differently. Although not all animals become infertile after consuming soy isoflavones at normal dietary levels for restricted periods, feeding at such levels does result in profound endocrine effects in all animals species studied to date.

ADM also claim that 'soy isoflavones have been widely consumed and are recognised to be non-toxic' citing Petrakis et al. (21) and Setchell et al. (22). In fact, nowhere in either of these papers do the authors state that soy isoflavones are recognised as non-toxic.

Petrakis et al. found that consumption of soy protein has a stimulatory effect on the premenopausal breast. Although Setchell et al. state that 'there is no evidence to suggest that ingestion of isoflavones...has adverse effects in human beings', they acknowledge 'the potential effect that these bioactive compounds may produce...is unknown'.

It is incorrect to state that there is no evidence of harmful effects of soy isoflavones on humans. In fact there is mounting evidence that dietary levels of soy isoflavones cause thyroid disease and may increase the risk of breast cancer.

Goitre and hypothyroidism were reported in infants fed soybean diets until the early 1960's (23). In fact recent reports indicate that thyroid disorders may be attributable to feeding soy-based infant formulas (24-25). Further, a study on 37 adults showed that diffuse goitre and hypothyroidism appeared in half of the subjects after consuming 30 g per day of pickled roasted soybeans for three months (26). These findings are consistent with the recently proposed mechanism by which soy isoflavones affect thyroid hormone synthesis (27).

It is concluded that soy isoflavones can be the cause of thyroid disorders in soy consumers and, hence, there is every indication that cases of goitre and hypothyroidism in infants were caused by the soy isoflavones. Unless diets that include soy isoflavones

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are adequately supplemented with iodine, goitre will result. In this regard Kay et al. discuss the minimum safety iodine requirement for a soybean diet (28).

However, even if iodine supplementation does occur, under conditions of high chronic doses of isoflavones persistent inhibition of thyroid hormone synthesis could potentially lead to thyroid cancer (27).

With regard to breast cancer, Dees et al. have shown that dietary concentrations of genistein may stimulate breast cells to enter the cell cycle; this finding led these authors to conclude that women should not consume soy products to prevent breast cancer (29). This work is consistent with an earlier report by Petrakis et al. who expressed concern that women fed soy protein isolate have an increased incidence of epithelial hyperplasia (21).

There is no doubt that soy isoflavones are biologically active in humans. The first report of a definitive experiment which showed this involved the consumption of 60g of soy protein per day for one month by pre-menopausal women (30). The soy isoflavones disrupted the menstrual cycle during, and for up to three months after, administration. With regard to this study the ADM document claims 'no adverse effects were noted' but the authors of the original paper did not state this. It is appreciated that there are varying opinions in the scientific community as to what constitutes toxicity. In recent times, however, there has evolved a greater understanding of endocrine disrupters and their effects. Many now view the multiplicity of effects that endocrine disrupters can induce as toxic effects (8).

The inclusion of endocrine disrupters in human diets should not be taken lightly. With specific reference to soy-based infant formulas the high soy isoflavone intake of this population group has led Dr Sheehan to note that infants fed soy-based formulas have been placed at risk in a 'large, uncontrolled, and basically unmonitored human infant experiment' (31). If soy isoflavones are granted GRAS status this experiment would spread to the greater population and millions would be exposed to compounds which are increasingly being shown to have adverse effects.

Also, the synergistic effects that soy isoflavones may induce when combined with other xenoestrogens that the human population are exposed are beyond the scope of this document. However, there is a general thesis that because of the potential for synergistic effects, human exposure to all endocrine disrupters, such as the soy isoflavones, requires urgent reduction (8).

5 SOY ISOFLAVONES: BENEFITS

In recent times there have been numerous claims that isoflavones prevent hormone related diseases such as breast cancer. Under some conditions genistein has been found to inhibit breast cancer cell growth (32). However, there is no consensus amongst scientists that isoflavone ingestion reduces breast cancer risk.

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Recently the UK government published a definitive review assessing the effects of phytoestrogens in the human diet (33). This study found that there was almost no evidence linking health benefits from foods containing isoflavones to the isoflavones themselves.

Similarly in their review of phytoestrogens and western diseases, Adlercreutz and Mazur assert that any benefits from soy products are not due to isoflavones specifically. They conclude that the combination of a high phytoestrogen intake with a western diet may not be beneficial (34).

ADM state that 'epidemiological studies between Western and Far Eastern populations suggest that components of soybeans may contribute to important health effects'. However an epidemiological study in China has shown that high soy intake is not protective against breast cancer (35).

Based on evidence to date it is concluded that there is little evidence for the beneficial effects of soy isoflavones. Indeed authorities in the field do not support the ADM thesis that soy isoflavones 'provide positive health maintenance benefits'.

6 SUMMARY AND CONCLUSIONS

In conclusion, the recognition by the Archer Daniels Midland Company that soy isoflavones are generally recognised as safe (GRAS) is seriously flawed. The supporting document entitled 'An information document reviewing the safety of soy isoflavones used in specific dietary applications' contains factual errors, misrepresents cited authors and does not present the full body of current scientific evidence. The conclusions reached in the ADM document are not based on fact:

- There is no evidence of a long and safe history of use or that 'these isoflavone components...have been consumed by millions of humans for over two thousand years'.
- It is not correct that 'published epidemiology and feeding studies in both animals and humans indicate no toxic effects at normal dietary levels' or that 'soy isoflavones, as part of a soybean based diet, are not associated with reports of adverse health effects'.
- Benefits of dietary intake soy isoflavones have not been proven.

To the consumer, dietary soy isoflavones represent a clear risk whereas the benefits are highly questionable. Rather than accept that soy isoflavones are GRAS, it is my opinion that regulatory agencies such as the FDA should give full attention to consumer protection and deny GRAS status to soy isoflavones.

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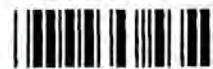
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Dr. Linda Kahl
Office of Pre Market Approval
Food and Drug Administration

FAX (202) 418 3131

~~Anthony E. Jones~~

R. D. L.
WILSON GREENE
NEW ZEALAND
April 24 1998

Dear Dr Kahl

E. R. N. # 000001

When I first communicated with your office on March 25 1998, I asked how one would make submissions.

I can't see that I have ever had clear guidance.

It seems to me that to fail to have an open debate is unconstitutional. The procedure is inappropriate for the subject matter involved.

Anyway, for what it is worth, my submission is simple:

- ① Isoflavones have been shown by your own National Center for Toxicological Research to be carcinogenic:

"ANTI-THYROID ISOFLAVONES FROM SOYBEAN":

Divi, Chang, and Doerge: 1997 Biochem. Pharm 54 1087-1096

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- ② Genistein, a soy isoflavone has been shown by the Oak Ridge, TX, Health Science Center to proliferate breast cancer cells at dietary doses:

"DIETARY ESTROGENS STIMULATE HUMAN BREAST CANCER CELLS TO ENTER THE CELL CYCLE" Dees, Foster Ahmed and Wimasalene: EHP 1997 105(3)

Therefore they cannot be regarded as "SAFE". In fact they are forbidden by the Food Safety Act 1997

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detection, and when peaks were not present, fractions of 1 min were collected across the entire elution profile. The collected fractions were dried *in vacuo* and dissolved in 100–200 μ L of eluent 1. A 50 μ L aliquot was then added to the tyrosine iodination assay to determine inhibition of TPO activity (see below).

Reagents

HPLC was used to monitor TFC catalyzed oxidation of tyrosine to MIT and DIT. Because the UV absorbance of the isoflavones interfered with the normal spectrophotometric assay [14], The same NovaPak C18 HPLC column was used with solvent system consisting of $\chi =$ acetonitrile, $B = 0.2\%$ trifluoroacetic acid in 5% methanol-water, and a gradient of 10% χ to 40% χ in 12 min with a flow rate of 1.5 ml/min and UV detector at 232 nm. Retention times for tyrosine, MIT, and DIT were 15, 16, and 14 min, respectively.

Preparation of Soybean Extracts

A whole sylvianus (100 g) was cut in two and fed by a tungsten wire electrode from a 100 V battery to a grounded metal powder. The powder sample (5 g) was extracted by stirring with 25 ml of 0.5% methanol (2 N HCl to pH 10.0) for 24 h and filtered at 0.45 μ m. The mixture was centrifuged at 1500 g for 10 min; the supernatant stopped in a tube and the residue dissolved in 10 ml of 0.5% methanol.

[illegible]

A fraction of the above extract was fractionated by column chromatography using methanol and 1.25% diethylamine as eluents. The extract was converted to a solid phase HPLC system using a 15 × 15 cm, 10-μm, 100 Å, Waters (Milford, MA) using a 500M grade silica particle packing (Chemex, Summit, CT). The column was conditioned with a solvent system consisting of 30% acetonitrile and 1 liter of water, increasing to 100% A in 20 min at a flow rate of 1.5 ml/min [A = acetonitrile, B = water (solvent front) with 10.5% (94% by vol.)]. The yield was monitored using a Spectra-Energy variable wavelength photodiode array (spectral type 1, San Jose, CA) at 20 min intervals by observing UV spectra every 20 min. The fraction HPLC system was used to separate the components of the sample. Elution was carried out with a mobile phase gradient starting with 50% A and increasing linearly to 100% A in 20 min at a flow rate of 1.5 ml/min. Peaks were detected using 275 nm absorbance.

For determination of ^{14}C efficiency, all ^{14}C labeled identifiable peaks were collected after emerging from the

Inhibition of TPP-Catalyzed Reaction.

Different amounts of soy extract (1–100 μ g mL⁻¹) were added to different concentrations (2–80 μ M) of TPC in order to determine the concentration of soy extract that inhibited TPC inhibition. The solution was incubated for 5 min and the reaction stopped by adding it onto the HPLC column. Tyrosine (100 μ M) and ascorbic acid (100 μ M) or gallic acid (2.5 mM) were incubated with TPC (10 nM) plus inhibitor, and the reaction was initiated by the addition of H₂O₂ (100 μ M) at 22 \pm 0.1°C. HPLC was used to follow the concentration of 3,4-DHT, and as the solvent system was acetonitrile containing TPC, the H₂O₂ generating system consisting of

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Antithyroid Activity of Soybean

glucose (125 mM), pH 7.0, glucose oxidase (10 mM) and gentamicin (100 mM).

Inhibition of thyroid peroxidase (Sigma Chemical Co.) was carried out using iodobeads (Pierce Chemical Co., Rockford, IL) using 10 beads in 5 mL MES buffer, pH 7.0, that contained 250 μ M iodide to generate I_2^- for 1 min. Then a 10- μ L aliquot of enzyme solution (1.25 mg/mL) in the same buffer was added. After incubation for 10-15 min at room temperature, the solution was dialyzed overnight. The degree of iodination was estimated spectrophotometrically using the change in absorbance at 290 nm as a measure of DIT formation ($\Delta A = 0.92/\text{nmol I}_2^-$, see Ref. 15). The content of MIT and DIT in iodinated casein was confirmed for one sample using HPLC analysis after proteolytic digestion of iodinated casein; reasonable agreement with the spectrophotometric determination was seen (not shown).

Measurement of coupling in an *in vitro* assay of thyroid hormone synthesis was carried out in the presence of TPO (Sigma) using chemically iodinated casein (1.25 mg/mL containing approximately 50-60 nmol I/mol) as the source of iodotyrosines [16]. Xenopus concentrations of 10 nM and 100 nM (100 μ M) for 1 h in 0.25 M MES buffer, pH 6.5, at $37 \pm 0.1^\circ$. Bovine insulin, alkaline phosphatase (50 units, Sigma Chemical Co.) was added to hydrolyze phosphate groups, including phosphotyrosines. This treatment increased yield of T_4 by approximately 25%. The reaction mixture was digested under a nitrogen atmosphere using pepsin (250 μ g/mL final concentration) for 1 h and then with leucine aminopeptidase (50 μ g/mL) for 3 h [17]. Thyroid hormones in the reaction mixture were extracted three times with ethyl acetate, and the extract was dried in vacuo and dissolved in a 100- μ L aliquot of starting HPLC solvent. Extraction efficiency for T_4 was estimated at 92-95%. Thyroid hormones were measured by HPLC using a Hamilton PRP11 reversed phase column with a solvent system of A = acetonitrile, B = trifluoroacetic acid in water (0.25:0.75, 1% v/v) starting with 10% A in B and increasing linearly to 50% A in 20 min at a flow rate of 1.0 mL/min. The peaks were detected and iodothyronines quantified using 230 nm absorbance.

Measurement of TPO catalyzed coupling was also carried out using human γ -over Ty, essentially as described by Tait et al. [13] except that the products of HPLC analysis described above were used to quantify iodothyronines. Samples were analyzed in triplicate for at least four different concentrations of soybean fractions.

Inactivation of TPO by Isoflavones

TPO was inactivated by isoflavones by incubating enzyme with 50 μ M daidzein or 50 μ M genistein in 0.25 M MES buffer, pH 7.0, at $25 \pm 0.1^\circ$ for 24 h. The remaining activity was determined by measuring the formation of T_4 in the presence of iodinated casein and TPO.

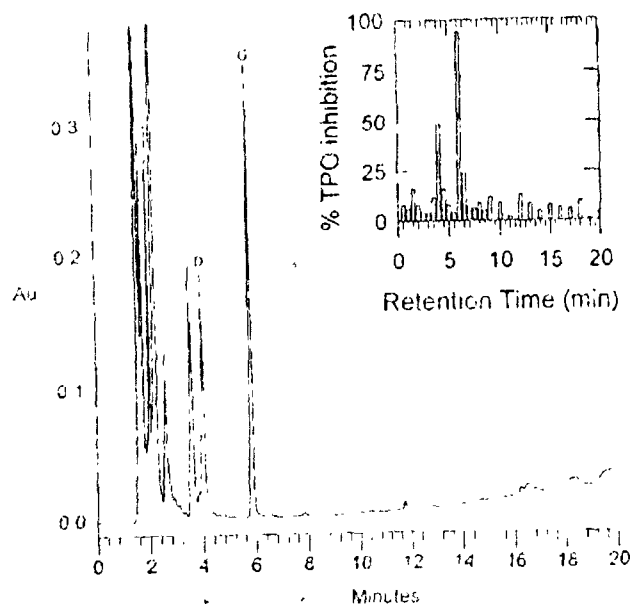


FIG. 1. HPLC fractionation of a soybean extract and inhibition of TPO-catalyzed tyrosine iodination. The soybean extract was fractionated using HPLC and UV detection (260 nm) as described in Materials and Methods, and the individually collected fractions were tested for inhibition of TPO activity (inset). The maximal TPO activity was 9 nmol MIT formed/min.

inactivation of TPO by centrifugal dialysis or extensive dialysis as previously described [14]. In separate experiments to study its protective effect on iodination, iodide (0.1 to 5.0 mM) was also included in the reaction mixture.

RESULTS

Characterization of Compounds Inhibiting TPO in Crude Soybean Extract

The presence of a crude extract from soybean was investigated using a heated, acidic methanol hydrolysis and extraction procedure. This procedure was selected to liberate the respective glycone from a glycoside conjugate, are the predominant form in whole soybean [19, 20]. It was determined that this procedure completely converted genistein and daidzein conjugates to the respective aglycones (data not shown). When the extract was fractionated using HPLC, two distinct peaks at UV absorbance retention times 4.2 and 5.8 min (see Fig. 1) were found to contain most of the inhibitory activity (Fig. 1 inset). Peaks labeled B and C showed UV absorption maxima at 251 and 259 nm, respectively (data not shown). These chromatographic and spectral properties were identical to those observed from authentic standards of daidzein and genistein, respectively. The extract fractionated with the extract showed no evidence for inhomogeneity. The genistein and daidzein content based on HPLC analysis of the acidic hydrolyzed extract was determined to be 0.3 and 0.7 μ g/g, respectively, using external standard calibration.

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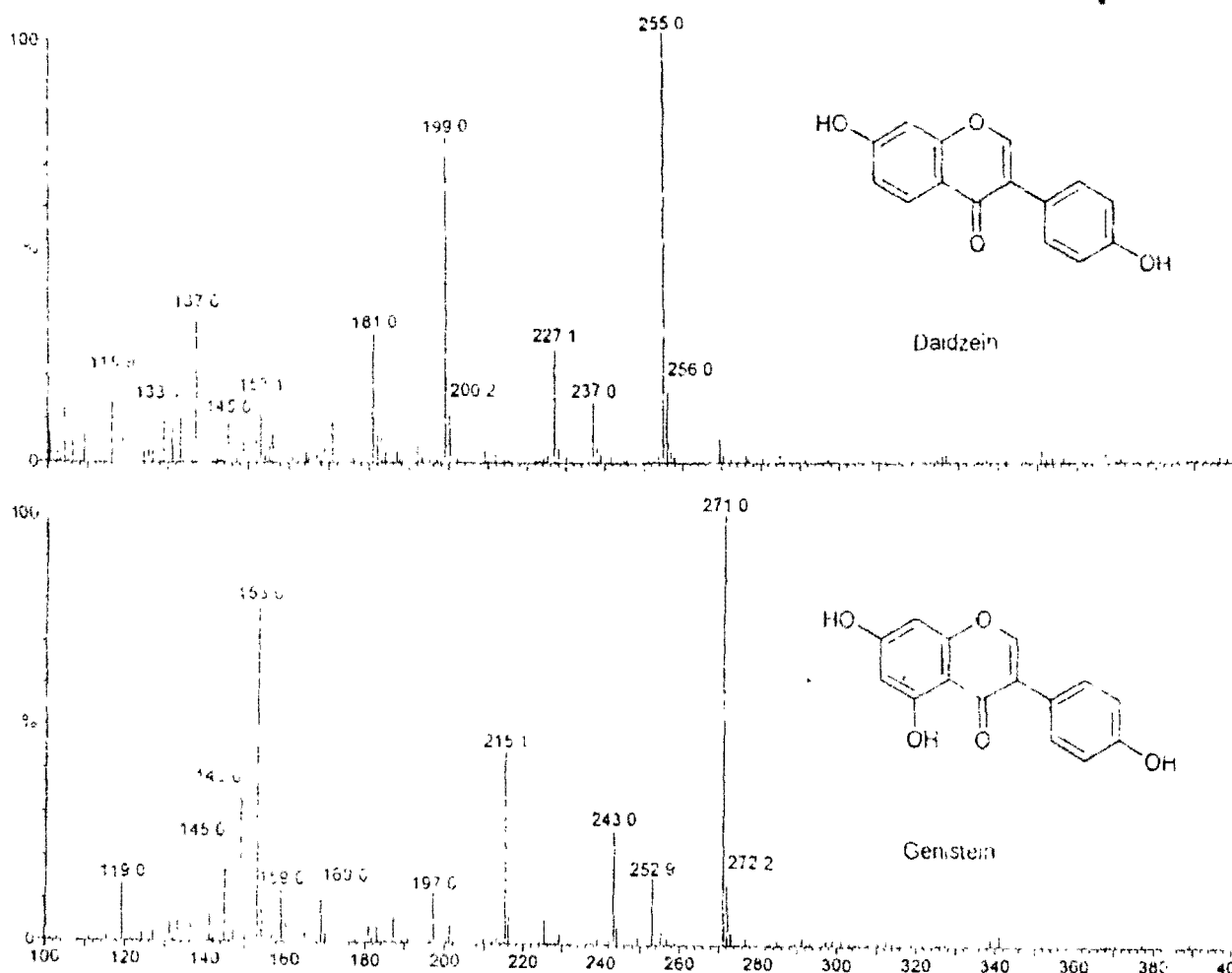


FIG. 2. On-line LC-APC/MS analysis of genistein and daidzein from a soybean extract. Mass spectra were obtained following on-line LC separation of genistein ($M + H^+ = 271$ Da, bottom panel) and daidzein ($M + H^+ = 255$ Da, top panel) in the soybean extract as described in Materials and Methods. The spectra were obtained using a sampling cone skimmer potential of 50 V.

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These values are consistent with the total isoflavone content in soybean reported previously [21, 22].

The soybean extract was analyzed further by on-line APC/MS under conditions that produced mass spectra containing protonated molecules ($M + H^+$) and fragment ions (see Fig. 2). Mass spectra from peaks D and A contained protonated molecules corresponding to the masses predicted for daidzein and genistein (255 and 271, respectively). Diagnostic fragment ions were also observed. Not only were the observed fragment ion masses and fragment ion chemical structures (from retention time standard in a library) but the even-odd mass pattern in the CID spectra previously reported for daidzein and daidzein using the same procedure (see above) and electrospray spectrometry [21, 22]. Since the chromatographic, spectroscopic, and TPV inhibitory properties were found to be identical with those isolated by authentic isoflavone, subsequent mechanistic studies were carried out with pure isoflavones.

Inhibition of TPV-Catalyzed Iodination and Coupling by Isoflavones

Genistein and daidzein were found to inhibit TPV-catalyzed iodination of tyrosine. The IC_{50} values for these reactions were estimated to be 32 and 22 μ M, respectively. These values were similar to those reported previously for related flavonoids [14]. The glycoside genistein was approximately 10-fold less potent than the aglycone with an IC_{50} value of 38 μ M, and HPLC-UV analysis showed the commercially product to be of the aglycone (OD_{280} of 0.25 μ l aliquot of the crude extract corresponds to 500 μ g of isoflavone powder) produced 50% inhibition of TPV-catalyzed tyrosine iodination activity (data not shown). It was possible to compare the inhibition of TPV activity by the crude extract with that predicted from the measured isoflavone content. The extract aliquot contained 0.99 μ g genistein and 0.37 μ g daidzein, and these amounts are predicted to produce approximately 67% inhibition of

TABLE 2. Inhibition of TPO-catalyzed coupling in iodinated casein by genistein

Conc. test. (μ M)	I_4 Residues (% control), average, $N = 2$
0	100
1	93.8
2	56.3
4	32.7
8	23.8
16	20.3

Hydroxy- β -M is reduced to β -M by a lithium chloride-catalyzed (25 mg/ml) alkaline phosphate-coupled enzymatic system in HEPES (pH 7.4) at 37°C for 1 h. The final and reduced form is sweet, light blue, practically colorless, and is stable in water, even at higher temperatures. Hydroxy- β -M is found in the form of a 1:1 adduct with the common food dyes present in the vegetables. The detection limit (2.76 μ g/g) is very low (5 ng/ml) and is constant. In the case of the detection of β -M, the detection limit is 0.2 μ g/g, even at 28°C, and the detection is very stable.

TPD catalyzed iodination in the 1:0 vol. incubation. This correlates closely with the 50% inhibition of TPD catalyzed tyrosine iodination. Absence of iodination of the soybean extract. These results also provide evidence that no major inhibitor of thyroid compound is present in the soybean extract.

Cinnemine was also tested for the ability to inhibit iodohyamine formation by using iodinated cinnemine model substrate that permits measurement of TPO catalyzed coupling [16]. In this reaction, cinnemine bound iodotyrosyl residues are oxidatively converted by TPO in the presence of H_2O_2 to protein bound T_4 and tT_4 . The formation of prominent amounts of tT_4 , but not T_4 , is not observed in vitro in model systems that use human galectin-1 as a coupling substrate [18, 23]. However, in all experiments, T_4 formation mirrored T_4 formation, and inhibition by anti-thyroid chemicals was similar for both products. Bovine milk casein is a complex multimeric aggregate (~300 kDa) consisting of subunits with an average monomer molecular mass of 23.3 kDa [24] and contain 3.5 tyrosyl residues/mol [25]. Before treatment with TPO, the amount of total iodohyamines detected by HPLC in hydrolyzed iodocasein was below the detection limit ($T_4 = 3026 \mu\text{g/mg}$ casein, or 16 ng on column). In the absence of an inhibitor, TPO catalyzed the formation of approximately 1

μ , T_4 /mg casein (approximately 0.85 residues of T_4 , 0.35 residue of rT, and ca. 0.17 residues of T_3 per mol of aggregated casein assuming a molecular mass of 312 kDa, see Ref. 24) under the conditions described. Because the small amounts of T_4 observed were constant and unaffected by inhibitor concentration, we concluded that this resulted from artifactual degradation of T_4 during sample preparation. Table 1 shows the concentration-dependent inhibition of T_4 synthesis in isolated casein by genistein for duplicate experiments. Inhibition of rT₃ formation showed similar results (not shown). Using the combined data set for the two separate experiments, the IC_{50} value for genistein was approximately 3 μ M.

Additional experiments were performed using human goiter Tg to measure the formation of thyroid hormones and its inhibition by isoflavones in a simultaneous iodine/coupling procedure [18]. When using Tg as the substrate, T_4 was the predominant product (1.00 ± 0.03 newly formed residues/nucleic (Tg)), and only trace amounts of T_3 and FT were observed. These studies confirmed the inhibition of coupling alone by genistein described above for iodinated casein. Genistein, daidzein, and genistin inhibited the formation of T_4 in a concentration-dependent manner (see Table 2), and the IC_{50} values for genistein, daidzein, and genistin were approximately 2.0, 8.8, and 40.6 μ M, respectively. Genistein and daidzein also inhibited TPO-catalyzed oxidation of guinacol. The IC_{50} values for these reactions were 3.7 and 12.4 μ M, respectively.

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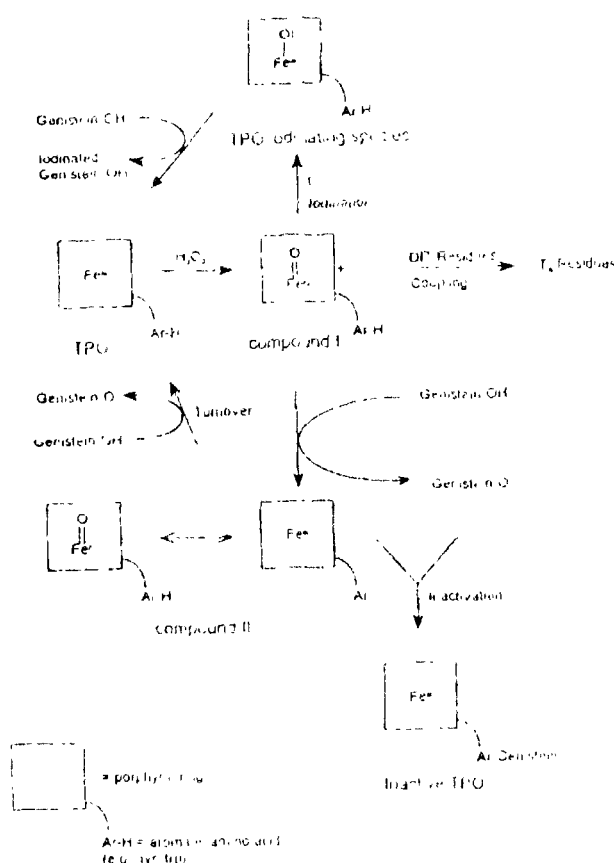
Characterization of Iodinated Isoflavones

Genistein and androgen were potent inhibitors of TPO-catalyzed tyrosine iodination (Table I above). Genistein inhibition of TPO-catalyzed tyrosine iodination produced kinetics consistent with die nature of state inhibition previously described for chlorogenic acid [14]. In this mechanism (see Scheme 1), two tyrosine molecules are oxidized by TPO compound I produces an oxidizing intermediate that is the equivalent of enzyme-bound hydrogen peroxide [26]. Iodination of tyrosine by the enzymatic oxidizing intermediate is blocked by flavonoids because of preferential iodination of

TABLE 2 Isoflavone inhibition of LPO catalyzed coupling in human gastric TG

Gemstem (μ M)	T ₁ Residues (% control)	Dand. an. (μ M)	T ₁ Residues (% control)	Gemstem (μ M)	T ₁ Residues (% control)
0	100	0	100	0	100
1	52.4 \pm 7.6	2.5	76.9 \pm 6.5	10	98.3 \pm 12.0
2.5	44.0 \pm 6.0	5	78.5 \pm 2.6	25	70.8 \pm 10.8
5	19.8 \pm 5.1	10	30.5 \pm 4.8	50	46.9 \pm 9.5
10	12.2 \pm 2.4	25	12.2 \pm 1.7	100	44.1 \pm 8.7

HAU-04-3M was incubated with HRP at pH 7.0 in 0.1 M glucose, 5 mM dithiothreitol, 5 mM glucose oxidase, 0.76 μ M T₆, and various concentrations of H₂O₂ for 10 min at 37°C. Topical and internal reaction mixtures were incubated with proteinase and the



SCHEME 1. Proposed mechanisms for genistein inhibition of TPO catalyzed reactions.

the flavonoid, presumably by forming a ground-state complex with the electrophilic enzyme species.

Figure 3 shows the conversion of genistein to three products under such conditions. Analysis of reaction products using on-line LC-APX-MS gave mass spectra consistent with derivatives containing either one, two, or three iodine atoms. The positive-ion mass spectra are characterized by abundant protonated molecular ion (M^+) as observed for the parent isoflavone (see Fig. 1). In addition, the oxidation products show fragmentation as characteristic losses of successive iodine atoms ($M-127$, $M-254$, $M-381$) indicating the presence of mono-, di-, and triiodinated genistein. For example, the triiodinated genistein spectrum consists of M^+ (m/z 642) and fragment ions corresponding to the protonated diiodo (m/z 523) and monoiodo derivative (m/z 397), as well as genistein (m/z 271). Similar results were obtained with androgen where not 100% consistent with 1,4,5-triiodo-3,7,8-trimethoxy derivative (M^+ m/z 642) and fragment ions corresponding to the protonated diiodo (m/z 523) and monoiodo derivative (m/z 397), as well as androgen (m/z 271). Similar results were obtained with androgen where not 100% consistent with 1,4,5-triiodo-3,7,8-trimethoxy derivative (M^+ m/z 642) and fragment ions corresponding to the protonated diiodo (m/z 523) and monoiodo derivative (m/z 397), as well as androgen (m/z 271).

[11] By analogy we propose that genistein is mono- and

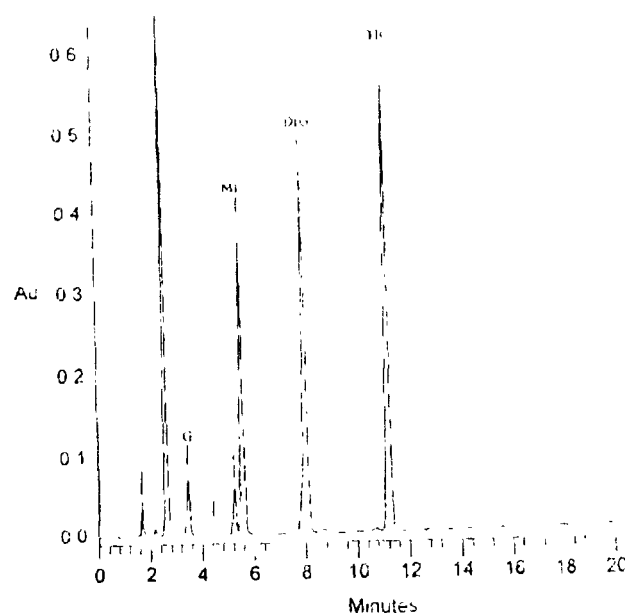


FIG. 3. TPO-catalyzed iodination of genistein. Genistein (25 μ M) was incubated with TPO (50 nM) and iodide (100 μ M) as described in Materials and Methods. The reaction was initiated by the addition of H_2O_2 (250 μ M) and after 5 min the reaction mixture was analyzed using HPLC with UV 275 nm detection. Abbreviations: G, genistein; MIG, monoiodo-genistein; DIC, diiodo-genistein; and TIC, triiodo-genistein.

diiodinated in the resorcinol ring (C6,S), and the third site for iodination is presumably the ortho position on the phenol ring (C3').

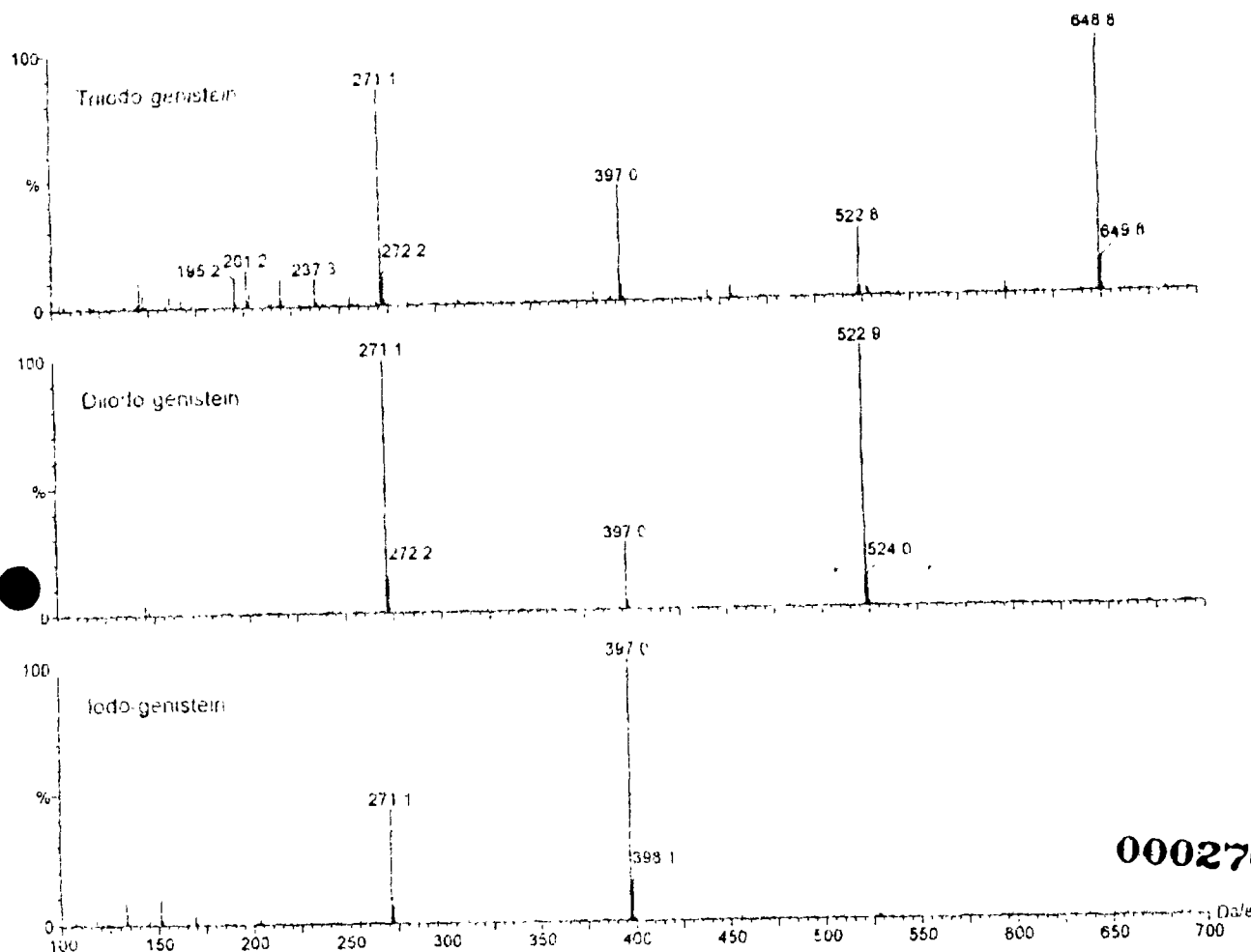
Inactivation of TPO by Isoflavones 000269

In the presence of H_2O_2 , genistein and diadene inactivated TPO by 60–70% (see Table 3). However, the glucoside genistin produced no loss of enzymatic activity under these conditions (data not shown). The inactivation was time dependent, increased with increasing concentrations of isoflavone or H_2O_2 , and was enhanced when the incubation was conducted under an aerobic condition. The inactivation was concentration dependent with a 60–80% loss of Soret absorbance (λ 419 nm) and shift of the absorbance maximum from 412 nm (native TPO) to 415 nm (genistein-inactivated TPO) or 419 nm (diadene-inactivated TPO) (Fig. 5). The presence of at least 150 μ M iodide in the incubation mixtures completely protected against genistein- or diadene-mediated TPO inactivation (see Table 3).

DISCUSSION

The results presented here demonstrate that isoflavones genistein and diadene are the compounds contained in a hydrolyzed extract of soybean that inhibit TPO catalyzed reactions. The hydrolytic procedure was required to convert the predominantly conjugated isoflavones present in soybean to aglycones as previously described [19, 20]. The

Anti-thyroidal isoflavones from Soybean



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FIG. 4. Mass spectra of iodinated isoflavones. Incubations of TPO with genistein were conducted as described in Fig. 4 except LC-APCI/MS with a sampling cone skimmer potential of 15 V was used to generate mass spectra of the genistein-derived products.

extract was fractionated by reversed-phase HPLC separation (see Fig. 1), and the peaks containing inhibitor, acetylcholinesterase, and spectroscopic properties (UV, APCI/MS) identical to authentic genistein and

TABLE 3. H₂O₂-dependent inactivation of TPO by isoflavones

Reaction conditions	Activity (% of control)
TPO + H ₂ O ₂	99 ± 1
TPO + daidzein	96 ± 7
TPO + genistein	97 ± 6
TPO + daidzein + H ₂ O ₂	95 ± 5
TPO + genistein + H ₂ O ₂	98 ± 9
TPO + I ⁻	97 ± 5
TPO + daidzein + I ⁻ + H ₂ O ₂	94 ± 9
TPO + genistein + I ⁻ + H ₂ O ₂	95 ± 9

TPO (1.0 μM) was incubated with 50 μM daidzein or 50 μM genistein, 150 μM H₂O₂, and 200 μM H₂O₂ in the combinations indicated at 25 ± 0.1° in 0.1 M MES buffer (pH 7.0). After 4 min, aliquots were withdrawn and diluted 1000 times in 0.1 M MES buffer. For the control, the maximal TPO activity was 9.0 and 10.0 μM H₂O₂ (to 1.0 μM H₂O₂) respectively.

daidzein (see Fig. 2). All of the TPO inhibitory activity present in the extract was accounted for by the measured amounts of genistein and daidzein. Determining total isoflavones in daidzein glycosides after hydrolysis can only give the maximum possible in thyroid potential of soy products because glycoside conjugates, which are the predominant forms in soybean, are weakly inhibitory. The mixture of glycoside conjugates and aglycones present in soybean have been shown to be bioavailable through identification of glucuronide and sulfate conjugates of isoflavones in plasma from humans consuming soy products [22]. This suggests that the conjugates are hydrolyzed during absorption from the gut as the aglycones. However, this does not give information about uptake into the thyroid, another critical factor for assessing goitrogenic potential that must be determined in future studies in vivo.

Genistein also inhibited TPO-catalyzed phenolic oxidations including guaiacol oxidation and coupling of diiodotyrosyl residues in casein and thyroglobulin to form diiodotyrosines (see Tables 1 and 2). These reactions proceed by phenoxyl radical intermediates, and the pres-

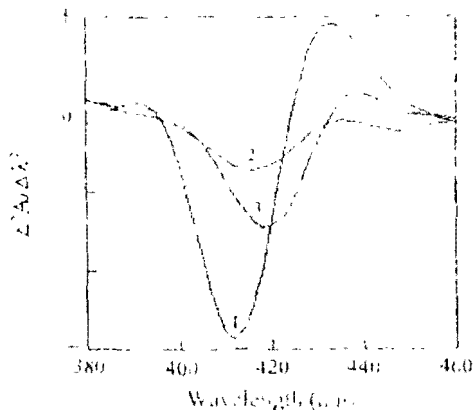


FIG. 5. Visible spectra for TPO and collagen-inactivated TPO. TPO (1 μ M) was incubated with genistein or daidzein (50 μ M) and H_2O_2 (200 μ M) as described in Materials and Methods. After a 4-min incubation, second derivative visible spectra were recorded. The spectra are shown for native TPO (1), genistein-inactivated TPO (2), and daidzein-inactivated TPO (3).

ence of phenolic group in the isoflavone could be predicted to react with extracellular peroxidase produced intermediate, or both, of H_2O_2 coupling [11, 15, 17]. The degree of inhibition of coupling in a simultaneous reaction, coupling is a strong T_2 was the same as that observed in a coupling only assay system using previously oxidized casein as the substrate. Because of the ability of the thyroid body to produce T_2 and to concentrate iodide, it is likely that coupling in vivo occurs in a manner more comparable to the simultaneous assay. Furthermore, the λ_{max} values obtained for isoflavones were similar for inhibition of iodination and coupling. This suggests a primary interaction between isoflavone and TPO that inhibits coupling and iodination activity in a similar manner. Furthermore, the levels of total isoflavones observed in human plasma following consumption of soy foods (c. 1 μ M; see Ref. 22) approach the concentrations reported to inhibit or inhibit TPO catalyzed reactions.

In the absence of iodide, genistein and daidzein showed time-dependent increases in the negative absorbance of TPO, consistent to distinct changes in the visible spectrum of the heme prosthetic group (see Fig. 5). These observations are similar to those made for inactivation of TPO by other flavonoid (flavonolignan, quercetin, rutin, and kaempferol; see Ref. 14) although the spectra of inactivated TPOs had different long wavelength absorption maxima (428 nm for rutin, 419 nm for kaempferol, 419 nm for quercetin, and 415 nm for genistein). These data are consistent with the covalent modification mechanism for casein, as previously proposed (previously proposed for TPO, H₂O₂, and C₁ [19, 28]). Despite the dramatic changes in the heme visible spectrum, the results to date are consistent with enzyme inactivation through covalent linkage to the heme prosthetic chain and not to the heme prosthetic group (see Scheme 1 and Ref. 29).

The inhibition of TPO catalyzed iodination and coupling *in vitro* is consistent with the numerous reports of anti-thyroid effects in human and animals from consumption of soy products, especially in cases of iodine deficiency. Many issues regarding the bioavailability of soy isoflavone conjugates and uptake into the thyroid remain unanswered. However, the demonstrated effects of the isoflavones presented here, and the well documented goitrogenic effects of soybeans in humans and animals, do provide a logical starting point from which possible anti-thyroid mechanisms can be examined. The different mechanisms reported for inhibition *in vitro* of the enzymatic reactions in thyroid hormone biosynthesis by isoflavones are useful for predicting potential anti-thyroid effects in animals and humans under several different dosing circumstances.

(A) In the normal case of iodine sufficient individuals receiving intermittent or low doses of soy isoflavones, alternate substrate iodination would consume the inhibitory compounds after which T_2 iodination and coupling reactions would resume unaffected. Since the normal thyroid contains significant amounts of iodide, its high substrate activity should prevent inactivation of TPO.

(B) In the case of iodine deficiency, low or intermittent doses of isoflavones could further deplete iodide levels by covalent incorporation of iodide into iodinated products. Also, enzymatic oxidation of the isoflavone would increase as the intrathyroidal iodide level decreased. Under these conditions, it is possible that inactivation of TPO could occur. This would produce a more long-lasting inhibition of hormone synthesis because enzymatic activity could be replaced only through de novo protein synthesis. However, either the alternate substrate inhibition or enzyme inactivation outcome is consistent with the anti-thyroid effects from soy observed in rodents maintained on an iodide free diet [7, 9] and with the ability of added iodide to reverse the goitrogenic effect of a soy can diet in rats [7]. There are also reports of goiter and hypothyroidism in a human infant receiving soy based formulas [3, 4] and evidence for elimination of such effects upon addition of iodide to the diet [3]. For this reason, it appears that iodide supplementation of formula during infancy is necessary to complement [1].

(C) In proposed rodent carcinogenicity bioassays, high doses of isoflavone will be administered chronically in a normal iodide containing diet. Under these conditions, complete blockade of iodination and coupling is possible even with normal dietary iodide because alternate substrate inhibition would dominate. Since the body burden of isoflavone is replenished continuously through feeding, the inhibition of thyroid hormone synthesis would persist throughout the lifetime of the animal. This hypothesis is consistent with observations of the hypothyroid condition that occurs in human consuming principal foodstuffs (e.g. millet) that contain large amounts of anti-thyroid flavonoids [30, 31]. It is possible that these anti-thyroid effects could be reversed by the addition of iodide to the diet through universal iodination programs.

Finally, other possible toxicologic consequences from ingestion of soy isoflavones come from the demonstrated estrogen receptor binding activity of genistein and daidzein [33, 34]. Although anti-mutagenic properties from soy bean isoflavones have been suggested [33, 35], the dose-response relationships that may separate toxic and beneficial responses are not clear. A possible consequence of TPO-mediated soy isoflavone modulation is modulation of such estrogenic receptor binding activity or changing the pharmacokinetics of elimination. For example, McCann *et al.* [36] reported an increased estrogen receptor binding affinity as well as a decrease in metabolism for 4-hydroxytamoxifen relative to the parent compound. Further experimentation will be required to assess the importance of TPO-mediated modulation in the biological activity and excretion of isoflavones.

We appreciate the assistance of Mr. J. C. Karch, of the NCHD, and many nurses, doctors, and the F. D. Research Department of Pediatrics, Children's Hospital Medical Center, in making it possible for me to participate in this study and to learn the details of the contributions by the African American Community of Detroit, who provided the human material through their kind and very helpful donation. R. L. D. and H. C. S. also supports this child's ship from the end. R. L. D. and H. C. S. would like to thank, also, the staff of the child and interagency committee for their use of the F. D. Department of Pediatrics, the Child and Family Administration. This research was supported by the National Agency for Negro Children, NCHD, and the National Institute of Child Health and Human Development, National Training Program.

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Clinical and Laboratory Pearl

Abnormal Thyroid Function Tests in Infants with Congenital Hypothyroidism: The Influence of Soy-Based Formula

1998 APR 27 A 9:33

Mohammad A. Jabbar, MD, Jennifer Larrea, RN, and Renee A. Shaw, BS

Department of Pediatrics (M.A.J., J.L.) and the Department of Research (R.A.S.), Hurley Medical Center, Evans, Michigan

Key words: soy formula, thyroxine, thyrotropin, hypothyroidism

Objective: To assess the etiology of hyperthyroxinemia or hyperthyrotropinemia in infants with congenital hypothyroidism who are on replacement therapy with L-thyroxine.

Methods: These infants were treated with recommended doses of L-thyroxine following the diagnosis of congenital hypothyroidism. Because of hyperthyroxinemia (2 patients) and hyperthyrotropinemia (3 patients), medication compliance and dietary intake/formula type/dilution and discontinuation or change of the formula were assessed. Clinical evaluation was also performed.

Results: Elevated thyroxine level in 2 infants was associated with discontinuation of soy formula 4 weeks previously. Serum thyroxine was normalized when both of these infants. In the third infant who received soy formula for 1 week, thyroxine level remained elevated despite incremental L-thyroxine dose of 10 µg/kg/day. Discontinuation of soy formula was followed by normalization of the TSH in 3 weeks and helped in a subsequent decrement of L-thyroxine dose to 8.5 µg/kg/day. Neither the hyperthyroxinemia nor hyperthyrotropinemia in these infants was associated with any adverse behavioral/developmental consequence.

Conclusion: When initiating soy formula feeding in infants with congenital hypothyroidism, the L-thyroxine dose should be increased because of significant reduction in intestinal absorption. Conversely, when a preceding reduction in the L-thyroxine dose should be decreased.

INTRODUCTION

Infants with congenital hypothyroidism require adequate thyroxine replacement therapy as a prerequisite for a good outcome. In infants and young children, overtreatment with thyroxine preparations may result in enhanced linear growth, precocious puberty, and behavioral changes. On the other hand, undertreatment of congenital hypothyroidism may lead to poor linear growth, excessive weight gain, and impaired development. While the daily dose requirement and adequate treatment primarily depend on the severity of the disease and patient compliance, dietary commitments may affect the various indicators of thyroid function [1-3].

Since the dietary factors involved are important for the treatment, it has been reported to cause jitter and hyperthyroidism [4]. In clinical and animal studies [5-8], soy-based formulas are often chosen by parents and pediatricians

for feeding of infants with presumed milk allergy [9]. In this report, we describe a sequential relationship between soy formula feeding and abnormalities in thyroid function tests in three infants with congenital hypothyroidism. The clinical course is also described. We conclude that soy formula feeding increases the thyroxine dose requirement. While a change from soy to cow's formula reduces the thyroxine dose requirement.

METHODS

All infants with congenital hypothyroidism were followed monthly for 6 months and twice monthly thereafter, unless an abnormality was detected. This follow-up included clinical and laboratory studies. Because of elevated thyroxine (T₄) or thyrotropin (TSH) level, we analyzed the L-thyroxine dose and

clinical course of feeding and the dietary intake of these infants with congenital hypothyroidism.

Based at full term, none of these infants had a documented history of thyroid disease before or during pregnancy. The diagnosis of congenital hypothyroidism was suggested from abnormal newborn screening TSH level (100 µU/mL) and was later confirmed by clinical laboratory test (T₄ 0.1 µg/dL and TSH 10.1 mIU/L) before the initiation of therapy. The patients were 6.5 and 10.0 (patient 1 and 2) and 1.0 (patient 3) and 7.7 (patient 3). During the initial 6 weeks of therapy, all infants with hyperthyroxinemia and hyperthyrotropinemia. These studies excluded other etiologies such as congenital goiter. All of the infants were given L-thyroxine (Eli Lilly, Kroll Pharmaceuticals, Mount Olive, NC) 100 µg/mL to T₄ daily as replacement therapy. The dosage of 50 µg/day was calculated at the time of initiation and followed until compliance to the medication and adherence to the diet. Dilution was also determined during these visits. Serum T₄ and TSH levels were measured by the radioimmunoassay laboratory of our institution.

RESULTS

T₄ and TSH Levels

Abnormal serum T₄ and TSH levels in relation to the change of formula are shown in Table 1 for two of the infants and children who were receiving L-thyroxine replacement therapy. Two infants (patients 1 and 2) had elevated serum T₄ levels. Parental compliance of the recommended medication schedule was adequate. However, dietary history revealed that feeding was switched from soy formula (Nutrilac) to cow's milk formula 4 weeks prior to the abnormal test, accounting for elevated serum thyroxine level. Decreasing the L-thyroxine dose from 9.2 µg/kg/day to 8.5 µg/kg/day in patient 1 and from 8.5 to 7.6 µg/kg/day in patient 2 normalized serum thyroxine level in 3 weeks. Serum thyroxine level were within high normal limits in both patients prior to soy formula discontinuation.

One infant (patient 3) had elevated TSH with normal serum T₄ that during abnormality was 10.1 mIU/L. This infant, who was on soy formula (Soylent) since 1 week of age, was started on a thyroxine 6 µg/kg/day at 2 weeks of

age. This dose was started on an empiric basis, was within recommended guidelines and was similar to the dose given other infants with congenital hypothyroidism at that time. Thyroxine remained high despite a gradual increase in L-thyroxine dose to 10 µg/kg/day during the next 4 months. At 5 months of age, feeding was changed from soy formula to cow's milk formula, which normalized TSH level. Subsequently L-thyroxine dose was decreased to 14 µg/kg/day, and TSH level remained normal during the follow-up.

Seventeen of our 22 patients (including infants and children) who received L-thyroxine replacement therapy did not have any abnormalities in their T₄ and TSH level during follow-up. None of these patients received soy formula during infancy.

Feeding History

These three infants with thyroid function abnormal were fed soy formula because of parental concern about milk allergy. Soy formula was started at 2 months, 2 weeks at week of age and was continued until 6 months, 2.5 months, 5 months of age, respectively. At that time, soy formula was discontinued and cow's milk formula and supplements were introduced. None of the three infants had obvious behavioral/clinical alterations attributable to the low thyroxine levels. However, long-term, comprehensive follow-up is not available.

The timing of soy introduction related to thyroid replacement therapy was also reviewed in these patients. In patient 1, discontinuation of soy feeding was preceded by initiation of replacement therapy. In patients 2 and 3, it was vice versa. In patients 1 and 2, the serum T₄ and TSH normalized 4 weeks of start of therapy, while in patient 3, TSH was normalized until soy feeding was discontinued 5 months later. This suggests that thyroid function test abnormalities originate from the timing of soy feeding introduction.

DISCUSSION

The effect of soy formula feeding (or its withdrawal) on T₄ and TSH levels is shown in three congenital hypothyroid infants who were on replacement therapy. The timing of soy formula feeding increases the dose require-

Table 1. Change of Thyroid Function Tests and Adjusted L-Thyroxine Dose Requirement During and After Discontinuation of Soy Feeding

Patient number	Thyroid function test results	Age at soy introduction (months)	Age at soy discontinuation (months)	Maximum dose while on soy (µg/kg/day)	Dose after discontinuation (µg/kg/day)
1	22.0 (normal) to 42.5 (elevated)	5	9	9.2	8.5
2	10.1 (normal) to 14.5 (elevated)	4	2.5	9.3	8.5
3	10.1 (normal) to 14.5 (elevated)	1	5	10	14

Abbreviations: T₄, thyroxine; TSH, thyrotropin.



To: Linda Kahl@OPA@FDA.CFSAN
From: "Dave Woodhams" <woodhams@iprolink.co.nz>
Certify: N
Subject: GRAS Notice GRN 000001
Date: Tuesday, April 28, 1998 at 11:20:34 pm EDT
Attached: None

Dear Dr Kahl

NOT ORIGINAL COPY

On 31 March 98 I sent you an Email relating to GRAS Notice GRN 000001.

Would please confirm receipt of this and advise what action is being taken by FDA on the issue.

I advise that I am today mailing a letter to you with copies of my correspondence with the NZ Nutrition Foundation attached. As ADM quote conclusions from the Foundation's position paper on soy infant formula this correspondence is relevant to ADM's GRAS determination.

Also enclosed with the letter is a hard copy of this and my previous Email, together with a copy of the Japanese paper, and its translation, referred to in the latter communication: (Y. Ishizuki, Y. Hirooka, Y. Murata and K. Togashi "The effects on the thyroid gland of soybeans administered experimentally in healthy subjects" Nippon Naibunpi gakkai Zasshi, 67, 622-629 [1991]).

have also enclosed a brief CV to place my credentials on record.

Under separate cover I am mailing copies of the first three newsletters of the Soy Information Network which cover the basis of our concerns, critiques of the soy industry response to our concerns and an invited article from Dr Messina, a soy proponent, in response to previous issues of the newsletter.

Under the same cover I am also sending a copy of my March 1995 paper on infant formula which is derived from the original Aspell Report by Dr Mike Fitzpatrick. This latter report, my introduction and my derivative paper on soy infant formula are available from the NCTR Library in Jefferson, Arkansas. My own paper was never intended to be submitted for independent publication and thus has not been peer reviewed but Dr Fitzpatrick's reports were independently reviewed before they were placed before the NZ Ministry of Health in November 1994. The three page independent review by a University of Auckland toxicologist is also included in the volume held by the NCTR Library.

Yours sincerely

000424

Apr 29, 1998

Dr David J Woodhams CEng
Dairy Process Consultant
PO Box 32 236
2/47 Church Street
Devonport 1309
New Zealand
Phone: +64 9 445 8721
Fax: +64 9 445 9834
Email: woodhams@iprolink.co.nz

000425

Apr 29, 1998

To: Dr. Linda Kahl, Office of Premarket Approval, Center for Food Safety, Food and Drug Administration, 200 C Street, S.W. (H-FS-200)
Washington D.C. 20204.

At: Facsimile 001-202-418 3131

From: ~~Debbie T. [unclear]~~, Rural Del: July 4, Whangorei, NZ.

At: Facsimile 64-9-434 0567.

Date: Tuesday, April 28th, 1998.

Re: GRAS Notice # GRN 000001 (ADM)

Dear Dr. Kahl,

Today I mailed you copies of a number of published scientific papers from my study files. Because I realize ~~that~~ some misrepresentations may have been made to you about my convictions and affiliation, I have chosen to provide entire documents so that they can be independently assessed, rather than just provide quotes which can actually mislead as to the impact and message of the entire paper.

The ADM submission 000001 contains many mistakes of fact. The following documents have been mailed to you to illustrate just a few. It would take a "basket," rather than a "package" to provide documentation to illustrate all of the mistakes.

The papers I have selected are as follows, with reasons given for inclusion in the mailed "package"

1. "Genistin (an Isoflavone Glucoside) and its Aglycone Genistein from Soybeans"; Walter 1961. (Front page only). This shows that the ability to extract isoflavones from soy is ~~approx~~ 000430

40 plus years old and is not a newly developed technique.

2. One page from a "data base" showing that geisterin (from studies of the 1950's) when added as 0.2% of diet elicited estrogenic effects. (I apologize for poor quality of reproduction as the information forced from data base, arrived here like that)

3. "Effect of Geisterin on Reproduction of the Mouse."

Carter, Matrone and Smot. (This is a classic paper and often quoted) Diet 1, was control with geisterin removed, Diet 2, had 0.02% added back, Diet 3 made up of commercial soy bean meal, with approx 0.01% geisterin.

Summary Both commercial soybean oil meal and isolated geisterin significantly lowered the age at which the vaginas of immature mice opened. The principal effect on reproduction of 0.02% geisterin in the diet was a decrease in the number of litters born, whereas litter size was not affected. The commercial soybean oil meal (80% of diet) was a decrease in the number of litters.

4. "Effect of Geisterin on Growth and Development of the Male Mouse." Carter, ^{Smot} Matrone, Smot.

Results Ten mice died, 4 receiving highest level of geisterin, two receiving third and four were scattered among other levels. Males on highest level of geisterin lost weight. Geisterin also had depressing effect on testes weight. No spermatozoa present in the testes of groups receiving two higher levels of geisterin. The higher levels of geisterin appeared to be lethal. "It appears from these results that geisterin, in relation to its estrogenic activity, has a greater depressing effect than does stilbestrol."

5. "Metabolism of Estrogenic Isoflavones in Domestic

Animals" Lundh. Soc. for Exp. Bio and Med, 1995

This is reference #11 in A.O.M. presentation. It does NOT state "infertility effects are not general to all animals" as submitted by A.O.M. It does discuss reasons for differences between sensitivity between pigs, sheep and cattle. In cattle, daily intakes may reach up to 50 - 100 g ^{estrogenic phytoestrogens} per day which may result in temporary infertility in cattle. The paper discusses detoxification mechanisms of the rumen, and more serious effects in sheep because, it is suggested, cattle have fewer estrogen receptors in uterus. (2 to 4 times higher in sheep than cattle. Pigs are more sensitive than either.

P. 36. Glucuronidation is the major detoxification system for potentially toxic endogenous and exogenous substances including phytoestrogens."

Note that for cattle and sheep the phytoestrogens in this red clover silage were Formonesetin, daidzein and equol found mainly as conjugates in the blood (about 5% of total amount as unconjugated substances in the plasma.

6. "Hormone Studies in Ewes Grazing on ~~Estrogenic~~ (Parloup clover Pasture During the Reproductive Cycle" Obst, Semarb.

1975 Aust. J. Biol Sci 28 279-90.

"Clover Disease" described as a "syndrome of aberrant reproductive function" signs are "ewe infertility, dystocia, uterine prolapse resulting in high ewe and lamb mortality."

Study investigated "the possibility that the clover acts by causing disturbances in the endocrinology of reproduction." "The mean content of the isoflavones formonesetin, genistein and biochanin A in Parloup clover during July and August 1970

was 1.2, 2.5 and 0.54% respectively of dry leaf weight."

The result was that "performance of ewes grazing Parloup

is associated with marked disturbances in reproductive

endocrine function.

7. "Toxicants of Plant Origin" Edited by Peter Cheek, 1989.
(Chapter 2, Phytoestrogens by H.R. Adame.)

Isoklavones (Table 2) listed as: — Formononetin, Genistein, Biochanin A. plus a discussion of isoflavones which were "very low in the absence of infection". "Plant estrogens have less affinity for the receptor than steroidal estrogens and the ranking is similar across species of animals." "In every case that has been examined, phytoestrogens produce changes in the reproductive tract which are qualitatively the same as those produced by endogenous estradiol. Both steroidal and plant estrogens affect the ovary and mammary gland. Exogenous estrogen causes dysplasia of the ovarian granulosa cells, thus impeding the maturation of the ovarian follicles." Note p. 36 comment on DES | estradiol | coumestrol. "Sheep grazed on highly estrogenic pastures consisting of relatively pure stands of subterranean clover or red clover containing high concentrations (0.8%) of the isoflavone formononetin may suffer marked clinical signs. Ewes may be affected by severe infertility accompanied by gross pathological changes in the uterus, including hydrops uteri, pyometra, cystic hyperplastic endometritis, or massive adhesions resulting from metritis." "The infertility results from abnormal function of the pituitary/ovarian axis. The reproductive problem frequently resembles the abnormalities associated with cystic ovaries, including prolonged but irregular estrus, nymphomania, or even the development of masculine sexual characteristics".

8. "Organizational and Activational Effects of Phytoestrogens on the Reproductive Tract of the Ewe" Adame. Soc. Exp. Biol. 1985

"Isoklavone compounds including genistein, Biochanin A and formononetin

occur in subterranean clover or red clover. Genistein and Biochanin A are usually broken down by microbial activity in the rumen of the sheep while formononetin is converted to the isoflavan equol, which is rapidly absorbed and is responsible for most of the estrogenic activity in ruminants."

"Temporary infertility results from actions of estrogen that are similar to the activation effects of estrogen in most species of mammals. The permanent infertility results from changes to the cervix which are analogous to the organizational effects of estrogen reported in other species treated during organogenesis."

9. "Phytoestrogen Content and Estrogenic Effect of Legume Fodder", Schorini, Wähli, Nykänen-Kurki, Karela and Saastomoinen Soc. for Exper. Bio and Med. 1995.

"All red clover studied contained estrogenic isoflavones, especially formononetin and Biochanin A. The phytoestrogen content varied from 1.0% to 2.5% of dry matter."

"Interest in phytoestrogens has generally been aroused by their adverse properties. They may, however, also be beneficial by increasing the growth of animals and the milk yield of cows." "Biochanin A and genistein, which in monogastric animals have an estrogenic effect, are broken down in the rumen of ruminants. daidzein and formononetin are converted by ruminal microbes into the active equol." The chart on p. 15 shows phytoestrogen content of red clover. That content consists of

Daidzein, Genistein, Formononetin, Biochanin-A.

"Red clover has the highest phytoestrogen content of Finnish legume fodder plants, varying from 1% to 2.5% of dry matter. The biological effect on the uterus of formononetin is large."

10. "Clinical Changes in Ovariectomized Ewes Exposed to Phytoestrogens and 17 β -Estradiol Implants",

Nwānenna, Lundh, Madej, Fredriksson, and Björnøy. Soc. for Exp. Bio. Med. 1987.

"Genistein and other isoflavones daidzein, biochanin A, and formononetin were isolated from clover and proved to be the cause of the disorder." "Mammary glands were noticeably more voluminous, and palpation revealed the presence of irregular, solid lumps of various sizes towards the end of the feeding period and long afterwards. The udder gradually changed from ^{pale to} pink, ~~and~~ then to red with increasing edema." "In this experiment, the irregular solid lumps of tissue found during clinical examinations looked like developing tumors.

Genistein, but not formononetin, and the estrogenic metabolite of formononetin called equol, can stimulate the growth of estrogen-dependent breast cancer cells in vitro. The estrogenic activity of biochanin-A and genistein in ruminants is limited to the first few days of exposure when the unadapted rumen microbes cannot convert them to their non estrogenic metabolites p-ethyl phenol and phenolic acid. formononetin... considered to be the overall cause of clinical changes observed."

000435

11. "The Effects of Plant Oestrogens on Animal Reproduction"
Donald A. Shaw, Endeavour 1976.

Note that this paper is very commonly cited as an authority. "The isoflavones genistein, biochanin A and formononetin occur in clover and many clover species."

"The reason why formononetin is oestrogenically more active than genistein in the sheep was found to lie in the digestion of plant constituents in the modified digestive system of the sheep." "It has been shown that the degradation of

biodanin A and genistein to inactive phenols in the rumen of the sheep becomes ^{increasingly} more efficient over a period of about five days." "There appears to be a shorter adaptive period in the cow and the detoxification and excretion of

the absorbed isoflavones and metabolites are more efficient."

"Physiologically, clover-induced infertility is brought about by a combination of factors, including interference with spermatozoan transport through the genital tract of the ewe, abnormal transport of ova and interference with implantation. There is also evidence that the neuro-endocrine

centres in the brain controlling the reproductive cycle of the ewe are suppressed." "However, the techniques of

identification and quantitative assessment of oestrogens in plants should allow a quicker screening for possible

oestrogenic compounds when they are suspected of reducing fertility in other animals, or having a contraceptive action in

women." "From the wider ~~point of~~ viewpoint of evolution it is interesting that compounds have evolved in plants that not only give the plant some protection from foliar pathogens, but also reduces the fertility of animals ingesting the plant!"

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"Plant products have been used in folk medicine from ancient times as aphrodisiacs, aids in childbirth, abortifacients and promoters of fertility. Both increases and decreases in fertility in animals have also been attributed to specific compounds in plants." "Clinical effects

"It became apparent that plant estrogens could reduce the fertility of ewes in the absence of obvious clinical signs."

If ewes are exposed to estrogenic pasture for more than two seasons they may suffer a decline in fertility from which they never recover. --- However permanent subfertility usually occurs in the absence of obvious clinical signs.

13. Effects of Plant Estrogens in Ruminants

H.F. McDonald. Proc. of the Nutr. Soc. of NZ. 1995 Vol 20.

"Fortunately management plans can be devised to avoid feeding oestrogenic herbage at critical times to breeding livestock." "The isoflavones are mainly found in the

clovers" "The effects of ingesting oestrogenic compounds in the ruminant should be anticipated with a variety of animal responses. These include anabolic and lactation responses as well as effects on reproduction. A spurt in mammary growth and udder secretion is often noticed in immature sheep." "Embryo mortality - Growing

000437

ewes on oestrogenic clover may cause oviductal hyperplasia and uterine oedema with a likely effect on the developing embryo." "Feeding Red clover for 8 days before and during the first cycle of mating resulted in higher service times than in control animals. The effects on conception persisted for at least 3 weeks after removal from red clover, but an earlier recovery of oovulation

The reason that say based formulae sales dropped in N.Z. when it became apparent that they contained phytoestrogens was because New Zealanders were from farming stock (pastoral) and so both benefits and risks of phytoestrogen exposure from plants ~~was~~ commonly understood. (ie Nothing to do with the imagined "lobby groups". !!!)

Every issue of this magazine, provided to farmers through New Zealand, contains comments on the effects of phytoestrogens because an understanding of them is basic to good animal husbandry. The copies include → An advertisement for a new strain of clover with lower oestrogen levels to "give fewer fertility problems with breeding stock".

→ An advertisement showing proportion of clover to ryegrass seed for pasture. Note that proportion of clover to total feed is rather low, normally.

c) Information 0000438

Effects of various levels of clover in pasture and relation to milk yield (increases yield) But cautions exist e.g. "Phyto-oestrogens in the new cultivars are 65% lower than in the older cultivars, greatly reducing the risk of problems with animal production"

d) Letter to the editor,

showing level of interest in and understanding of estrogen exposure within the N.Z. community. "Estrogens are involved in thyroid function, which suggests a link..."

involved in thyroid function, which suggests a toxic estrogen is likely to be a thyroid poison. It is time to find out. Estrogen has been known to induce serious

000439

reproductive failure and failure of beta development."

15. Letter to the Editor of *ATDOG* in Pediatric

Forum. Keri Montague-Brown. "The authors found positive statistical associations between premature melanoma and consumption of various meat products and soy-based formula, and a maternal history of ovarian cysts."

16. Second page of "Short paper" Frances Patterson.

(Front page faced previously) Effects (estrogenic) were noted when soy was 5% of diet. Later studies done when soy was 10% of diet. Diet gives DES equivalents.

000440

17. "Phytoestrogens: Toxicology and regulatory recommendations"

Robertson. Proc. of the Nutr. Soc. of NZ. 1995 Vol 20.

"It must be stressed that beneficial and adverse effects are not mutually exclusive." "Whatever the implications for fertility in women, it is clear that addition of quite

modest amount of soy protein to a complex diet had physiological effects, which lasted for several months after soy was removed from the diet." "The main effects are those due to possible alteration of endocrine dependent regulation. These might include carcinogenic, reproductive and developmental outcomes, including effects on sexual maturation and behaviour, which would not necessarily be readily apparent either in time or in appearance"

18. "Dietary Intervention Study to Assess Estrogenicity of Dietary Soy among Postmenopausal Women" Baird, Umbach, Liss, et al.

Hughes, Setchell, Weinberg, Henry, Wilcox and McLachlan.

"nor did sex hormone binding globulin increase" "Phytoestrogens do not bind well to SHBG."

Note that these results have been repeated in many studies. The claim of ADH that phytoestrogen administration increases SHBG is not due to be substantiated. I'll follow these notes with a page from the "Final Report to MAFF" which also states this.

19. Journal of Pharmaceutical Sciences "Potential Value of Plants as Sources of new antifertility agents" (Parts I & II, 1975. Note that soy was included in these considerations (because of isoflavone content.)

20. "The Case for Expanded Phytoestrogen Research"

Sheehan, Report of introduction to 2nd Internat. Phytoestrogen Conference. 1995 Proc. Soc. for Exp. Bio & Med. 1995.

"A sequence of findings in experimental animals demonstrated that phytoestrogens possess the same wide range of biological activities previously found with traditional estrogens." "As with any biologically active chemical, it is crucial to define adverse versus beneficial effects of the phytoestrogens".

Thank you for considering the above!

1872



Institute for Environment
and Health

IEH assessment on

PHYTOESTROGENS IN THE HUMAN DIET

FINAL REPORT TO THE
MINISTRY **OF** AGRICULTURE, **FISHERIES AND** FOOD

November 1997

000442

The Institute for Environment and Health (IEH) was established by the Medical Research Council at the University of Leicester in 1993. The Institute is partly funded by the various UK government departments and agencies by way of specific research and consultancy contracts.

This literature review has been prepared by IEH for the Ministry of Agriculture, Fisheries and Food. The principal focus of this document is on the potential beneficial effects of phytoestrogens on adults. Potential detrimental effects on adults and the influence on other life stages were specifically excluded from consideration. It also contains an assessment of the factors influencing the phytoestrogen content of food and the relative potencies of the various phytoestrogens. The assessment incorporates the output of a workshop held in Leicester in March 1997 which was chaired by Professor Lewis Smith, IEH. The Institute gratefully acknowledges the contribution of all those who attended the workshop and provided material for inclusion in the assessment but assumes no endorsement from these scientists for the conclusions and recommendations contained herein.

The Ministry of Agriculture, Fisheries and Food has provided funding for this project but has not conducted the research or written this report. The views expressed here do not necessarily represent those of any government department or agency.

Prepared by:

Dr Charles Humphrey and Mr Philip Holmes, IEH

* Literature search to November 96, supplemented by additional papers to June 97

000443

producing a number of effects which have been associated with a reduced breast cancer risk. In premenopausal women, soya has been shown to increase the length of the menstrual cycle and/or delay menstruation, and to reduce the levels of LH, FSH and progesterone at various stages of the cycle. The reported effects of soya on blood levels of 17β -oestradiol have not been consistent, one study reporting a reduced level throughout the cycle (Lu *et al.*, 1996), another reporting an increase in the follicular phase only (Cassidy *et al.*, 1994) and another finding no change in levels (Baird *et al.*, 1995). Based on the studies reviewed, the evidence for changes in levels of the adrenal androgen DHEAS is conflicting and further investigations would be required to clarify these opposing findings. Plasma levels have been shown to vary with energy intake; for example, a recent study in premenopausal women found that for each additional 1MJ (239kcal) consumed, levels of DHEAS decreased by 5.1% (Dorgan *et al.*, 1996). To ensure that energy intake does not confound intervention studies using soya products, it would seem sensible to ensure that all diets investigated are isocaloric.

Both lignans and isoflavones have been reported to increase the levels of plasma SHBG, which may decrease the blood levels of biologically active sex hormones and thus influence cancer risk. Again, however, epidemiological studies are conflicting; levels were lower in postmenopausal women with breast cancer than in vegetarians or omnivores (Adlercreutz *et al.*, 1989 and 1992), although an earlier study found no difference between postmenopausal breast cancer patients and controls (Bruning *et al.*, 1985). In more informative controlled trials, in which lignans (linseed) or soya (as textured vegetable protein and miso) was added to the diets of premenopausal women, a small, but significant, decrease in the level of SHBG was seen only in women consuming the linseed supplement. From these studies, isoflavones do not appear to have any effect on SHBG levels.

Although preliminary, the potentially important finding of Petrakis *et al.* (1996) that soya consumption may have an oestrogenic effect by increasing the incidence of hyperplastic epithelial cells in the nipple aspirate fluid of pre- and postmenopausal women constituting a risk factor for breast cancer, should be the subject of further investigation.

Overall, these studies show that phytoestrogens are biologically active in women and can affect the levels of sex hormones and potentially therefore contribute to a reduced breast cancer risk. The effects produced have not always been consistent between studies, although this may relate to the use of different doses, types of product used, study design or the generally small numbers of women studied. In order to elucidate, the potential beneficial effects of phytoestrogens in breast cancer risk reduction, further controlled studies in larger populations of premenopausal women are warranted.

6.5 POST-MENOPAUSAL SYMPTOMS

The reported incidence of hot flushes, one of the most common symptoms of the menopause, varies markedly between different countries, with high levels in Europe (from 70-80% of postmenopausal women), intermediate levels in Malaysia (57%),

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FAXED & MAILED

Dr Linda Kahl
Office of Pre-Market Approvals
U. S. F. D. A. # HFS 806
200 C St SW
Washington DC 20204

~~R. F. Jones~~
R. D. H.
WIRANGAREI
NEW ZEALAND
APRIL 29 1998

FTN 64. 9 -4340567

Dear Dr Kahl

GRN # 000001

I have today airmailed to you a number of published research reports to support my previous correspondence.

Dr Archer Daniels Midland has used a historic definition of "toxicity" to equate with the LD-50 standard for rat baits, and has ignored chronic toxicity, hormonal toxicity, and carcinogenicity. I have submitted slightly different material from that listed in my first communication of 3/25/98

This is what is coming:

000445

1. Dietary Estrogens: Probable Cause of Liver Disease and Infertility in Captive Cheetahs - Setchell et al.
2. Causes of Adverse Responses to Soybean Milk Replacers in Young Calves. - Gardner et al
3. Roasted Soybeans and an Estrogenic Growth Promoter Affect the Thyroid Status of Beef Steers - Rumary et al
4. Anti Thyroid Isoflavones from Soybean: Isolation Characterization and Mechanism of Action - Dai et al
5. Effects on the Thyroid Gland of Soybean Administered Experimentally in Healthy Subjects - Ishizuki et al
6. Breast and Soy-Formula Feedings in Early Infancy and the Prevalence of Autoimmune Thyroid Disease in Children. - Fort et al

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- 9) Phytoestrogens in Soy Based Infant Foods:
Concentration, Daily Intake and Possible Biological
Effects — Irvine et al
- 10) Herbal Medicines, Phytoestrogens and Toxicity:
Risk Benefit Considerations — Sheehan
- 11) Dietary Genistein Exerts Estrogenic upon the Uterus,
Mammary Gland, and the Hypothalamic/Pituitary
Axis in Rats — Santell et al

In each of the enclosed research reports, I have placed arrows alongside other reports in the reference lists. I wish those other reports to be regarded as an integral part of this submission.

Your namesake, Dr Stanislaw Kahl, a co-author of paper # 3 above, seems to be a scientist whom you should consult.

Sincerely

~~Richard J. Jones~~

e

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Computer Tecnnology Services, Inc.

To: Dr. Linda Kahl, Office of Premarket Approval,
Center for Food Safety, Food and Drug Administration
200 C Street, S.W. (HFS - 206) Washington D.C.
20204

At For. 001 - 202 - 418 - 3131.

From: ~~Walter James~~ at For 64 - 9 - 434 - 0567.

Re GRAS GRN 000001 (A.O.M)

Date: April 29th. 1998.

Dear Dr. Kahl,

000451

I completed the following very late last night. Please excuse the writing but since most of it is exact quotes from papers mailed, the original text will be clearer. Please note however, that AOM's assertion that "clover disease" was caused by coumestrol rather than isoflavones in clover is false. Also the effects were more severe than just a reduction in fertility.

Thankyou for your continuing communications.

Yours sincerely,

~~Walter James~~

To: Dr. Linda Kahl, Office of Pre-market Approval, Center for Food Safety, Food and Drug Administration, 200 C Street, S.W. (HFS-206)
Washington D.C. 20204.

At: Facsimile 001-202-418 3131

From: ~~Dorothy Barnes~~, Rural Del: July 4, Whangarei, NZ.

At: Facsimile 64-9-434 0567.

Date: Tuesday, April 28th, 1998.

Re: GRAS Notice # GRN 000001 (ADM)

Dear Dr. Kahl,

Today I mailed you copies of a number of published scientific papers from my study files. Because I realize ~~that~~ some misrepresentations may have been made to you about my convictions and affiliation, I have chosen to provide entire documents so that they can be independently assessed, rather than just provide quotes which can actually mislead as to the impact and message of the entire paper.

000452

The ADM submission #000001 contains many mistakes of fact. The following documents have been mailed to you to illustrate "just a few". It would take a "basket," rather than a "package" to provide documentation to illustrate all of the mistakes.

The papers I have selected are as follows, with reasons given for inclusion in the mailed "package"

1. "Geistin (an Isoflavone Glucoside) and its Aglycone Geistin from Soybeans"; Walter 1961. (Front page only). This shows that the ability to extract isoflavones from soybeans

40 plus years old and is not a newly developed technique.

2. One page from a "data base" showing that genistein (from studies of the 1950's) when added as 0.2% of diet elicited estrogenic effects. (I apologize for poor quality of reproduction as the information forced from data base, arrived here like that)

3. "Effect of Genistein on Reproduction of the Mouse."

Carter, Macrone and Smart. (This is a classic paper and often quoted) Diet 1, was control with genistein removed, Diet 2, had 0.02% added back, Diet 3 made up of commercial soybean meal, with approx 0.01% genistein.

Summary Both commercial soybean oil meal and isolated genistein significantly lowered the age at which the vaginals of immature mice opened. The principal effects on reproduction of 0.02% genistein in the diet was a decrease in the number of litters born, whereas litter size was not affected. The commercial soybean oil meal (80% of diet) was a decrease in the number of litters.

4. "Effect of Genistein on Growth and Development of the Male Mouse." Carter, ^{Smart} Macrone, Smart. 000453

Results Ten mice died, 4 receiving highest level of genistein, two receiving third and four were scattered among other levels. Males on highest level of genistein lost weight. Genistein also had depressing effect on testes weight.

No spermatozoa present in the testes of groups receiving two higher levels of genistein. The higher levels of genistein appeared to be lethal. "It appears from these results...that genistein, in relation to its estrogenic activity, has a greater depressing effect than does stilbestrol."

5. "Metabolism of Estrogenic Isoflavones in Domestic

Animals" Lundh. Soc. for Exp. Bio and Med., 1995

This is referenced in A.O.M. presentation. It does NOT state "infertility effects are not general to all animals" as submitted by A.O.M. It does discuss reasons for differences between sensitivity between pigs, sheep and cattle. In cattle, daily intakes may reach up to 50-100g ^{estrogenic phytoestrogens} per day which may result in temporary infertility in cattle. The paper discusses detoxification mechanisms of the rumen, and more serious effects in sheep because, it is suggested, cattle have fewer estrogen receptors in uterus. (2 to 4 times higher in sheep than cattle. Pigs are more sensitive than either.

p.36. Glucuronidation is the major detoxification system for potentially toxic endogenous and exogenous substances including phytoestrogens. Note that for cattle and sheep the phytoestrogen in this red clover silage were Formononetin, daidzein and genistein found mainly as conjugates in the blood (about 5% of total amount as unconjugated substances in the plasma.

6. "Hormone Studies in Ewes Grazing on ~~Estrogenic~~ (Yarloop clover pasture During the Reproductive Cycle" Obst, Semark. 1975 Aust. J. Biol Sci 28 279-90.

"Clover Disease" described as a "syndrome of aberrant 000454 reproductive function" signs are "ewe infertility, dystocia, uterine prolapse resulting in high ewe and lamb mortality."

Study investigates "the possibility that the clover acts by causing disturbances in the endocrinology of reproduction."

"The mean content of the isoflavones formononetin, genistein and biochanin A in Yarloop clover during July and August 1970 was 1.2, 2.5 and 0.54% respectively of dry leaf weight." The result was that "performance of ewes grazing Yarloop

endocrine function."

- 7. "Toxicants of Plant Origin" Edited by Peter Cheek, 1989.
(Chapter 2; Phytoestrogens by HK Adams.)

Isolflavones (Table 2) listed as: — Formononetin, Genistein, Biochanin A. plus a discussion of coumestrols which were "very low in the absence of infection". "Plant estrogens have less affinity for the receptor than steroidal estrogens and the ranking is similar across species of animals." "In every case that has been examined, phytoestrogens produce changes in the reproductive tract which are qualitatively the same as those produced by endogenous estradiol. Both steroidal and plant estrogens affect the ovary and mammary gland.

- Exogenous estrogen causes dysplasia of the ovarian granulosa cells, thus impeding the maturation of the ovarian follicles." Note p. 360 comment on DES | estradiol | coumestrol. "Sheep grazed on highly estrogenic pastures consisting of relatively pure stands of subterranean clover or red clover containing high concentrations (0.8%) of the isolflavone formononetin may suffer marked clinical signs. Ewes may be affected by severe infertility accompanied by gross pathological changes in the uterus, including hydrops uteri, pyometra, cystic hyperplastic endometritis, and massive adhesions resulting from metritis." "The infertility results from abnormal function of the pituitary/ovarian axis. The reproductive problem frequently resembles the abnormalities associated with cystic ovaries, including prolonged but irregular estrus, nymphomania, or even the development of masculine sexual characteristics".

- 8. "Organizational and Activational Effects of Phytoestrogens on the Reproductive Tract of the Ewe" Adams. Sci. Exp. Biol Med 1985
"Isolflavone compounds including genistein, Biochanin A and formononetin

Occur in Subterranean clover or Red clover. Geristein and Biochanin A are usually broken down by microbial activity in the rumen of the sheep while formononetin is converted to the isoflavan equol, which is rapidly absorbed and is responsible for most of the estrogenic activity in ruminants."

"Temporary infertility results from actions of estrogen that are similar to the activation effects of estrogen in most species of mammals. The permanent infertility results from changes to the cervix which are analogous to the organizational effects of estrogen reported in other species treated during organogenesis."

9. "Phytoestrogen Content and Estrogenic Effect of Legume Fodder", Seleniemi, Wähälä, Nykinen-Kurki, Karela and Saastomoinen Soc. for Exper. Bio and Med. 1995.

"All red clover studied contained estrogenic isoflavones, especially formononetin and Biochanin A. The phytoestrogen content varied from 1.0% to 2.5% of dry matter."

"Interest in phytoestrogens has generally been aroused by their adverse properties. They may, however, also be beneficial by increasing the growth of animals and the milk yield of cows." "Biochanin A and geristein, which in monogastric animals have an estrogenic effect, are broken down in the rumen of ruminants. daidzein and formononetin, are converted by ruminal microbes into the active equol." The chart on p. 15 shows phytoestrogen content of Red clover. That content consists of

Daidzein, Geristein, Formononetin, Biochanin-A.

"Red clover has the highest phytoestrogen content of Finnish legume fodder plants, varying from 1% to 2.5% of dry matter. The biological effect on the uterus of immature rats is large."

10. "Clinical Changes in Ovariectomized Ewes Exposed to Phytoestrogens and 17 β -Estradiol Implants", Nwamenne, Lundh, Madej, Fredriksson, and Björnray. Soc. for Exp. Bio. Med. 1987.

"Genistein and other isoflavones daidzein, Biochanin A, and formononetin were isolated from clover and proved to be the cause of the disorders." "Mammary glands were noticeably more voluminous, and palpation revealed the presence of irregular, solid lumps of various sizes towards the end of the feeding period and long afterwards. The udder gradually changed from ^{pale to} pink, ~~and~~ then to red with increasing edema." "In this experiment, the irregular solid lumps of tissue found during clinical examinations looked like developing tumors. Genistein, but not formononetin, and the estrogenic metabolite of formononetin called equol, can stimulate the growth of estrogen-dependent breast cancer cells in vitro. The estrogenic activity of Biochanin-A ⁰⁰⁰⁴⁵⁷ and genistein in ruminants is limited to the first few days of exposure when the unadapted rumen microbes cannot convert them to their non estrogenic metabolites p-coumyl phenol and phenolic acid. formononetin... considered to be the overall cause of clinical changes observed."

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Note that this paper is very commonly cited as an authority.

"The isoflavones genistein, Biochanin A and formononetin occur in variable concentrations in the leaves of many clover species."

"The reason why formononetin is oestrogenically more active than genistein in the sheep was found to lie in the digestion of plant constituents in the modified digestive system of the sheep." "It has been shown that the degradation of biochanin A and genistein to inactive phenols in the rumen of the sheep becomes ^{increasingly} more efficient over a period of about five days." "There appears to be a shorter adaptive period in the cow and the detoxification and excretion of the absorbed isoflavones and metabolites are more efficient." "Physiologically, clover-induced infertility is brought about by a combination of factors, including interference with spermatozoan transport through the genital tract of the ewe, abnormal transport of ova and interference with implantation. There is also evidence that the neuro-endocrine centres in the brain controlling the reproductive cycle of the ewe are suppressed." "However, the techniques of identification and quantitative assessment of oestrogens in plants should allow a quicker screening for possible oestrogenic compounds when they are suspected of reducing fertility in other animals, or having a contraceptive action in women." "From the wider ~~point of~~ viewpoint of evolution it is interesting that compounds have evolved in plants that not only give the plant some protection from foliar pathogens, but also reduces the fertility of animals ingesting the plant!"

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000459

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14. Pages from recent "Dairy Exporter" NZ. I believe that

The reason that say based formula ^{9'} was dropped in N.Z. when it became apparent that they contained phytoestrogens was because New Zealanders came from farming stock (pastured) and so soon benefited and risks of phytoestrogen exposure from plants ~~was~~ commonly understood. (i.e. Nothing to do with the imagined "lobby groups". "11")

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000460

c) Information on

effects of various levels of clover in pasture and relation to milk yield (increases yield) but cautions avoid e.g. "Phyto-oestrogens in the new cultivar are 65% lower than in the older cultivar, greatly reducing the risk of problems with animal production".

d) Letter to the editor, showing level of interest in and understanding of estrogen exposure within the N.Z. community. "Estrogens are involved in thyroid function, which suggests a toxic estrogen is likely to be a thyroid poison. It is time to find out. Evidence has been known to induce serious

10

reproductive failure and failure of fetal development."

1. Letter to the Editor of *ATDC* in *Pediatric Forum*. Kerla Montague-Brown. "The authors found positive statistical associations between premature melanoma and consumption of various meat products and soy-based formula, and a maternal history of ovarian cysts."

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000461

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"It must be stressed that beneficial and adverse effects are not mutually exclusive." "Whatever the implications for fertility in women, it is clear that addition of quite modest amount of soy protein to a complex diet had physiological effects, which lasted for several months after soy was removed from the diet." "The main effects are those due to possible alteration of endocrine dependent regulation. These might include carcinogenic, reproductive and developmental outcomes, including effects on sexual maturation and behaviour, which would not necessarily be readily apparent either in time or in appearance."

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000462

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Thank you for considering the above!

Yours,

~~Harvey~~



Institute for Environment
and Health

IEH assessment on

PHYTOESTROGENS IN THE HUMAN DIET

FINAL REPORT TO THE
MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

000462.001

November 1997

The Institute for Environment and Health (IEH) was established by the Medical Research Council at the University of Leicester in 1993. The Institute is partly funded by the various UK government departments and agencies by way of specific research and consultancy contracts.

This literature review* has been prepared by IEH for the Ministry of Agriculture, Fisheries and Food. The principal focus of this document is on the potential beneficial effects of phytoestrogens on adults. Potential detrimental effects on adults and the influence on other life stages were specifically excluded from consideration. It also contains an assessment of the factors influencing the phytoestrogen content of food and the relative potencies of the various phytoestrogens. The assessment incorporates the output of a workshop held in Leicester in March 1997 which was chaired by Professor Lewis Smith, IEH. The Institute gratefully acknowledges the contribution of all those who attended the workshop and provided material for inclusion in the assessment but assumes no endorsement from these scientists for the conclusions and recommendations contained herein.

The Ministry of Agriculture, Fisheries and Food has provided funding for this project but has not conducted the research or written this report. The views expressed here do not necessarily represent those of any government department or agency.

Prepared by

Dr Charles Humfrey and Mr Philip Holmes, IEH

000462.002

* Literature search to November 96, supplemented by additional papers to June 97.

producing a number of effects which have been associated with a reduced breast cancer risk. In premenopausal women, soya has been shown to increase the length of the menstrual cycle and/or delay menstruation, and to reduce the levels of LH, FSH and progesterone at various stages of the cycle. The reported effects of soya on blood levels of 17β -oestradiol have not been consistent, one study reporting a reduced level throughout the cycle (Lu *et al.*, 1996), another reporting an increase in the follicular phase only (Cassidy *et al.*, 1994) and another finding no change in levels (Baird *et al.*, 1995). Based on the studies reviewed, the evidence for changes in levels of the adrenal androgen DHEAS is conflicting and further investigations would be required to clarify these opposing findings. Plasma levels have been shown to vary with energy intake, for example, a recent study in premenopausal women found that for each additional 1MJ (239kcal) consumed, levels of DHEAS decreased by 5.1% (Dorgan *et al.*, 1996). To ensure that energy intake does not confound intervention studies using soya products, it would seem sensible to ensure that all diets investigated are isocaloric.

Both lignans and isoflavones have been reported to increase the levels of plasma SHBG, which may decrease the blood levels of biologically active sex hormones and influence cancer risk. Again, however, epidemiological studies are conflicting; levels were lower in postmenopausal women with breast cancer than in vegetarians or omnivores (Adlercreutz *et al.*, 1989 and 1992), although an earlier study found no difference between postmenopausal breast cancer patients and controls (Bruning *et al.*, 1985). In more informative controlled trials, in which lignans (as linseed) or soya (as textured vegetable protein and miso) was added to the diets of premenopausal women, a small, but significant, decrease in the level of SHBG was seen only in women consuming the linseed supplement. From these studies, isoflavones do not appear to have any effect on SHBG levels.

Although preliminary, the potentially important finding of Petrakis *et al.* (1996) that soya consumption may have an oestrogenic effect by increasing the incidence of hyperplastic epithelial cells in the nipple aspirate fluid of pre- and postmenopausal women constituting a risk factor for breast cancer, should be the subject of further investigation.

Overall, these studies show that phytoestrogens are biologically active in women and can affect the levels of sex hormones and potentially therefore contribute to a reduced breast cancer risk. The effects produced have not always been consistent between studies, although this may relate to the use of different doses, types of product used, study design, or the generally small numbers of women studied. In order to elucidate potential beneficial effects of phytoestrogens in breast cancer risk reduction, further controlled studies in larger populations of premenopausal women are warranted.

000462.003

6.5 POST-MENOPAUSAL SYMPTOMS

The reported incidence of hot flushes, one of the most common symptoms of the menopause, varies markedly between different countries, with high levels in Europe (from 70-80% of postmenopausal women), intermediate levels in Malaysia (57%),

and low levels in China (18%) and Singapore (14%) (Knight & Eden, 1995 and 1996). As a result of dietary differences between these countries, particularly the significant variations in consumption of soya products, several studies have investigated the possible role of these products in modulating these symptoms. It is of interest to note that many plants of the family Leguminosae, which contain high levels of isoflavones and their glucosides, have long been used to treat menopausal symptoms such as hot flushes, sweating and depression by Chinese herbal doctors (Lien & Lien, 1996). Examples include alfalfa, dandelion, red clover and liquorice.

Wilcox *et al.* (1990) showed that consumption by 25 postmenopausal women of a diet supplemented with soya flour (45g/day), red clover sprouts (10g dry seed/day), and linseed (25g/day), each for two weeks, had no effect on LH or FSH levels when analysed after each individual two week supplement, but had a marginal cumulative effect on FSH levels over the six week study. No control group was included in this study. However, oestrogenic effects were observed when measured as cytological maturation of the vaginal epithelium. This was not confirmed in subsequent studies by Murkies *et al.* (1995) and Baird *et al.* (1995). In the former study, postmenopausal women with more than 14 hot flushes per week consumed a diet supplemented with either soya flour (45g/day, n=25) or wheat flour (45g/day, n=22) for 12 weeks. No effect on vaginal cell maturation was seen in women consuming either supplement, although hot flushes were significantly reduced at six weeks in the women consuming soya flour and by 40% and 25% at the end of the study in women consuming the soya flour or wheat flour, respectively. A subjective assessment of menopausal symptoms also showed significant reductions in both groups by the end of the study. Urinary levels of daidzein, equol and enterolactone were significantly higher at the end of the study in the soya flour group but not in the wheat flour group (although wheat flour also contains phytoestrogens). As a result of the decrease in flush frequency over the study period, a placebo effect could not be discounted. In the study of Baird *et al.* (1995), groups of postmenopausal women consumed either a normal diet (control, n=25) or a diet supplemented with soya foods (equivalent to 165mg isoflavones/day, n=66) for four weeks. Despite an average 105-fold increase in urinary excretion of isoflavone phytoestrogens in the soya diet group and an average two-fold increase in the control group (which was not significant), no significant difference in vaginal maturation index was noted between the two groups. In addition to these observations, there were no significant differences in the levels of FSH, LH and SHBG between the two groups or comparing levels before and after the dietary intervention. A slight decrease in serum oestradiol levels was noted in both groups during the study, but neither was significant. The use of a different sampling technique in this study to the others above may have falsely lowered the estimate of vaginal maturation (Knight & Eden, 1996). However, the lack of measurement of biological dose in this study makes it difficult to determine whether the lack of effect was really simply due to the dose not being high enough to have a significant effect.

Further studies are being undertaken throughout the world to assess the effectiveness of isoflavones in treating symptoms of the menopause. The current evidence suggests that there may be some beneficial effects

000462.004

To: Linda Kahl@OPA@FDA.CFSAN
From: "Ian & Yvonne Clapperton" <safetywize@clear.net.nz>
Certify: N
Subject: RE: Response to your submissions
Date: Tuesday, April 28, 1998 at 5:57:49 am EDT
Attached: attach1

OU



Could we please hve the names of those scientists. Please treat this as a
Freedom of Information Act request. Dick James

From: Linda Kahl
Sent: Tuesday, 28 April 1998 02:24
To: safetywize@clear.net.nz
Subject: Response to your submissions

Thank you for the information that you have provided regarding soy
isoflavones. I am passing it along to the scientists who are reviewing
GRAS

Notice GRN #000001 from Archer Daniels Midland.

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C St., SW, Washington DC 20204
hone: (202)418-3101 Fax: (202)418-3131
Internet:LKAHL@BANGATE.FDA.GOV

000463

Apr 28, 1998

OU



Dr. Linda E. Kahl
Office of Pre Market Approvals
U.S. F. D. A
FAX (202) 418 3131

1998 APR 28

A 10-1000
R. D. 4

WILMINGTON N. C.

4/28/97

Dear Dr. Kahl

G. R. N. # 000001

Thank you for your Memorial Day E-MAIL to Yvonne Clapperton, which she sent on to me. Thank you for keeping her informed so well. It is most helpful.

I'm sorry, but I thought I understood you to have advised that there was no provision for review or submissions.

There seems to be a prevailing atmosphere of secrecy and unwillingness to face public scrutiny in the F. D. A.'s actions to date. I have made it clear that I believe the law requires such important decisions to be made in a transparent environment.

So. If a decision is made in favor of G. R. A. S. Determination # G. R. N. 000001 without open public hearings, it will be brought before Federal Court, and those who were party to the determination will be required to give their evidence under oath, and on penalty of perjury.

Please make this known to your colleagues

Sincerely

000465

~~Richard F. Jones~~

Dr Linda Kahl

001 (202) 418 3131

~~A. K. Tamm~~

R.D. 4

Whangarei

April 28 1998

Dear Dr Kahl.
Gullible!! Some idiot on a daybreak
breakfast T.V. program said Wall St was
closed for Memorial Day. We believed it, half
asleep. It didn't sound quite right. Of course it
wasn't.

Another month to go.
Can't believe a thing you hear
nowadays.. Sorry about that

Cheers anyway

~~[Signature]~~

1998 APR 28 A 10:16

000466

**SOY INFORMATION NETWORK**

PO Box 32 236
Devonport
North Shore City 1309
New Zealand
Telephone: +64 9 445 8721
Facsimile: +64 9 445 9834
Email: woodhams@iprolink.co.nz

28 April 1998

Dr Linda S. Kahl
Regulatory Policy Branch HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C Street SW
Washington DC 20204
United States of America

Dear Dr Kahl

I have received a full copy of the Archer Daniels Midland Company's GRAS Notice Number GRN 000001 from Yvonne Clapperton, to whom you sent it.

I have not yet had the opportunity to study it in detail. However, I note that on page 32 there is a reference to a conclusion of the New Zealand Nutrition Foundation's Scientific Advisory Committee and Council "...that there is no credible human data to support the hypothesis that soy infant formula ... has adverse effects on the sexual development of the fetus, infants or children."

A year ago, when the Foundation's position paper first became available, I engaged in some correspondence with the Foundation over some rather startling omissions from their coverage of the Cassidy study¹ and on a couple of other points which appeared to give their treatment of soy infant formula a less than objective slant. I attach copies of the full correspondence for your information. In my opinion I raised some valid points, admitted to be such by the Foundation which it failed to address adequately in its responses.

By July 1997 I was engaged in a demanding contract and was unable to take the matter further. However, I would comment briefly on the final responses I received from Dr Cliff Tasman-Jones (18 Jul 97) and Dr John Birkbeck (17 Jul 97).

Apparently the fact that "This was a position paper and NOT a scientific review" excuses the omission of any mention of the 67% and 47% reductions in LH and FSH from the discussion of Cassidy's work while including comment on the 5.5% increase in menstrual cycle length and the 16.7% increase in the follicular phase length. (The

1 Cassidy, A., Bingham, S. and Setchell, K.D.R. [1994], "Biological Effects of a Diet of Soy Protein Rich in Isoflavones on the Menstrual Cycle of Premenopausal Women" *Am. J. Clin. Nutr.* **60**: 333

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latter have been suggested as the basis for the benefit of possible protection against breast cancer while the former suggest the risk of temporary or permanent infertility.)

Apparently the same fact ("This was a position paper NOT a scientific review") excuses the omission of any mention of the Fort study² of infant feeding and autoimmune thyroid disease and also justifies the misinformation that "It is not known if this (high dose of estrogens) differs drastically from the estrogen exposure of the developing fetus or from human and/or cow milk in infancy."

Tasman-Jones repeats the common statement that "most of the published work gives soy a positive role in nutrition...". This implies that a drug which has a benefit cannot simultaneously have adverse side effects, which I would have thought to be a relatively common phenomenon. It also implies that the benefit:risk balance can be derived from counting the number of published pages on the subject (and that therefore the balance is strongly in favour of the benefits and against the risks) I suggest, however, that such an assessment actually reflects the balance and direction of the **funding** of research projects rather than the relative potential benefits and risks.

In this regard, it is interesting to note that the bulk of the research reporting beneficial effects has been published from the late 1980's onward. This period postdates the publication of three significant papers^{3,4,5} suggesting the potential for harm to infants from soy formula. However, in the decade 1985-95 there is almost no published work that attempts to answer the serious questions raised in these publications, either by the authors or by other institutions. Why not? Was it perhaps because such studies were not funded while studies of the potential benefits of soy isoflavones were?

To the best of my knowledge there has been no retrospective study of infertile women to establish whether or not there might be some connection with soy formula consumption as infants. Why not?

Tasman-Jones also notes that paediatricians have been alerted to the possibilities for "damage" but have not reported any. However, a number of the potential problems (infertility, menstrual irregularities) will not be observed until after puberty, by which time the paediatrician is no longer involved.

-
- 2 Fort, P., Moses, N., Fasano, M., Goldberg, T. and Lifshitz, F. 'Breast and soy-formula feedings in early infancy and the prevalence of autoimmune thyroid disease in children' *Journal of the American College of Nutrition* 9(2) 164-7, 1990 Apr
 - 3 Murphy, P. A., [1982], "Phytoestrogen Content of Processed Soybean Products", *Food Tech* 36, 60
 - 4 Setchell, K. D. R., Borriello, S. P., Hulme, P. and Axelsson, M. [1984] 'Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease' *The American Journal of Clinical Nutrition* 40, 569-78
 - 5 Setchell, K. D. R. [1985]. Naturally occurring non-steroidal estrogens of dietary origin. In 'Estrogens in the Environment' (Ed J. A. McLachlan) pp 69-85 Elsevier Science Publishing Co Inc

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Similarly, in his letter Birkbeck comments on the lack of reports of feminising effects. But again, to the best of my knowledge there has been no retrospective study of the male homosexual community to establish whether or not there might be some connection with soy formula consumption as infants

If the questions have not been asked, it is hardly surprising that the answers have not been reported.

My intentions in writing this letter were twofold.

The first was to acquaint you with my reservations about the NZ Nutrition Foundation's Position Paper, quoted by Archer Daniel Midland in support of their GRAS notification.

The second was to provide a hard copy of my Email of 31 March together with a copy of the original and translation of the Japanese research paper⁶ mentioned in it.

I also enclose a brief CV for your information

Yours sincerely

David J Woodhams (Dr)
Soy Information Network

Enclosures:

Correspondence with NZ Nutrition Foundation (12 pages)
Copy of Email D Woodhams to Dr L Kahl, 31 Mar 98
Parallel original and translation of Japanese paper (ref 6)
Curriculum Vitae, D J Woodhams

6 Ishizuki, Y , Hirooka, Y , Murata, Y & Togashi, K ' The effects on the thyroid gland of soybeans administered experimentally in healthy subjects' *Nippon Naibunri gakkai Zasshi* **67** 622-29 (1991)

000547

Dr David J Woodhams CEng
Soy Information Network
PO Box 32 236
Devonport 1309
NEW ZEALAND

Fax # +64 9 445 9834
Tel # +64 9 445 8721

COPY

*Dr Linda Kay
USFDA*

17 April 1997

Dr J Birkbeck
Nutrition Foundation
12-14 Northcroft Street
Takapuna
North Shore City 9

Copy: Pamela Williams

Dear Dr Birkbeck

My sister Pamela Williams was kind enough to send me a copy of the New Zealand Nutrition Foundation's position paper on "Soy Foods in Human Nutrition".

It is not my purpose or desire to debate the issues addressed in a comprehensive way but I am puzzled. I find it very curious that the paper talks about Cassidy's measurements of the increase in the follicular phase of ovarian function and of the lengthening of the menstrual cycle but does not mention the suppression of midcycle luteinising hormone and follicle stimulating hormone levels measured at the same time. When I compare the data from the Cassidy study I find:

	Control Diet	Soy Diet	% Change
Menstrual cycle length, days	27.5	29.0	5.45
Follicular phase length, days	15.0	17.5	16.7
Luteinising hormone, units/litre	21.2	7.1	-66.5
Follicle Stimulating Hormone, units/litre	14.6	7.8	-46.6

Clearly, by far the greatest of the measured physiological changes brought about by the change from the control to the soy diet are the changes in the levels of the LH and FSH. Why is this effect not mentioned in the Foundation's position paper? I can understand the commercial motives behind the omission of this dominant effect from the advertising of Bean Supreme, even if I disagree with them. But I am at a loss to understand how such an omission has occurred from a science-based position paper authorised by the Nutrition Foundation.

There are a couple of other comments I wanted to make about the position paper.

In section 5.3 the Foundation says:

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"Calculations of the oestrogen-like substances in soy-based infant formula suggests that infants fed these as the sole or principal component of the diet risk severe effects due to the high dose of oestrogens. It is not known if this differs drastically from the oestrogen exposure of the developing foetus or from human and/or cow milk in infancy."

Clearly I agree with the first sentence. I did the first bodyweight dose calculations.

As for the second, I note that it is very easy to lose sight of the fact that the isoflavones are not actually estrogens; they are phytoalexins which, among other actions, can bind to estrogen receptors. This fact alone makes exposure of infants to soy isoflavones differ substantially from their exposure to estradiol etc, the human estrogens that developed as an integral part of the evolution of human beings. Thus, in this sense, the difference is known. They are different in nature.

It is also possible to quantify how different the exposure (dose and duration) of the soy formula fed infant is from the exposure of the breast fed baby:

During days 3 to 5 *post partum*, human breast milk contains around 200 pg/ml estradiol. Assuming a daily consumption of 250 ml, the infant's dose of estradiol is around 50 ng/day. (Weaker estrogens omitted)

For soy formula babies less than 4 kg, the can labels recommend about 65 g powder/day containing about 95 mg genistein/kg (expressing genistein as the genistein equivalent). The infant's dose of genistein is then about 6.2 mg/day. (Weaker phytoestrogens omitted)

Assuming the relative potencies of estradiol and genistein to be 1200:1, then the soy fed infant's dose is equivalent to 5200 ng estradiol per day.

Thus the dose ratio, as at 3 to 5 days *post partum*, is about 100:1 soy fed to breast fed.

The estradiol content of breast milk virtually disappears during the first 2 weeks. The dose of the soy fed baby remains approximately the same (bw basis) throughout perhaps 12 months. The duration of the exposure is thus around 25 times longer.

Thus, in a comparison of soy-fed and breast-fed infants, the estrogenic dose is about 100 times greater and the exposure is about 25 times longer. For this comparison at least, the New Zealand Nutrition Foundation would be justified in stating in its position paper: "It is known that the exposure differs drastically - in nature, in dose and in duration."

Finally, there is no mention in Section 5.2 of the Fort epidemiology (J. Amer. Coll. Nutr. 9(2): 164-67, 1990), with which I presume you are familiar. There may be a

good scientific reason for omitting any mention of a study about which the authors claim in the summary:

"Therefore this retrospective analysis documents the association of soy formula feedings in infancy and autoimmune thyroid disease."

If so, I would appreciate knowing what it is.

As a food professional of 34 years' standing and as a Chartered Engineer, I stand by what I write and what I publish under the aegis of the Soy Information Network. I am not responsible for what others may say or write.

I would be surprised if you have not seen copies of SIN Newsletters 1 to 4 and I presume that you can access the most recent versions of the Introduction and the Soy Infant Formula paper, published with the Aspell Reports. (I gave a copy of the 3rd Edition (Mar 95), standard issue to purchasers for the last 12 months, to Cliff Tasman-Jones about a year ago. He was among the first to receive it.)

I understand that you had some harsh words to say about the earlier versions of my contributions. I invite your review and rebuttal of the later ones. Comments which address the issues raised will be considered with due respect.

I attach, for your information, a copy of my translation of an article from the Swiss Bulletin de l'Office fédéral de la santé publique by Schlatter and Zimmerli. I was particularly interested in their general introductory comments and their conclusions regarding the relative risks posed by artificial and natural pesticides. I attach also a section of the Annual Report of the Swiss Public Health Bureau together with its translation.

I am able to understand the reluctance of the New Zealand Nutrition Foundation and the NZ Ministry of Health to espouse the sensible and logical position of the Swiss, as set out in the first sentence of their final paragraph, only from a political or commercial viewpoint. On technical and scientific grounds, feeding soy formula to normal babies is without medical benefit and subjects them to unnecessary risk.

I look forward to receiving your response.

Your sincerely

David J Woodhams.
Soy Information Network



Dr David Woodhams
Soy Information Network
Devonport

April 28, 1997

Dear Dr Woodhams,

Your letter of April 17 has been forwarded. (As a part-time staff member, I do not operate from the Foundation office).

Although I will respond to some points you raise, you should know that I did not write the Position Paper in question. Council felt that because of my commercial involvements [although I do not actually work for any of the soy industry], it might be better if it was written by someone else.

Your points about the Cassidy et al work are valid, although the trophin effects are of course the cause of the changes in physiological variables.

I would agree with your second series of statements about soy formula versus human milk, until your paragraph commencing "Assuming...". This is the difficult part. There are very clearly, in the literature, vast differences in apparent potency of phytoestrogens compared to a mammalian hormone standard [estradiol]. While the few human studies such as Cassidy et al give us some inkling, we really have almost no information in humans, certainly not young humans. This is one of the major gaps in our knowledge which makes the issue so contentious. Also, the effects are not simply those of a weaker estrogen; I understand that phytoestrogens bind more tightly to estrogen receptors and hence reduce the binding of endogenous estrogens.

With regard to effects of soy components other than phytoestrogens, I doubt if the writer wished to expand the paper to cover those. One great difficulty is that the actual composition of the "soy protein isolate" used in formulas has changed over the years. This has meant that trying to define which of the numerous potentially deleterious components of native soybeans are actually present in the protein fraction in amounts sufficient to cause problems is very difficult. For example, the trypsin inhibitors in soybeans appear to cause pancreatic enlargement in rats, but no such effect is reported in humans (to my knowledge) who consume soy foods. There were I believe one or two papers which raised the issue of thyroid effects of soy formulas, but they were written in the 1960s when the formulas were very different from those used today.

I understand that your letter has been copied to the writer of the Position Paper, who no doubt will respond to your questions about why certain matters were or were not dealt with.

Yours sincerely,

*John Birkbeck, MB, ChB, FRCPC, MNZIFST
Medical/Scientific Director*

000551

New Zealand Nutrition Foundation, 12-14 Northcote Street, PO Box 33-1409, Takapuna, Auckland 9, New Zealand.
Telephone 0-9-486 2036 Facsimile 0-9-486 2038.

Dr David J Woodhams CEng
Soy Information Network
PO Box 32 236
Devonport 1309
NEW ZEALAND

Fax # +64 9 445 9834
Tel # +64 9 445 8721

27 May 1997

Dr J Birkbeck
Nutrition Foundation
12-14 Northcroft Street
Takapuna
North Shore City 9

Copy: Pamela Williams

Dear Dr Birkbeck

The Foundation office has provided me with a copy of your 28 April letter. With that copy to hand, I would like to recast my letter of 11 May.

Thank you. I am indeed grateful for your prompt response to my 17 April letter. I still have not yet heard from the author of the position paper. I wrote to you assuming that you would be at least a member, if not the convener of the Foundation's Scientific Committee that reviewed the paper before it was authorised.

Now that I have read your letter again, I find that my comments were at times on target and at times a little off beam. As I said previously, there are some comments to be made. I will make them again, in full, with the intention of replacing my previous letter with this one.

It seems to me that my "valid" points about the work of Cassidy *et al* need to be answered, both scientifically and ethically, by both the author of the position paper and by the scientific committee that recommended its authorisation to the Foundation. My valid points cannot be just dismissed by an off-hand, half sentence,

"...although the trophin effects are of course the cause of the changes in physiological variables."

How does the NZ Nutrition Foundation justify omitting any reference to 67% LH suppression and 47% FSH suppression from its position paper on "Soy Foods in Human Nutrition"? There is no reference at all to measured 2:1 and 3:1 changes in the two ovulation hormones? I would have thought that, from a position of scientific objectivity, this called for at least a mention in an authorised position paper from a professional body such as the Foundation.

000552

A food scientist, technologist, manager or general practitioner who reads the Foundation's position paper without reference to the original paper would have no knowledge of the potential for a soy diet to affect ovulation. They would thus be misled regarding the extent of knowledge of the physiological effects of a soy diet on humans. This is a valid point, regardless of whether this effect is regarded as a potential risk, as a potential benefit or merely as a now-established fact.

I am of course aware of the vast differences reported in comparing the estrogenic potency of the soy isoflavones with estradiol. I am also aware of some pretty shonky reporting of the data. [Please see the articles on pages 7 and 9 of the SIN Newsletter #2 enclosed with my 11 May letter.] The factor 1/1200 in my calculation is drawn from the work of Markiewicz *et al* [J. Steroid Biochem. Molec. Biol. 45[5] 399, (1993)]. I understand from Dr Michael Fitzpatrick that this factor was generally accepted at the Little Rock conference, even by strong soy supporters.

The Foundation's own position paper quotes a factor of "at least 1/1000", although the author did not use the word "oestradiol" specifically in the comparison. Had I used the NZ Nutrition Foundation's factor instead of Markiewicz's 1/1200, the calculation of relative potency would have yielded a daily dose equivalent, for an infant weighing less than 4 kg, of 6200 ng estradiol per day, over 120 times the potency of natural estradiol exposure one week after birth, for something like 25 times as long. Even if your writer were out by a factor of 10 and the factor was 1/10,000 (which I do not concede and use only for the purpose of the argument) the dose would still be 12 times as much for 25 times as long. If this were the case the Foundation would still be justified in saying that this differs substantially, even "drastically", from the exposure of the breast fed baby to natural estradiol.

You say: "...we really have almost no information in humans, certainly not young humans." This is precisely my point. I agree with you. Take that as read and accept the calculation above, with all its uncertainties.

How can you justify feeding 12 times the natural estrogen dose (let alone 120 times) to a normal neonate, as an uncontrolled part of its total diet for over six months in the absence of this information? We know that the effects of the isoflavones will be dependent on both dose and duration.

Where is the benefit that justifies the NZ Nutrition Foundation assuming this risk for normal babies without telling their mothers or their GP's either that the risk exists or that there is no information about the extent of the risk?

The "...major gaps in our knowledge..." should not make the issue of open sales of soy infant formula contentious; they should remove all contention from the issue. There is no need for New Zealand infants to be subjected to this risk other than in exceptional medical circumstances. No contention. Restrict it to pharmacies.

The reference to my two contributions supporting the Aspell Report was not necessarily intended to be regarded as comment on the Foundation's position paper. It was an invitation to make your criticisms of my treatment of the subject to

me directly so that I could understand and learn from your position on the other "...numerous potentially deleterious components of native soybeans...". Nevertheless, it is unclear why the writer and reviewers of a position paper dealing with soy foods and human nutrition have restricted their attention to the isoflavones and have omitted, just as an example, the possible effects on zinc metabolism of the heat-stable, uncontrolled and unmeasured phytate content of soy infant formula.


I am aware, in general terms, of the possibility of processing changes in the production of soy protein over the years. However, as the soy industry did not provide this information to the Ministry in response to its 1994 request, I do not know the extent or nature of such changes. It may well be that, if soy protein concentrate had been used in the past, an alcohol wash may have had an influence on the isoflavone content of soy infant formula. This possibility has not apparently restricted industry claims of 40 years' safe use for the present product.

Finally, I am aware of several papers describing case studies in the 1960's which link soy infant formula to thyroid disease and cretinism. I did not refer to them in my letter and did not suggest that they should have been included in the Foundation's position paper. The Fort paper mentioned [J. Amer. Coll. Nutr. 9(2): 164-7 (Apr 1990)], however, was published in 1990. It is the only epidemiology available in the literature that I am aware of and it surely cannot be dismissed, without reference or discussion, from an objective review of soy foods and human nutrition.

It is my understanding of the scientific process that an example which is clearly counter to a prevailing theory or belief requires investigation and is often the trigger for new understanding. Surely this is especially necessary where the health and safety of human infants are involved. To ignore the Fort paper is to condone and encourage such untrue statements as:

"...there is no scientific evidence to suggest that these formulas can cause any health problems." (Sanitarium Nutrition Education Service, supermarket leaflet, April 1996).

Your sincerely



David J Woodhams.
Soy Information Network

Dr David J Woodhams CEng
Soy Information Network
PO Box 32 236
Devonport 1309
NEW ZEALAND

Fax # +64 9 445 9834
Tel # +64 9 445 8721

15 July 1997

Dr J Birkbeck
Nutrition Foundation
12-14 Northcroft Street
Takapuna
North Shore City 9

Copy: Pamela Williams
Cliff Tasman-Jones

Dear Dr Birkbeck

I refer to my letter of 27 May to which I have had no reply. Nor have I yet heard from the author of the Nutrition Foundation's position paper.

I write again following the publication of an "Early Report" in the 5 July issue of Lancet, "Exposure of infants to phyto-estrogens from soy-based infant formula", reporting work carried out by Dr Ken Setchell.

Analysis of five random samples of each of five brands of US soy infant formulas leads him to the conclusion that a four month old baby receives a dose which is 6 to 11 times higher than the body weight adjusted intake found to cause significant modifications to the hormonal regulation of the menstrual cycle of western women. I note that this is twice the dose rate that I calculated for NZ babies in 1994.

In addition, he compared the plasma concentrations of genistein and daidzein, and the intestinally derived metabolite, equol, in 21 four month old infants fed exclusively on soy infant formula (n = 7), cows' milk formula (n = 7) or breast milk (n = 7) from birth. He reports that:

"Circulating concentrations of isoflavones in the seven infants fed soy-based formula were 13 000 - 22 000 times higher than plasma oestradiol concentrations in early life, and may be sufficient to exert biological effects. whereas the contribution of isoflavones from breast milk and cow milk is negligible." (p 23)

Dr Setchell does not discuss the comparative duration of exposure and I have not followed up his reference for the oestradiol data to determine the stage of life (birth or neonatal which may differ by two orders of magnitude) to which they refer or the duration.

In his discussion of the results Dr Setchell says:

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"Plasma total isoflavone concentrations in these seven [soy formula fed] infants (range 552 - 1775 ng/ml, mean 980 ng/ml) is 2 - 5 fold higher than the peak plasma concentrations observed in adults after a single oral dose of 50 mg of the pure compounds (300 ng/ml), and significantly greater than reported concentrations (50 - 200 ng/ml) for adults consuming diets of soy-based foods containing similar amounts of isoflavones. These values are also much higher than plasma isoflavone concentrations of Japanese adults, which range from 40 to 240 ng/ml." (p 26)

Later he makes clear that the potential effects that the bioactive soy isoflavones may produce, by creating steroid hormone imbalance, by competition with enzymes that metabolise steroids, drugs and xenobiotics, or by influencing gonadal function are unknown.

The issue of bioavailability to infants, previously "unknown" and first put to me by Dr Cliff Tasman-Jones in late 1995, is now clearly resolved. The issue of the soy isoflavone content of the breast milk of soy-consuming, lactating mothers, first raised by Dr Messina in December 1994, has also been clearly resolved. In both cases the scientific data have refuted these arguments, originally advanced as reasons why the concerns expressed by the Soy Information Network should not be acted on or else should be ignored pending their scientific resolution.

In view of Dr Setchell's published work, and in light of my previous (apparently unanswerable) critique of the Foundation's position paper, I presume that the Foundation will now reconsider its position.

As a food professional, I look forward to your professional response to my letter of 27 May and to the Foundation's reaction to the implications of Dr Setchell's work.

Your sincerely

David J Woodhams.
Soy Information Network



July 17, 1997

Dr D Woodhams
P O Box 32236
Devonport

Dear Dr Woodhams,

This is a reply to your communication of 15 July. I had referred your earlier letter to the writer of the Foundation's position paper; I understand you have now received a response.

I have examined the summary at least of the Setchell paper in *Lancet* 350:23-27. It is good now to have some objective evidence on this issue, which has been so clouded by speculation. It is now for the first time clear first, that young [four months] human infants do indeed metabolise the isoflavone glycosides in soy formulas, and second that the resulting aglycones are absorbed. This gives us some quantitative information on the aglycones; it does not of course give us any information about the possible hormonal effects of those compounds in the particular hormonal environment of a young infant, which is very different from that of an adult woman. Even the issue of the quantitative relationship between the values found and the levels of plasma estradiol which is quoted in the paper is not really helpful because we do not know what the relative potencies are.

As I have stated before, I have no personal axe to grind regarding soy formulas. If there is any objective evidence of lack of safety I would be the first to call for their removal from the market. However I consider it is odd, if these products have either significant estrogenic effects, or for that matter significant antithyroid effects, that nobody has reported them. The lack of reports of feminising effects is odd if such effects occur. It had been hoped that the breast tissue ultrasound study organised by Dr Tuohy of the Plunket Society might have provided some answers. The last time I was in contact with him, although he had some data on infants who were *not* fed soy formula, he had none on those so fed. So that question remains unanswered.

I am not sure whether you have the same interest in antithyroid effects as some of your colleagues, but I have just received a photocopy, (with some unsigned comments, from Whangarei), of a paper in *J Trop Pediat* June 1988 on this issue. It is perfectly true that native soybeans contain goitrogenic agents, but the evidence is that the level present in soy protein isolate can be counteracted by dietary iodine. There are many very common vegetables, especially the brassica family such as cabbage, and especially Brussels sprouts, which also contain quite potent goitrogens. We do not however recommend that people should not eat these. The level of blockage of iodine uptake is readily counteracted by a normal level of dietary iodine. Soy formulas of course contain iodine at a level believed to be suitable for infants. In the rat study reported in that paper, the rats were fed soy protein equal to over one-third of the total mass of their diet. Such rats, provided they had normal iodine intakes, had normal plasma thyroxin levels, albeit in some cases with some histological evidence of thyroid hypertrophy.

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New Zealand Nutrition Foundation, 12-14 Northcroft Street, PO Box 33-1409, Takapuna, Auckland 9, New Zealand.
Telephone 0-9-486 2036 Facsimile 0-9-486 2038.

Unless some further scientific evidence comes to hand, I do not feel that further debate on the issue will be fruitful.

Yours sincerely,

~~~~
John Birkbeck

000558



NEW ZEALAND
NUTRITION
FOUNDATION

18 July 1997

Dr David Woodhams
Soy Information Network
PO Box 32 236
Devonport 1309
AUCKLAND

Dear David

POSITION PAPER - SOY FOODS IN HUMAN NUTRITION

This was a position paper and NOT a scientific review. As such the aim is to take a moderate line raising the major benefits and adverse reactions so that lay members can get the thrust of where the Foundation stands. As such it makes no attempt to be comprehensive but does give selected scientific references. As it happens most of the published work gives soy a positive role in nutrition with food intolerances forming the bulk of adverse reactions. I have reread the paper and consider that it faithfully addresses its intended role.

The position paper recognises the need for further research particularly in the area of infant feeding with soy and it is very encouraging that some information is coming through in the scientific literature.

Since soy has been raised as a potential hazard for infants, paediatricians have been alerted to the possibilities of "damage" by infant soy formulas. In spite of this awareness there have not been any reports or anecdotal cases to support the hypothesis of hormone induced harm in infants being caused by soy formulae.

We must continue to be vigilant and encourage good research studies which investigate the metabolism of absorbed phytoestrogens. Dr Setchell's paper is a good step along the way but considerably more research is necessary.

Yours sincerely

CLIFF TASMAN -JONES
Bsc, MB, ChB, FRCP, FRACP, FICN
Chairman

New Zealand Nutrition Foundation
12-14 Northcroft Street, PO Box 33-1409,
Takapuna, Auckland 9, New Zealand.
Telephone 0-9-486 2036. Facsimile 0-9-486 2038
Email nznf@cybernet.co.nz

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Patron
His Excellency
The Right Honourable
Sir Michael Hardie Boys GNZM, GCMG
Governor-General of New Zealand

To: Linda Kahl
From: Dave Woodhams <woodhams@iprolink.co.nz>
Subject: GRAS Notice GRN 000001
Cc:
Bcc: re
Attachment:

HARD
COPY

Dear Dr Kahl

I am given to understand that Arner Daniels Midland has provided notice in accordance with a proposed regulation at 61 Fed. Reg. 18938 (April 17, 1996) that the substance "soy isoflavone" is generally recognised as safe for use as a micronutrient in food.

Would you please advise which substances are in fact included in the term "soy isoflavone" as this is a generic term covering different forms of several isoflavones and it is not a single "substance".

In a recent publication (Cassidy A, Bingham S, Setchell K D P 1994, "Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women" Am J Clin Nutr. 60: 333-340) evidence was presented in the physiological effects of 45mg/day of "soy isoflavones" from 40g/day soy protein isolate in the diet on the menstrual cycle of premenopausal women. Principal among these were the suppression of the midcycle surge of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by 67 and 50 respectively. These are the hormones that trigger ovulation and the data were measured for a soy diet during only one cycle for each woman.

In my professional opinion, there is a danger that providing GRAS classification for these substances could result in temporary infertility at dietary doses on prolonged exposure. The use of greater doses as dietary supplements or through direct addition to conventional foods, in addition to doses received from both soy foods and processed foods having soy protein isolate as a constituent, could have more lasting effects. To the best of my knowledge the effects of higher doses and longer exposure than 30 days on fertility have not been assessed in humans.

I am also concerned that there appears to have been little examination of the potential immune suppressant action of genistein in the diet. This action of genistein was reported by G. and C. Adluru "Evidence that Genistein, a Protein-Tyrosine Kinase Inhibitor, Inhibits CD39 Monoclonal-Antibody-Stimulated Human T-Cell Proliferation", Transplantation 61 148-50 (1991).

There is also the fascinating study of adult Japanese males (I. Ishibuchi, T. Hiroawa, T. Murata and H. Togashi, "The effects on the thyroid gland of soybeans administered experimentally in healthy subjects" Nippon Naikunpi Sakka Gasshi, 67, 411-19 (1981). This study showed that in the groups of subjects fed 30g soybeans per day for 3 months, half the subjects exhibited hypometabolic symptoms (malaise, constipation, sleepiness and dizziness. These symptoms disappeared the month after cessation of soybean ingestion. I have a parallel Japanese-English translation of this paper which I would be happy to copy and send you, should you wish to see it, translated by the Japanese Communication Service in Wellington, New Zealand.

For the above reasons I would oppose the granting of GRAS status to "soy isoflavone".

Yours sincerely,

BEST ORIGINAL COPY

* attached.

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ORIGINAL COPY

Translation of
The Effects on the Thyroid Gland of Soybeans Administered
Experimentally in Healthy Subjects
Y. Ishizuki, Y. Hirooka, Y. Murata, K. Togashi
(Nippon Naibunpi gakkai Zasshi, 67, 622-629, 1991)

Japan Communication Service
PO Box 3857
Wellington
Tel +64 4 232 8434
Fax +64 4 232 4308
Mobile 021 631 051
E-mail 100246 2672@Compuserve.com

健康者甲状腺機能への大豆の効果 —大豆投与実験—

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石突甲状腺研究所 (株)

石 突 吉 持

愛知医科大学第四内科

広 岡 良 文

名古屋大学環境医学研究所

村 田 善 晴

SMI ブリストル (株) 北里バイオケミカルラボラトリー

富 樫 和 美

The Effects on the Thyroid Gland of Soybeans Administered Experimentally in Healthy Subjects

Ishizuki Thyroid Clinic

Yoshimochi ISHIZUKI

The Fourth Department of Internal Medicine, Aichi Medical University

Yoshifumi HIROOKA

The Research Institute of Environmental Medicine, Nagoya University

Yoshiharu MURATA

SMI-Bristol, Kitazato Biochemical Laboratories

Kazuyoshi TOGASHI

To elucidate whether soybeans would suppress the thyroid function in healthy adults, we selected 37 subjects who had never had goiters or serum antithyroid antibodies. They were given 30g of soybeans everyday and were divided into 3 groups subject to age and duration of soybean administration.

In group 1, 20 subjects were given soybeans for 1 month. Groups 2 and 3 were composed of 7 younger subjects (mean 29 y.o.) and 10 elder subjects (mean 61 y.o.) respectively, and the subjects belonging to these groups received soybeans for 3 months. The Wilcoxon-test and t-test were used in the statistical analyses. In all groups, the various parameters of serum thyroid hormones remained unchanged by taking soybeans, however TSH levels rose significantly although they stayed within normal ranges. The TSH response after TRH stimulation in group 3 revealed a more significant increase than that in

group 2, although inorganic iodide levels were lowered during the administration of the soybeans. We have not obtained any significant correlation between serum inorganic iodide and TSH.

Hypometabolic symptoms (malaise, constipation, sleepiness) and goiters appeared in half the subjects in groups 2 and 3 after taking soybeans for 3 months, but they disappeared 1 month after the cessation of soybean ingestion.

These findings suggested that excessive soybean ingestion for a certain duration might suppress thyroid function and cause goiters in healthy people, especially elderly subjects.

緒 言

大豆には甲状腺腫誘発物質¹⁾が含まれており、実験ラットに与えると、短期間でも血中T値が低下²⁾し、I¹³¹摂取率が抑制³⁾されるといわれている。人では豆乳で育てた乳児に甲状腺腫、甲状腺機能低下症が発症した数症例が報告^{4)~6)}されている。吾々は大豆が慢性甲状腺炎の甲状腺機能を軽度抑制する事を示し、成人甲状腺にも影響を及ぼす事を報告した。しかし常食の大豆が健康成人の甲状腺機能を抑制するかどうかについて、系統的に検討した報告がない。

そこでこの研究では健康者に大豆を投与し、成人甲状腺機能に変化をうえるかどうかを検討した。そして甲状腺機能検査値を判読する時、食事の影響を無視して判定して良いかどうか考察する事を研究目的とした。

対象並びに方法

対象には、甲状腺疾患の既往や甲状腺腫がなく、血中抗甲状腺抗体陰性で、TBG変動を招く薬剤服用がなく、通常の仕事に従事している人を健康者とし、22~76才の男8、女46計54例を選んだ。

5群に分けた。第1群は大豆1ヵ月投与の短期投与群で、22~60才の男7、女13例計20例である。大豆3ヵ月投与の長期投与群を年齢により更に2群に分けた。第2群は22~39才平均29才の女性若年群7例で、第3群は46~76才平均61才の男1、女9計10例の高年群である。第2、3群と夫々に同年令分布、同平均年齢、同数例を、対照群に選んだ。第4群が7例、第5群が10例である。

酢大豆は炒った大豆(高山産)を米酢に漬け、蒸えたもので、30g/日を2分割し毎日経口投与した。豆腐、味噌、海藻摂取には制限を加えず、通常生活を続行させた。

大豆投与実験は1989年8月から1年間に行い毎日摂取を怠ったものは除外した。

検査は大豆投与前、投与最終日、及び大豆中止3ヵ月以降日の午前中に施行し、問診、甲状腺の触診と、採血を行った。-80℃で保存した血清を用い大豆前後をペアで同時測定した。甲状腺腫を認めた例は超音波検査を行った。自覚症状は期間中の再三の問診から先ず全ての症状を取り上げた。その中から持続した症状のみを取り上げ、他病態によると判断される症状は除外した。大豆中止後に消失した症状を人々効果と見なして採択した。対象例中4例は投与前検査が、また2例は大豆中止後の検査が出来なかった。

血清T₄、T₃、FT₄、FT₃、rT₃、TBGはRIAで、TSHは高感度法で測定した。NEFAは酵素法で、CPK、LDH、GOT、GPTはUV法で測定した。TRHテストは500ug静注法を用い、30分後の

The Effects on the Thyroid Gland of Soybeans Administered
Experimentally in Healthy Subjects

Y. Ishizuki, Y. Hirooka, Y. Murata, K. Togashi

(Nippon Naibunpi gakkai Zasshi, 67, 622-629, 1991)

Introduction

It is said that soy beans contain a goitrogenic substance ¹⁾ and that the administration of soy beans to experimental rats, even for a short period, lowers serum T₄ levels ²⁾ and suppresses the I¹³¹ intake rate ³⁾. In the case of humans, there have been several cases in which the onset of goitres and hypothyroidism in infants fed with soy milk were reported ^{3) - 8)}. These writers reported that soy beans had slightly suppressed the thyroid function in chronic thyroiditis, and that they affected the thyroid gland in adults ⁹⁾. However, there has been no systematic study on whether soy beans in the normal diet suppress the thyroid function or not.

In this study, soybeans were administered to normal healthy adults, and it was investigated whether they affected thyroid function in adults or not. The study also examined whether the effects of diet on the thyroid function can be ignored or not, when reading the values of various parameters.

Subjects and methods

The subjects were selected from healthy working adults who had never had thyroid disorders or goitres, had no serum antithyroid antibodies, and were not on medications which influenced TBG fluctuation. Eight males and forty six females, 54 in total, aged between 22 and 76, were divided into 5 groups. Group 1 was the short duration group. Seven males and thirteen females, 20 in total, aged between 22 and 60 were given soy beans for 1 month. The long duration (3 months) group was divided into 2 groups (Groups 2 and 3) by age. Group 2 comprised younger females aged between 22 and 39 (average 29), and Group 3 comprised an older male (1) and females (9) aged between 46 and 76 (average 61). Control groups comprising the same age distribution, average age and number of subjects as Groups 2 & 3 were selected as Groups 4 & 5. Hence, Group 4 comprised 7 subjects, and Group 5 comprised 10 subjects.

Vinegared soy beans were prepared by pickling roasted soy beans (Produce of Takayama) in rice vinegar. 30 g of this preserve was administered orally every day, twice a day. Soy bean curd, miso (soy bean paste), and seaweed were given as usual without any restriction.

The administration of was continued for a year (sic) from August 1989, the subjects who neglected daily intake of soy beans were excluded.

Examinations were carried out before administration, on the last day of administration, and on a morning more than 3 months after the cessation of soy beans. Examinations comprised an interview, palpation of the thyroid gland, and blood tests. Serum was stored at minus 80 deg C, and the serums before and after the administration of soy beans were measured simultaneously. Those in whom goitre was detected were examined by ultrasonic scan. All symptoms during the period were recorded. Only sustained symptoms were selected, and the symptoms which were assumed to be caused by other causes were excluded. The symptoms which disappeared after the cessation of the administration of soy beans were considered to be the result of the administration of soy beans, and so recorded. 4 subjects missed the pre-administration examination. 2 subjects missed the post-administration examination.

Serum T₄, T₃, FT₃, gammaT₃, and TBG were measured by RIA, and TSH was measured by the high sensitivity method. NEFA was measured by the enzymic method. CPK, LDH, GOT, and GPT were measured by the UV method. The TRH test used a 500 micro g venous injection method. The TSH value after 30 minutes was treated as TSH, and T₃ was measured at the same time. Serum total iodine was measured by the alkali incineration method. The inorganic iodide value was calculated by subtracting the iodine value in the T₄ value measured by RIA from total iodine. The t test, the Mann-Whitney method, and the order addition test using Wilcoxon, the X² test, and the direct probability method were employed in the statistical analyses.

Results

1) Thyroid function of the short duration group

Serum T₃ values, and T₄ values of Group 1 showed a tendency to drop after soy bean intake over one month, but the drop was not significant. FT₄, FT₃, gammaT₃, TBG, the FT₄/T₄ ratio, and the FT₃/T₃ ratio showed no significant changes.

Inorganic iodide values showed no difference before and after the treatment, but TSH levels significantly increased after soy bean intake (P(U) < 0.01 Table 1).

2) Thyroid function of the long duration group

Serum T₄, T₃, FT₄, FT₃, Inorganic iodide, the FT₄/T₄ ratio, and the FT₃/T₃ ratio in Group 2 of the long duration (3 months) group showed no significant changes, while TSH levels clearly increased (P(U) < 0.01), though the rise was slight. Serum T₄, T₃, FT₄, FT₃, Inorganic iodide, the FT₄/T₄ ratio, and the FT₃/T₃ ratio in Group 3 showed no significant changes before and after soy bean intake, while TSH levels clearly increased (P < 0.05) although they stayed within the normal range. Inorganic iodide levels in the older group were higher than those in the younger group, but the inorganic iodide values when

TSH 値を Δ TSH とし、 T_3 も同時に測定した。血中総ヨード量はアルカリ灰化法で測定し、RIA で測定した T_4 値中ヨード量を総ヨード値より差し引き、その値を無機ヨード値とした。統計処理は t テスト, Mann-Whitney 法, Wilcoxon による順位検定, X^2 テスト, 直接確率法を用いた。

結 果

1) 短期大豆投与群の甲状腺機能

第1群の血清 T_4 値、 T_3 値は大豆服用1ヵ月後に低下傾向を示したが、有意でなく、 FT_4 、 FT_3 、 rT_3 、 TBG 、 FT_4/T_4 比、 FT_3/T_3 比も有意な変動を示さなかった。

無機ヨード値も前後で差がなかったにも拘わらず、TSH は大豆服用後有意な ($P(U) < 0.01$) 増加を示した (Table 1)。

Table. 1 Thyroid function in healthy subjects before and after soybean ingestion

		Group 1			Group 2			Group 3		
		No. of Cases	pre-treatment	3M after soybean ingestion	No. of Cases	pre-treatment	3M after soybean ingestion	No. of Cases	pre-treatment	3M during soybean ingestion
T_4	$\mu g/dl$	20	96 ± 0.5	90 ± 0.5	7	92 ± 0.8	94 ± 0.8	6	79 ± 0.9	79 ± 0.9
T_3	ng/dl	20	143.2 ± 5.0	140.2 ± 5.6	7	139.9 ± 8.8	146.3 ± 11.2	6	137.3 ± 11.3	137.0 ± 10.7
FT_4	ng/dl	20	14 ± 0.07	14 ± 0.07	7	14 ± 0.1	15 ± 0.1	6	10 ± 0.07	10 ± 0.1
FT_3	pg/ml	20	40 ± 0.3	41 ± 0.1	7	40 ± 0.2	44 ± 0.2	6	34 ± 0.3	34 ± 0.3
rT_3	ng/dl	20	25.5 ± 1.7	26.6 ± 1.8	7	25.5 ± 1.5	26.8 ± 2.8	6	18.4 ± 2.1	23.9 ± 2.3
TBG	$\mu g/ml$	12	23.1 ± 1.3	22.2 ± 1.1						
inorg iodide	$\mu g/dl$	20	2.4 ± 0.9	2.1 ± 1.0	7	1.5 ± 0.4	1.6 ± 0.3	6	9.5 ± 2.3	7.1 ± 2.2
TSH	$\mu U/ml$	20	$1.2 \pm 0.1^*$	$1.6 \pm 0.3^*$	7	$1.1 \pm 0.1^*$	$2.1 \pm 0.6^*$	6	$2.0 \pm 0.3^*$	$3.3 \pm 0.5^*$
$FT_4/T_4 \times 10^4$		20	14.9 ± 0.5	15.5 ± 0.6	7	15.4 ± 1.1	15.3 ± 0.7	6	13.4 ± 1.0	15.0 ± 1.3
$FT_3/T_3 \times 10^4$		20	2.8 ± 0.1	2.9 ± 0.1	7	2.9 ± 0.2	3.0 ± 0.1	6	2.5 ± 0.2	2.6 ± 0.3

P(U) *--TSH < 0.01 *--TSH < 0.01 *--TSH < 0.01 *--TSH < 0.05
FT₄ < 0.025
TSH < 0.05

2) 長期大豆投与群の甲状腺機能

大豆を3ヵ月投与した第2群では血清 T_4 、 T_3 、 FT_4 、 FT_3 、無機ヨード、 FT_4/T_4 比、 FT_3/T_3 比、いずれも有意な変動を示さなかったが、TSH は軽微ながら明らかに ($P(U) < 0.01$) 増加した。第3群では T_4 、 T_3 、 FT_4 、 FT_3 、 rT_3 、 FT_4/T_4 比、 FT_3/T_3 比も大豆前後で有意な変動を見なかったが、TSH が正常域内で明らかに増加 ($P < 0.05$) した。高年齢群無機ヨード値は若年群より高かったが、大豆を服用して、TSH の増加した時の無機ヨードは前値より低値であった。第3群の大豆後 TSH は第2群の大豆後より高い傾向 ($P(U) = 0.05$) にあり、第5群 (対照) の平均 $1.7 \pm 0.2 \mu U/ml$ より明らかに ($P < 0.02$) 高かった。 Δ TSH で見ると、第2群の Δ TSH は $20.5 \pm 2.6 \mu U/ml$ で、第4群の平均 $22.4 \pm 2.9 \mu U/ml$ と変わりなかった。第2群の TRH 後 T_3 は前値 $147.3 \pm 6.7 ng/dl$ が TRH により $140 \pm 10.3 ng/dl$ と増加しなかった。第3群の Δ TSH は $30.3 \pm 2.6 \mu U/ml$ で、第5群の平均 $19.0 \pm 2.2 \mu U/ml$ より明らかに ($P < 0.05$) 高値であり、しかも第2群の Δ TSH より明らかに ($P(U) < 0.03$) 高値を示した (Fig 1)。

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TSH was raised by the intake of soy beans were lower than those before the rise of TSH. The TSH value after the treatment in Group 3 tended to be higher than that in Group 2 ($P(U) = 0.05$), and was clearly higher ($P < 0.02$) than that of Group 5 (Control), being on average $1.7 \pm 0.2 \mu U/ml$. Δ TSH in Group 2 was $20.5 \pm 2.6 \mu U/ml$, and was no different from that in Group 4, being on average $22.4 \pm 2.9 \mu U/ml$. The post-TRH T_3 in Group 2 was $140 \pm 10.3 ng/dl$, which did not rise by TRH from the pre-TRH T_3 of $147.3 \pm 6.7 ng/dl$. Δ TSH in Group 3 was $30.3 \pm 2.6 \mu U/ml$, and was clearly higher ($P < 0.05$) than Group 5, being on average $19.0 \pm 2.2 \mu U/ml$, and also higher than that of Group 2 ($P(U) < 0.03$) (Fig 1).

The T_3 in Group 3 at the time of the TRH test was $139.9 \pm 3.8 ng/dl$, and became $128.9 \pm 10.2 ng/dl$ after 30 minutes, showing no increase.

8 subjects in Group 3 who showed a large fluctuation in TSH were studied after the cessation of the administration of soy beans. T_4 and T_3 showed a rising trend, and FT_4 and FT_3 clearly increased ($P(U) < 0.05$, < 0.025). The FT_4/T_4 ratio and the FT_3/T_3 ratio showed no significant fluctuation. However, TSH levels clearly dropped ($P(U) < 0.05$). The Δ TSH of seven subjects was on average $19.0 \pm 4.7 \mu U/ml$, showing an obvious decrease ($P(U) < 0.01$), while inorganic iodide values showed no significant difference between before and after soy bean intake.

The correlation between the post-administration T_4 value and T_3 value was significant, the correlation coefficient being $+0.67$ ($N=37$, < 0.01), while there was no correlation between T_4 and TSH, and between inorganic iodide values and TSH, the correlation coefficient being $+0.07$ (Fig 2).

3) Fluctuations of serum substances

Various substances which are considered to be affected by the thyroid function were studied. Serum albumin was not measured as it had already been accepted that it was not influenced by soy beans. CPK, NEFA, GOT, AND GPT in Groups 1 and 2 showed no significant difference between before and after the intake of soy beans, and indices in Group 3 showed no significant difference between during the intake of soy beans and after the cessation of soy bean intake. LDH in Group 1 alone showed an increase owing to the intake of soy beans ($P < 0.001$). LDH in Group 3 during the intake of soy beans was higher than that in Groups 1 and 2, while there was no significant difference between the former and LDH in Group 1 during the intake of soy beans (sic). LDH in Group 3 clearly dropped after the cessation of soy bean intake ($P(U) < 0.01$) (Table 2).

4) Symptoms and goitre

20 subjects in Group 1 complained of symptoms of diarrhoea (7 subjects 35%), abdominal inflation (5 subjects 25%), constipation (4 subjects 20%), fatigue, lethargy, and eczema (2 subjects each 10%). 17 subjects in Groups 2 and 3 complained of diarrhoea (11 subjects 64.7% increase), constipation (9 subjects 52.9%), fatigue

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第3群のTRHテスト、 T_4 は $139.9 \pm 3.8 \text{ ng/dl}$ であり、30分後 $128.9 \pm 10.2 \text{ ng/dl}$ となり増加を見なかった。

TSHの変動の大きかった第3群中8例について大豆中止後の検索を行うと、 T_4 、 T_3 は増加傾向で、 FT_4 、 FT_3 は共に明らかな ($P(U) < 0.05$, < 0.025) 増加を示した。 FT_4/T_4 比、 FT_3/T_3 比は大豆中止によっても有意な変動でなかった。しかし、TSHは明らかな ($P(U) < 0.05$) 低下を見た。7例の Δ TSHは平均 $19.0 \pm 4.7 \mu\text{U/ml}$ となり明らかに ($P(U) < 0.01$) 低下したが、無機ヨード値は大豆服用中と中止後とで有意差がなかった。

大豆投与後の T_4 値と T_3 値とは相関係数 $+0.67$ ($N=37$, < 0.01) と有意な相関であったが、 T_4 とTSHとの間に相関なく、無機ヨード値とTSHとの間にも相関係数 $+0.07$ と相関がなかった (Fig 2)。

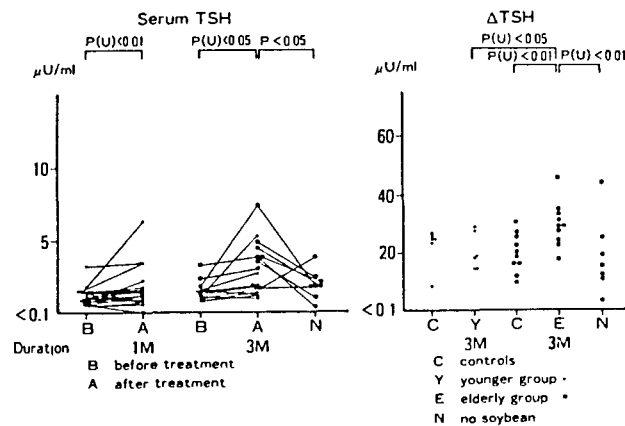


Fig 1 Serum TSH and Δ TSH in healthy subjects who have been administered 30g/day of soybean

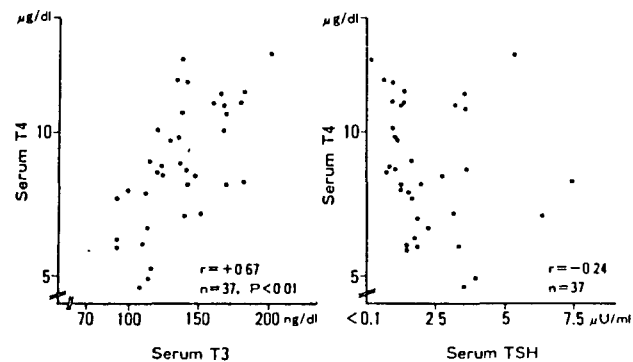


Fig. 2 Correlation between serum T_4 and T_3 , Correlation between serum T_4 and TSH

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3) 血清諸物質の変動

甲状腺機能により変動すると言われる諸物質の変動を見た。血清アルブミンは大豆により変動しない事を既に認めている¹²⁾ので本研究では測定しなかった。第1, 2群では大豆前後のCPK, NEFA, GOT, GPTに有意差がなく、また第3群では大豆服用中と中止後の指間間に有意差が見られなかった。第1群のLDHのみが大豆服用により増加した ($P < 0.001$)。大豆服用中の第3群LDHは第1, 第2群より高かったが、第1群大豆服用中の値との間に有意差がなかった。第3群のLDHは服用中止後明らかに ($P(U) < 0.01$) 低下した。(Table 2)

Table. 2 Serum enzyme levels in healthy subjects before and after soybean ingestion

		No of Cases	Group 1		No of Cases	Group 2		No of Cases	Group 3	
			pre-treatment	1M after soybean ingestion		pre-treatment	3M after soybean ingestion		during soybean ingestion	after the cessation of soybean
CPK	IU/l/37C	14	40.2 ± 2.1	42.0 ± 2.8	5	39.8 ± 2.1	45.6 ± 4.4	8	50.25 ± 12.1	46.0 ± 5.9
NEFA	mEq/l	17	0.96 ± 0.16	0.78 ± 0.15	5	0.89 ± 0.2	0.65 ± 0.14	8	1.02 ± 0.32	1.02 ± 0.31
GOT	IU/l	17	10.2 ± 2.6	12.4 ± 1.4	5	8.4 ± 2.8	9.8 ± 2.5	8	26.6 ± 11.0	27.3 ± 17.0
GPT	IU/l	17	2.9 ± 0.9	3.9 ± 0.7	5	1.8 ± 0.4	3.4 ± 0.8	8	10.4 ± 5.0	4.3 ± 0.3
LDH	IU/l	17	114.6 ± 10.5	185.3 ± 12.3	5	125.5 ± 23.1	147.8 ± 23.1	8	229.5 ± 33.3	189.7 ± 47.4

--- $P < 0.001$

--- $P(U) < 0.01$

4) 自覚症状と甲状腺腫

第1群20例では下痢7例35%, 腹部膨満5例25%, 便秘4例20%, 倦怠, 眠気, 及び浮腫がそれぞれ10%であったが、第2, 第3群の17例では下痢が1例と減少し、便秘9例52.9%, 倦怠9例52.9%, 眠気7例41.2%と増加した。甲状腺機能低下症を思わせる症状は第1群より夫々明らかに ($P=0.039$, 0.006 , 0.034) 多く見られた。大豆中止1ヵ月後全例の症状が消失した。

甲状腺腫はI~II度大のゴム様硬の慢性甲状腺腫で、第1群に3例、第2群+3群では8例47.1% (中3例は第3群) に出現し明らかに ($P=0.038$) 長期服用群に多かった。第1群の甲状腺腫3例中1例は大豆摂取3週間経て亜急性甲状腺炎を発症した。大豆中止1ヵ月後には、11例中2例の甲状腺腫の大きさが不変、他は縮小、消失した。不変の2例はT₄治療を行って2, 6ヵ月後に縮小し、治療中止とした。甲状腺腫が認められた例の超音波で hypoechoic 像は全例に見られなかった。

考 察

大豆投与実験に際して、特徴付けた事は先ず健康者を規定した事である。その上日常生活での食品大豆が甲状腺機能に与える影響を知ろうとしたため、大豆以外の食生活に制限を加えなかった事である。

人量の前大豆を3ヵ月間服用させると、健康者と言えども小さな甲状腺腫が半数に見られた。甲状腺機能低下症状も短期間より長期摂取群に多く見られた。Rippの乳児¹⁴⁾例も、大豆中止によ

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(9 subjects 52%, and lethargy (7 subjects 41.2%), the latter three showing an increase. Symptoms indicating hypothyroidism were more noticeable than in Group 1 ($P = 0.039$, 0.006 , 0.034). All symptoms disappeared one month after the cessation of soy bean intake.

This goitre was a diffuse goitre of Degrees I & II enlargement with rubber-like hardness. It appeared in 3 subjects in Group 1, and 8 subjects (47.1%, 3 subjects are in Group 3) in Groups 2 & 3, indicating clearly that there was higher incidence in the long duration group. One subject (out of 3 subjects) with goitre in Group 1 developed subacute thyroiditis three weeks after the commencement of soy bean intake. Two subjects (out of 11 subjects) with goitre showed no change in size one month after the cessation of soy bean intake, while others showed a reduction in size, then the disappearance of goitre. The two subjects with no change received T_4 treatment, and their goitres reduced in size over 2 and 6 months. The treatment was then ceased. No subjects with goitre showed a hypoechoic image on an ultrasonic scan.

Conclusion

The characteristics of this test were firstly to use healthy subjects. Secondly, there was no restrictions on diet except soy beans, since the effects of soy beans on the thyroid function was the purpose of this test.

A large amount of vinegared soybean intake over 3 months induced a small goitre in half the subjects, though they were healthy. In addition, more cases of hypothyroidism were found in the long duration group than in the short duration group. Infant subjects in the Ripp report⁴⁾ showed a size reduction in goitre following the cessation of soy bean intake, but an enlargement of goitre 80 days after the recommencement of the administration of soy beans. Therefore, there is a possibility of the onset of goitre even in healthy adults after a large amount of soy bean intake over a long duration. Despite a high incidence of hypothyroidism, there was no drop in thyroid hormone levels, and TSH alone increased within the normal range. The rise in TSH was greater in the long duration group, and more so in the older group. The TSH response rose more significantly following the administration of TRH. Though the T_3 values did not rise following the administration of TRH, it was not necessarily due to the suppression of the thyroid function, as it was measured after as a short time as 30 minutes. In the older group, there tended to be a rise in peripheral hormones and a drop in TSH and Δ TSH three months after the cessation of soy bean intake, indicating the thyroid function was suppressed during the intake of soy beans. Moreover, regardless of age, goitres which appeared during the intake of soy beans, reduced in size, and the raised TSH level dropped after the cessation of soy bean intake. Based on these facts and the report²⁾ that the height of rat's thyroid follicles is raised by the intake of soy beans, it is reasonable to conclude that soy beans affect the thyroid function rather than the secretion of thyroid hormone. Consequently,

In the case of chronic thyroiditis where the reserve tends to decrease, the intake of soy beans for more than 5 months clearly induced a drop in serum thyroid hormones, and a rise in TSH⁹⁾. However, the subjects in this test were healthy adults with an ample reserve. The duration of the test was as short as within three months. These factors were considered to have contributed to the result that the level of hormone reserve did not drop as much as to lower the concentration of serum thyroid hormones. In healthy adults, serum T_4 and T_3 correlated well, gamma T_3 and TBC did not fluctuate, and the thyroid hormone levels did not drop. These indicated that the effect of soy beans on the suppression of thyroid hormone synthesis and the slowing down of Tg hydrolysis in the follicles was weak/mild, and a longer duration of administration was required to cause hypothyroidism severe enough to be clinically noticed. In a report where soy beans were administered for 5 days⁸⁾, 2 subjects out of 14 showed a drop in PBI¹³⁾ despite the PBI remaining constant (sic). The difference between the two indices was considered to be due to the loss of thyroid hormones in the faeces. There is an animal experiment²⁾ where the addition of iodine to soy bean diet induced the disappearance of goitre which had occurred on a soy bean diet with iodine restriction. However, in the case of humans, goitre appeared with the normal intake of iodine, and a slight suppression of the thyroid function was detected. It was necessary to examine whether the drop in thyroid hormones and the rise in TSH in the elderly were caused by iodine or not. (Literal - Translator) The fact that the inorganic iodide levels in the older group were higher than those in the younger group indicated that the elderly had a higher intake of iodine than the young, and that soy beans did not inhibit the absorption of iodine. The higher intake of iodine in the older group was reflected on the high TSH levels before the administration of soy beans. Although the inorganic iodide levels after the administration of soy beans decreased compared with those before the administration, TSH increased, and Δ TSH was higher than that of the Control because of the intake of soy beans. However, the inorganic iodide levels did not correlate with TSH. This would indicate that the sporadic intake of a large amount of iodine in normal daily life is unlikely to affect the TSH fluctuation. There is a report in which older women showed a greater drop in serum T_3 and a greater rise in Δ TSH than young women, and it was concluded that this was due to lack of responsiveness of the thyroid gland caused by ageing¹⁰⁾. In the case of chronic thyroiditis, the majority⁹⁾ of the subjects aged in a wide range showed a high Δ TSH due to the intake of soy beans, despite their inorganic iodine levels being in the normal range. The recovery of the thyroid function observed some period after the cessation of soy bean intake was not considered to be purely due to the age factor. Chronic thyroiditis shows a high sensitivity to iodine, and a high sensitivity to goitrogen in soy beans is also a possibility. However, the result of this experiment on healthy subjects indicates that soy beans may influence on the thyrotropic hormone from the pituitary gland, rather than the suppression of (thyroid) functions due to iodine intake. It also indicates that the elderly are more susceptible than the young. Although there is a report¹¹⁾ which states soy beans encourage the elimination of thyroid hormones into faeces, thereby lowering the function, this is debatable as both peripheral thyroid hormone and TSH levels fluctuated within the normal range.

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Hypothyroidism was observed in half the subjects, and a rise in some enzymes in the blood were observed both the elderly and the young, and only during the intake of soy beans. Therefore, it is hard to conclude that they were induced by muscular exertion (Literal). Rather, it would indicate that the peripheral function of thyroid hormones was slightly and mildly suppressed by soy beans.

The normal range for hormone levels is wide. A value in the normal range of readings needs to be interpreted by taking clinical symptoms and the difference in sensitivity by age into account. In addition, the effects of dietary constituents such as soy beans and seaweed should be taken into consideration in order to give an accurate diagnosis of the thyroid function.

Summary

The changes in the thyroid function were studied by administering 30 g of vinegared soybeans per day to 37 healthy subjects.

20 subjects were given soy beans for 1 month, and no change was detected in the peripheral thyroid hormone levels, while TSH was slightly raised ($P(U) < 0.01$). 17 subjects were administered soy beans for 3 months, and no change in the thyroid hormone levels was detected, but a clearly raised level of TSH was observed after the intake of soy beans, regardless of the age factor ($P(U) < 0.01$ for the older group, ($P(U) < 0.01$ for the younger group). The TSH level in the older group (average age 61) rose significantly after the administration of TRH, compared with those in the Control group and in the younger group (average age 29) ($P(U) < 0.01$, $P(U) < 0.03$). In the older group, FT_4 and FT_3 levels rose, and Δ TSH and TSH levels dropped to those in the Control after the cessation of soy bean intake. No correlation was detected between serum inorganic iodide and TSH.

Diffuse goitre and hypothyroidism appeared in half the subjects after taking soybeans for 3 months, but they reduced and disappeared after the cessation of soy bean intake.

Serum CPK, NEFA, GOT, and GPT were unchanged by the administration of soy beans, while LDH alone rose. The LDH rise was more marked in the older group, but LDH levels dropped in all subjects after the cessation of soy bean intake.

The above results indicated that excessive intake of soy beans for a long duration might cause the enlargement of the thyroid gland, and slightly suppress thyroid function in healthy people.

て一時縮小した甲状腺腫が、再投与80日後再び増大している。大豆を大量、長期服用すると健康成人でも甲状腺腫発症の可能性があると云える。甲状腺機能低下症状の出現度が多かったにも拘わらず、甲状腺ホルモン値(甲ホ)の低下がなく、TSHのみが正常域内で増加した。しかも長期群殊に高年群程、TSH上昇度が大きく、TRH投与後TSH反応度も大きかった。TRH投与後の T_3 値は上昇しなかったが、投与30分後という短時間値であり、甲状腺機能抑制を示す結果とは言えなかった。高年群では大豆服用を中止すると、3ヵ月以上経て、末梢ホルモンが高くなる傾向とTSH、 Δ TSHの低下が見られ、大豆服用中の甲状腺機能は抑制されていた事が示唆された。しかも老若に拘わらず、大豆投与中に見られた甲状腺腫、TSH上昇は大豆中止後に縮小し、低下を示した。これら事実と、大豆投与ラットで甲状腺濾胞細胞の高さが増大したという報告²⁾を併せ考えると、大豆は甲ホ分泌よりもむしろ、ホルモン合成に影響を及ぼし、TSHを増加させたと思なし得る。

予備能の減退し易い慢性甲状腺炎では5ヵ月以上大豆を服用して血中甲ホが明らかに減少、TSHが高値を示した³⁾のとは異なり、本対象は予備能が充分にある健康者であり、しかも服用が3ヵ月内という短期間であった。その為に血中甲ホ濃度を低下させる程の貯蔵ホルモン量の減退がもたらされなかったであろう。健康者では血中 T_4 が T_3 とよく相関し、 rT_3 、TBGが変動せず、甲ホ値の低下が見られなかった事は、大豆が甲ホ合成を抑制させる力や濾胞内での T_4 水分解速度を減退させる作用が弱く、且つ緩やかである事を示唆したもので、臨床像として把握出来る程の機能低下状態を招くには更に長期間を要するものと考えられる。大豆を5日間与えた報告⁴⁾では、PBIが不変にも拘わらず14例中2例のPBI⁵⁾低下が見られたと言い、両指標の差は便中への甲ホ喪失によるものでないと思なしている。大豆食にヨードを加えると、ヨード制限下で発症した甲状腺腫が、見られなくなるという動物実験⁶⁾がある。しかし人では普通にコードを摂取した生活で甲状腺腫を認めると共に甲状腺機能の軽度抑制が見られた。むしろ高齢者での甲ホ低下、TSHの増加がヨード作用によるかどうかを考える必要があった。高年群無機ヨード値が若年群より高かった事は、高年者がヨード摂取量の多い生活をしている事を意味しており、大豆がヨードの吸収を阻止しないことを示している。ヨード摂取の多いことは高年群の大豆投与前TSHが高い事に反映されたと解される。大豆投与後の無機ヨードが前値より減少したにも拘わらず、大豆服用によってTSHが高くなり、 Δ TSHも対照群より高値を示したが、無機ヨード値はTSHと相関しなかった。この事は通常生活で散発的にヨードを多く摂取した程度では、ヨード効果がTSH変動に逆反映されにくい事を示したものであろう。高年女性では若年より血中 T_4 が低下し、 Δ TSHが上昇したという成績があり、加齢に伴う甲状腺の不応性による結果だろうとの報告⁷⁾がある。広い年齢域にあった慢性甲状腺炎の大多数例⁸⁾で無機ヨード値が正常域にあったにも拘わらず、 Δ TSHは大豆服用により高値を示した。大豆服用を中止し、暫く時を置いて見られた甲状腺機能の回復事実は年齢のみによると思えられなかった。ヨードへの感受性が高い慢性甲状腺炎は大豆内 goitrogen への感受性も高いと考えられるが、健康者で見た今回の成績は、摂取ヨードによる機能抑制ではなく、ヨード以外の要素として大豆が下垂体甲状腺系に影響を及ぼした事を示したものであろう。また高年者は若年者より影響を受け易いと解すべきであろう。大豆は甲ホの便中への排泄を促し、機能低下に導くとの報告⁹⁾もあるが、末梢甲ホ値及びTSHは正常域内の変動であった事から大きな効果を示すとは言えなかった。

略半数に見た低下症状や、一部酵素の血中増加は高年、若年共に見出され、しかも大豆服用時のみである事から筋労作による血中上昇のみは見なされない。むしろ大豆による甲ホの末梢作用が

軽度に, しかも緩やかに減退したことを示唆したものと考えたい。

ホルモン値の正常域は広い幅を示すものである。たとえ正常値が得られたとしても, 臨床症状や年齢による感受性の差を考慮して判断せねばならない。そのみならず, 大豆, 昆布など食餌の影響を加味して検査値を判定してこそ, 適確な甲状腺機能診断が可能となるものと考えられた。

ま と め

健康者37例に酢大豆30g/日を与え甲状腺機能変動を検索した。

1ヵ月投与の20例では, 末梢甲状腺ホルモン値に変化がなく, TSH が軽度 ($P(U) < 0.01$) 増加した。3ヵ月投与の17例では甲状腺ホルモン値に変化を見なかったが, 大豆服用後 TSH は服用前より高年, 若年共明らかに ($P(U) < 0.01$, $P(U) < 0.01$) 増加した。高年者 (平均61才) への TRH 投与に対しては同年, 同数の対照群及び若年大豆群 (平均29才) より明らかに ($P(U) < 0.01$, $P(U) < 0.03$) 大きく反応した。高年群では大豆中止後に FT_4 , FT_3 が増加し, ΔTSH , TSH は対照群の値に逐低下回復した。血中無機ヨードと大豆服用中 TSH とは相関がなかった。

大豆3ヵ月投与後には慢性甲状腺腫, 機能低下症状が夫々半数に見られたが, 大豆中止によって縮小, 消失した。

血清 CPK, NEFA, GOT, GPT は大豆投与によって変動しなかったが, LDH のみは増加した。LDH 増加は高年群に著しく, 大豆中止後全例が減少した ($P(U) < 0.01$)。

以上から健康者でも大量大豆を長期に摂取すれば, 甲状腺は腫大し, 甲状腺機能は軽度抑制される事が示唆された。

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CURRICULUM VITAE

DAVID JOHN WOODHAMS

Correspondence to:

Dr David J Woodhams CEng
2/47 Church Street
Devonport
North Shore City 1309
New Zealand

Telecommunications:

Telephone: +64 9 445 8721
Facsimile: +64 9 445 9834
Email: woodhams@iprolink.co.nz

Occupation:

Chemical Engineer, self employed as a Dairy Process Consultant, offering services to the NZ dairy industry in the management of research projects, technology transfer projects, process and product development projects, process assessment, quality and control documentation, strategic studies.

Date and place of birth:

(b) (6)

Academic Qualifications:

BE(Chem)(Hons), University of Canterbury, 1962
ME(Chem), University of Canterbury, 1963
PhD(Food Science), University of Wisconsin, 1970

Professional Qualifications:

Chartered Engineer
Member, Institution of Chemical Engineers
Member, Institution of Professional Engineers, NZ
Member, NZ Institute of Chemistry

Other Professional Organisations:

Society of Chemical Engineers (NZ) (was Chemical Engineering Group of IPENZ) (Hon Secretary 1971-74)
Dairy Industry Association of NZ (was NZ Society of Dairy Science and Technology) (President 1976-77)

000569

Languages:

Fluent reader of technical French.

Professional Experience:

Mar 63 - Dec 65: Chemical Engineer, NZ Dairy Research Institute.
Jan 66 - Oct 69: NZDRI Research Fellow, University of Wisconsin.
Nov 69 - Jan 70: Seconded to NZ Dairy Board. Soluble whey protein processing investigations in USA.
Feb 70 - Oct 71: Research Officer, NZDRI. Whey ultrafiltration, whey protein concentrate.
Nov 71 - Feb 75: Section Head, Milk Powder and Drying, NZDRI.
Mar 75 - May 80: Technical Officer, Northern Wairoa Coop Dairy Company.
Travelled to USA and Europe in June/July 1978 as member of NZ Dairy Factory Managers' Study Tour.
Jun 80 - Dec 82: APV Bell Bryant (NZ) Ltd, Assistant General Manager.
Jan 83 - Dec 84: APV Bell Bryant (NZ) Ltd, General Manager.
Jan 85 - Aug 85: APV Engineering Ltd, Divisional General Manager, Process Engineering and Packaging Division.
Sep 85 - Present: Dairy Process Consultant, self employed or contracted.

000570

OU



THE
FOOD
COMMISSION

Publisher of The Food Magazine

BY FAX (COPY BY MAIL)

28 April 1998

Linda S Kahl, PhD
Regulatory Policy Branch HFS-206
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
200 C St SW Washington DC 20204
USA

Dear Dr Kahl

GRN #000001

The Food Commission is a consumer organisation which campaigns for safer, healthier food. We publish a quarterly journal, *The Food Magazine*, and are well respected as a source of independent information and food policy analysis. We have taken an interest in phytoestrogens in the human diet and the debate around their potential health benefits and risks and have published a number of articles in our journal on the subject. In addition I am a consumer representative to the Ministry of Agriculture, Fisheries and Food Working Party on Chemical Contaminants in Food which examines research into natural toxicants in food, including phytoestrogens.

We are concerned that the FDA is considering a petition by Archer Daniels Midland for soy isoflavones to be generally recognised as safe (GRAS). Given the conflicting and controversial nature of the research on the health risks and benefits of isoflavones we request that the FDA rejects this petition. The FDA cannot be unaware firstly that research shows that the claimed health benefits of isoflavones are inconclusive and inadequately researched; secondly, that evidence exists of potential health risks to humans from isoflavones and thirdly, that this is a highly controversial area of nutrition research. For these reasons alone, we consider it is inappropriate for isoflavones to be considered for GRAS status at this time.

Because of the growing interest in the claimed health benefits of phytoestrogens, the UK government commissioned an authoritative report into the subject from the Medical Research Council's Institute for Environment and Health¹. This concluded that 'though some epidemiological studies suggest that consumption of foods containing phytoestrogens may have beneficial effects, almost no evidence exists to link these effects directly to phytoestrogens'. It goes on to conclude that research would be needed to clarify whether

¹ Institute for Environment and Health, IEH Assessment on Phytoestrogens in the Human Diet, Final Report to the Ministry of Agriculture, Fisheries and Food, November 1997.

000713

Please note our new address - The Food Commission (UK) Ltd, 94 White Lion Street, London N1 9PF. Tel: 0171 837 2250 Fax: 0171 837 1141
e-mail: foodcomm@compuserve.com

Campaigning for safer, healthier food

The Food Commission (UK) Ltd, 94 White Lion Street, London N1 9PF. Tel: 0171 837 2250. Fax: 0171 837 1141

The Food Commission (UK) Ltd is a not for profit company limited by guarantee and publisher of the *Food Magazine*. Company Registration No. 2485176. Registered office as above. VAT No. 524 9331 48. The Food Commission (UK) Ltd campaigns for safer, healthier food and is funded by public subscriptions and donations.

consumption of phytoestrogens specifically confers any degree of protection against cancer, osteoporosis, post-menopausal symptoms and cardiovascular disease

In addition there is a growing body of evidence pointing to possible adverse effects of isoflavones. To quote just two examples of recent US research, low doses of phytoestrogens in dietary levels have been shown to cause breast cancer cells to proliferate² and research from the US government's National Center for Toxicological Research indicates that isoflavones could inhibit thyroid hormone synthesis, inducing goitre and even thyroid cancer³.

In 1996 the UK government's Department of Health issued warnings⁴ about infant soya formula following a review of literature by its expert Committee on Toxicity (CoT). The CoT concluded, 'The potential for phytoestrogens, including isoflavones, to affect adversely infants is of particular concern since it is possible that a hormonal imbalance in early life can permanently affect sexual development and fertility'. The Committee's report called for research as a matter of high priority to determine whether ingestion of soy based formulae carries any risk for infants and added, '*As a result of further research, it may be necessary to consider the potential risk of soy products to other sectors of the population*'.

We consider that the current state of knowledge into isoflavones cannot support Archer Daniel Midlands assurances of safety. We therefore request that the GRAS petition be withdrawn. Furthermore we request to be kept informed on this matter.

Yours sincerely



Mrs Sue Dibb
Co-director

58995

² Dees C et al (1997) Dietary estrogens stimulate human breast cells to enter the cell cycle, Environmental Health Perspectives, Vol 105, Supplement 3, April 1997.

³ Divi RL et al (1997) Anti-thyroid isoflavones from soybean, Biochemical Pathology, Vol 54, 1087-1096.

⁴ Phytoestrogens in soya infant formula milks, Public Health Link CEM/CMO/96/8, Department of Health, 17 July 1996.

000714

TOTAL P.02



To: Linda Kahl@OPA@FDA.CFSAN
From: "Dave Woodhams" <woodhams@iprolink.co.nz>
Certify: N
Subject: re: GRAS Notice GRN 000001
Date: Wednesday, April 29, 1998 at 7:55:00 am EDT
Attached: None

Dear Dr Kahl

Thank you for your prompt response.

I have just finished reading Dr Daniel Sheehan's paper to the 1995 Little Rock Conference: "Herbal Medicines, Phytoestrogens and Toxicity: Benefit Considerations". P.S.E.B.M. 1998, Vol 217 pp 379-385, which reached my desk only today. (Hence the lateness of the hour as I write!)

This is a very cogent argument that expresses, much better than I can, the view I have been trying to convey to our own Ministry of Health here for the last three and a half years. I trust that your scientific reviewers have this paper on top of their pile.

Dr David J Woodhams CEng
Dairy Process Consultant
PO Box 32 236
2/47 Church Street
Devonport 1309
New Zealand
Phone: +64 9 445 8721
Fax: +64 9 445 9834
Email: woodhams@iprolink.co.nz

000426

Apr 29, 1998

To: Dr. Linda Kahl, Office of Premarket Approval,
Center for Food Safety, Food and Drug Administration
200 C Street, S.W. (HFS-206) Washington D.C.
20204

OU



At For. 001-202-418-3131.

From: ~~Valerie James~~ at For 64-9-434-0567.

Re: GRAS GRN 000001 (A.O.M)

Date: April 24th. 1998.

Dear Dr. Kahl,

I completed the following very late
last night. Please excuse the writing but since most
of it is exact quotes from paper mailed, the original
text will be clearer. Please note however, that
AOH's assertion that "clover disease" was caused
by contamination rather than isoflavones in clover is
false. Also the effects were more severe than just
a reduction in fertility.

Thankyou for your continuing communications.

Yours sincerely,

~~Valerie James~~

000428

P.01

64+9+4340567

29-APR-98 13:01

To: Dr. Linda Kahl, Office of Pre-market Approval,
Center for Food Safety, Food and Drug Administration
200 C Street, S.W. (HFS - 206) Washington D.C.
20204

At Fax. 001 - 202 - 418 - 3131.

From: ~~V. Jones~~ at Fax 64 - 9 - 434 - 0567.

Re ERAS GRN 000001 (A.D.M)

000429

To: Dr. Linda Kahl, Office of Premarket Approval, Center for Food Safety, Food and Drug Administration, 200 C Street, S.W. (H-15-104) Washington D.C. 20204.

At: Facsimile 001-202-418 3131

From: ~~Ilse B. Jones~~, Rural Delivery 4, Whangarei, N.Z.

At: Facsimile 64-9-434 0567.

Date: Tuesday, April 28th, 1998.

Re: GRAS Notice # GRN 000001 (ADM)

Dear Dr. Kahl,

Today I mailed you copies of a number of published scientific papers from my study files. Because I realize ~~that~~ some misrepresentations may have been made to you about my convictions and affiliation, I have chosen to provide entire documents so that they can be independently assessed, rather than just provide quotes which can actually mislead as to the impact and message of the entire paper.

The ADM submission #000001 contains many mistakes of fact. The following documents have been mailed to you to illustrate just a few. It would take a "basket," rather than a "package" to provide documentation to illustrate all of the mistakes.

The papers I have selected are as follows, with reasons given for inclusion in the mailed "package"

1. "Genistin (an Isoflavone Glucoside) and its Aglycone Genistein from Soybeans"; Walter 1991. (Front page only). This shows that the ability to extract isoflavones from soy is approx 000430

40 plus years old and is not a newly developed technique.

2. One page from a "data base" showing that genistein (from studies of the 1950s) when added as 0.2% of diet elicited estrogenic effects. (I apologize for poor quality of reproduction as the information forced from data base, arrived here like that)

3. "Effect of Genistein on Reproduction of the Mouse."

Carter, Malone and Snot. (This is a classic paper and often quoted) Diet 1, was control with genistein removed, Diet 2, had 0.02% added Sack, Diet 3 made up of commercial soy bean meal, with approx 0.01% genistein.

Summary Both commercial soybean oil meal and isolated genistein significantly lowered the age at which the vaginas of immature mice opened. The principal effect on reproduction of 0.02% genistein in the diet was a decrease in the number of litters born, whereas litter size was not affected. The commercial soybean oil meal (80% of diet) was a decrease in the number of litters.

4. "Effect of Genistein on Growth and Development of the Male Mouse." Carter, ^{Snot} Malone, Snot.

Results Ten mice died, 4 receiving highest level of genistein, two receiving third and four were scattered among other levels. Males on highest level of genistein lost weight. Genistein also had depressing effect on testes weight. No spermatozoa present in the testes of groups receiving two higher levels of genistein. The higher levels of genistein appeared to be lethal. "It appears from these results that genistein, in relation to its estrogenic activity, has a greater depressing effect than does stilbestrol." 000431

5. "Metabolism of Estrogenic Isoflavones in Domestic

Animals" Lundh. Soc. for Exp. Bio and Med. 1995

This is referenced in A.O.M. presentation. It does NOT state "infertility effects are not general to all animals" as submitted by A.O.M. It does discuss reasons for differences between sensitivity between pigs, sheep and cattle. In cattle, daily intakes may reach up to 50-100g ^{estrogenic phytoestrogen} per day which may result in temporary infertility in cattle. The paper discusses detoxification mechanisms of the rumen, and more serious effects in sheep because, it is suggested, cattle have fewer estrogen receptors in uterus. (2 to 4 times higher in sheep than cattle. Pigs are more sensitive than either.

P.30. Glucuronidation is the major detoxification system for potentially toxic endogenous and exogenous substances including phytoestrogens.

Note that for cattle and sheep the phytoestrogen in this red clover silage was Formononetin, daidzein and genistein found mainly as conjugates in the blood (about 5% of total amount as unconjugated substances in the plasma.

6. "Hormone Studies in Ewes Grazing on Estrogenic (Yarloop clover) Pasture During the Reproductive Cycle" Obst, Semark.

1975 Aust. J. Biol Sci 28 271-90.

"Clover Disease" described as a "syndrome of aberrant reproductive function" signs are "ewe infertility, dystocia, uterine prolapse resulting in high ewe and lamb mortality."

Study investigated "the possibility that the clover acts by causing disturbances in the endocrinology of reproduction."

"The mean content of the isoflavones formononetin, genistein and biochanin A in Yarloop clover during July and August 1970 was 1.2, 2.5 and 0.54% respectively of dry leaf weight." The result was that "performance of ewes grazing Yarloop is associated with marked disturbances in reproductive

endocrine function."

7. "Toxicants of Plant Origin" Edited by Peter Cheek, 1989.

(Chapter 2; Phytoestrogens by NR Adams.)

Isoflavones (Table 2) listed as: — Formononetin, Genistein,

Biochanin A. plus a discussion of coumestrol which were
"very low in the absence of infection". "Plant estrogens have

less affinity for the receptor than steroidal estrogens and the
ranking is similar across species of animals." "In every

case that has been examined, phytoestrogens produce changes
in the reproductive tract which are qualitatively the same as

those produced by exogenous estradiol. Both steroidal

and plant estrogens affect the ovary and mammary gland.

Exogenous estrogen causes dysplasia of the ovarian
granulosa cells, thus impeding the maturation of the

ovarian follicles." Note p. 36 comment on DES |

estradiol | coumestrol. "Sheep grazed on highly

estrogenic pastures consisting of relatively pure stand of

subterranean clover or red clover containing high concentrations

(0.5%) of the isoflavone formononetin may suffer marked
clinical signs. Ewes may be affected by severe

infertility accompanied by gross pathological changes in the
uterus, including hydrops uteri, pyometra, cystic hyperplastic

endometritis, and massive adhesions resulting from metritis".

"The infertility results from abnormal function of the
pituitary/ovarian axis. The reproductive problem frequently
resembles the abnormalities associated with cystic ovaries,
including prolonged but irregular estrus nymphomania, or
even the development of masculine sexual characteristics".

8. "Organizational and Activational Effects of Phytoestrogens
on the Reproductive Tract of the Ewe" Adams. Sci. Exp. Biol Med 1985

"Isoflavone compounds including genistein, biochanin A and formononetin

000433

Occur in Subterranean clover or Red clover. Genistein and Biochanin A are usually broken down by microbial activity in the rumen of the sheep while formononetin is converted to the isoflavan equol, which is rapidly absorbed and is responsible for most of the estrogenic activity in ruminants."

"Temporary infertility results from actions of estrogen that are similar to the organizational effects of estrogen in most species of mammals. The permanent infertility results from changes to the cervix which are analogous to the organizational effects of estrogen reported in other species treated during organogenesis."

9. "Phytoestrogen Content and Estrogenic Effect of Legume Fodder", Saloniemi, Wähälä, Nykänen-Kurki, Karella and Saastamoinen Soc. for Exp. Bio and Med. 1995.

"All red clover studied contained estrogenic isoflavones, especially formononetin and Biochanin A. The phytoestrogen content varied from 1.0% to 2.5% of dry matter."

"Interest in phytoestrogens has generally been aroused by their adverse properties. They may, however, also be beneficial by increasing the growth of animals and the milk yield of cows!" "Biochanin A and genistein, which in monogastric animals have an estrogenic effect, are broken down in the rumen of ruminants. ... daidzein and formononetin, are converted by ruminal microbes into the active equol." The chart on p. 15 shows phytoestrogen content of Red clover. That content consists of

Daidzein, Genistein, Formononetin, Biochanin-A.

"Red clover has the highest phytoestrogen content of Finnish legume fodder plants, varying from 1% to 2.5% of dry matter. The biological effect on the uterus of immature rats is large!" 000434

10. "Clinical Changes in Ovariectomized Ewes Exposed to Phytoestrogens and 17 β -Estradiol Implants",

Nwānne, Lundh, Madej, Fredriksson, and Björnå. Soc. for Exp. Bio. Med. 1997.

"Genistein and other isoflavones daidzein, biochanin A, and formononetin were isolated from clover and proved to be the cause of the disorder." "Mammary glands were noticeably more voluminous, and palpation revealed the presence of irregular, solid lumps of various sizes towards the end of the feeding period and long afterwards. The udder gradually changed from ^{pale to} pink, and then to red with increasing edema." "In this experiment, the irregular solid lumps of tissue found during clinical examinations looked like developing tumors. Genistein, but not formononetin, and the estrogenic metabolite of formononetin called equol, can stimulate the growth of estrogen-dependent breast cancer cells in vitro. The estrogenic activity of biochanin-A and genistein in ruminants is limited to the first few days of exposure when the unadapted rumen microbes cannot convert them to their non estrogenic metabolites p-ethyl phenol and phenolic acid. formononetin... considered to be the overall cause of clinical changes observed."

000435

11. "The Effects of Plant Oestrogens on Animal Reproduction"

Donald A. Shaw, Endeavour 1976.

Note that this paper is very commonly cited as an authority. "The isoflavones genistein, biochanin A and formononetin occur in variable concentrations in the leaves of many clover species"

"The reason why formononetin is oestrogenically more active than genistein in the sheep was found to lie in the digestion of plant constituents in the modified digestive system of the sheep." "It has been shown that the degradation of biochanin A and genistein to inactive phenols in the rumen of the sheep becomes ^{increasingly} more efficient over a period of about five days." "There appears to be a shorter adaptive period in the cow and the detoxification and excretion of the absorbed isoflavones and metabolites are more efficient." "Physiologically, clover-induced infertility is brought about by a combination of factors, including interference with spermatozoan transport through the genital tract of the ewe, abnormal transport of ova and interference with implantation. There is also evidence that the neuro-endocrine centres in the brain controlling the reproductive cycle of the ewe are suppressed." "However, the techniques of identification and quantitative assessment of oestrogens in plants should allow a quicker screening for possible oestrogenic compounds when they are suspected of reducing fertility in other animals, or having a contraceptive action in women." "From the wider ~~point of~~ viewpoint of evolution it is interesting that compounds have evolved in plants that not only give the plant some protection from foliar pathogens, but also reduces the fertility of animals ingesting the plant!"

12. "Toxicants of Plant Origin" Checke, 1989, Introduction. 000436

"Plant products have been used in folk medicine from ancient times as aphrodisiacs, aids in childbirth, abortifacients and promoters of fertility. Both increases and decreases in fertility in animals have also been attributed to specific compounds in plants." "Clinical effects

"It became apparent that plant estrogens could reduce the fertility of ewes in the absence of obvious clinical signs."

If ewes are exposed to estrogenic pasture for more than two seasons they may suffer a decline in fertility from which they never recover. ... However permanent subfertility usually occurs in the absence of obvious clinical signs.

13. "Effects of Plant Estrogens in Ruminants"

M.F. McDonald. Proc. of the Nutr. Soc. of NZ. 1995 Vol 20.

"Fortunately management plans can be devised to avoid feeding estrogenic herbage at critical times to breeding livestock." "The isoflavones are mainly found in the clovers"

"The effects of ingesting estrogenic compounds in the ruminant should be anticipated with a variety of animal responses. These include anabolic and lactation responses as well as effects on reproduction. A spurt in mammary growth and even secretion is often noticed in immature sheep." "Embryo mortality - Grazing

ewes on estrogenic clover may cause oviductal hyperplasia and uterine oedema with a likely effect on the developing embryo."

"Feeding Red clover for 8 days before and during the first cycle of mating resulted in higher service times than in control animals. The effects on conception persisted for at least 3 weeks after removal from red clover, but an earlier recovery of oovulation

9.
The reason that say based formula sales dropped in N.Z. when it became apparent that they contained phytoestrogens was because New Zealanders came from farming stock (pastoral) and so both benefits and risks of phytoestrogen exposure from plants ~~was~~ commonly understood. (ie Nothing to do with the imagined "lobby groups" !!!)

Every issue of this magazine, provided to farmers through New Zealand, contains comments on the effects of phytoestrogens because an understanding of them is basic to good animal husbandry. The copies include a) An advertisement for a new strain of clover with lower oestrogen levels to "give fewer fertility problems with breeding stock".

b) An advertisement showing proportion of clover to rye grass seed for pasture. Note that proportion of clover to total feed is rather low, normally.

c) Information 0000438

Effects of various levels of clover in pasture and relation to milk yield (increases yield) But cautions exist e.g. "Phyto-estrogens in the new cultivars are 65% lower than in the older cultivars, greatly reducing the risk of problems with animal production"

d) Letter to the editor, showing level of interest in and understanding of estrogen exposure within the N.Z. community. "Estrogens are involved in Thyroid function, which suggests a th...

involved in thyroid function, which suggests a toxic estrogen is likely to be a thyroid poison. It is time to find out. Estrogen has been known to induce serious

000439

10

reproductive failure and failure of fetal development."

15. Letter to the Editor of *ATDOG* in *Pediatric Forum*. Kerie Montague-Brown. "The authors found positive statistical associations between premature menarche and consumption of various meat products and soy-based formula, and a maternal history of ovarian cysts."
16. Second page of "Short paper" Frances Patterson. (Front page faced previously) Effects (estrogenic) were noted when soy was 5% of diet. Later studies done when soy was 10% of diet. Diet gives DES equivalents. 000440
17. "Phytoestrogens: Toxicology and regulatory recommendations" Robertson. *Proc. of the Nutr. Soc. of NZ*. 1995 Vol 20. "It must be stressed that beneficial and adverse effects are not mutually exclusive." "Whatever the implications for fertility in women, it is clear that addition of quite modest amount of soy protein to a complex diet had physiological effects, which lasted for several months after soy was removed from the diet." "The main effects are those due to possible alteration of endocrine dependent regulation. These might include carcinogenic, reproductive and developmental outcomes, including effects on sexual maturation and behaviour, which would not necessarily be readily apparent either in time or in appearance"
18. "Dietary Intervention Study to Assess Estrogenicity of Dietary Soy among Postmenopausal Women" Baird, Umbach, Cresswell,

Hughes, Setchell, Weinberg, Henry, Wilcox and McLachlan.

"nor did sex hormone binding globulin increase" "Phyto-estrogens do not bind well to SHBG."

Note that these results have been repeated in many studies. The claim of ADH that phytoestrogen administration increases SHBG is not able to be substantiated. I'll follow these notes with a page from the "Final Report to MAFF" which also states this.

19. Journal of Pharmaceutical Sciences "Potential Use of Plants as Sources of new antifebrile agents" (Parts I & II, 1975. Note that soy was included in these considerations (because of isoflavone content.)

20. "The Case for Expanded Phytoestrogen Research"

Sheehan, Report of introduction to 2nd Internat. Phytoestrogen Conference. 1995 Proc. Soc. for Exp. Bio & Med. 1995.

"A sequence of findings in experimental animals demonstrated that phytoestrogens possess the same wide range of biological activities previously found with traditional estrogens." "As with any biologically active chemical, it is crucial to define adverse versus beneficial effects of the phytoestrogens".

000441

Thank you for considering the above!

Yours,

~~Dr. [Name]~~



Institute for Environment
and Health

IEH assessment on

PHYTOESTROGENS IN THE HUMAN DIET

FINAL REPORT TO THE
MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

November 1997

000442

United Kingdom

The Institute for Environment and Health (IEH) was established by the Medical Research Council at the University of Leicester in 1993. The Institute is partly funded by the various UK government departments and agencies by way of specific research and consultancy contracts.

This literature review^{*} has been prepared by IEH for the Ministry of Agriculture, Fisheries and Food. The principal focus of this document is on the potential beneficial effects of phytoestrogens on adults. Potential detrimental effects on adults and the influence on other life stages were specifically excluded from consideration. It also contains an assessment of the factors influencing the phytoestrogen content of food and the relative potencies of the various phytoestrogens. The assessment incorporates the output of a workshop held in Leicester in March 1997 which was chaired by Professor Lewis Smith, IEH. The Institute gratefully acknowledges the contribution of all those who attended the workshop and provided material for inclusion in the assessment but assumes no endorsement from these scientists for the conclusions and recommendations contained herein.

The Ministry of Agriculture, Fisheries and Food has provided funding for this project but has not conducted the research or written this report. The views expressed here do not necessarily represent those of any government department or agency.

Prepared by:

Dr Charles Humfrey and Mr Philip Holmes, IEH

^{*} Literature search to November 96, supplemented by additional papers to June 97

000443

P. 13

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29-APR-98 13:09

producing a number of effects which have been associated with a reduced breast cancer risk. In premenopausal women, soya has been shown to increase the length of the menstrual cycle and/or delay menstruation, and to reduce the levels of LH, FSH and progesterone at various stages of the cycle. The reported effects of soya on blood levels of 17β -oestradiol have not been consistent, one study reporting a reduced level throughout the cycle (Lu *et al.*, 1996), another reporting an increase in the follicular phase only (Cassidy *et al.*, 1994) and another finding no change in levels (Baird *et al.*, 1995). Based on the studies reviewed, the evidence for changes in levels of the adrenal androgen DHEAS is conflicting and further investigations would be required to clarify these opposing findings. Plasma levels have been shown to vary with energy intake; for example, a recent study in premenopausal women found that for each additional MJ (239kcal) consumed, levels of DHEAS decreased by 5.1% (Dorgan *et al.*, 1996). To ensure that energy intake does not confound intervention studies using soya products, it would seem sensible to ensure that all diets investigated are isocaloric.

Both lignans and isoflavones have been reported to increase the levels of plasma SHBG, which may decrease the blood levels of biologically active sex hormones and thus influence cancer risk. Again, however, epidemiological studies are conflicting; levels were lower in postmenopausal women with breast cancer than in vegetarians or omnivores (Adlercreutz *et al.*, 1989 and 1992), although an earlier study found no difference between postmenopausal breast cancer patients and controls (Bruning *et al.*, 1985). In more informative controlled trials, in which lignans (as linseed) or soya (as textured vegetable protein and miso) was added to the diets of premenopausal women, a small, but significant, decrease in the level of SHBG was seen only in women consuming the linseed supplement. From these studies, isoflavones do not appear to have any effect on SHBG levels.

Although preliminary, the potentially important finding of Petrakis *et al.* (1996) that soya consumption may have an oestrogenic effect by increasing the incidence of hyperplastic epithelial cells in the nipple aspirate fluid of pre- and postmenopausal women constituting a risk factor for breast cancer, should be the subject of further investigation.

Overall, these studies show that phytoestrogens are biologically active in women and can affect the levels of sex hormones and potentially therefore contribute to a reduced breast cancer risk. The effects produced have not always been consistent between studies, although this may relate to the use of different doses, types of product used, study design or the generally small numbers of women studied. In order to elucidate the potential beneficial effects of phytoestrogens in breast cancer risk reduction, further controlled studies in larger populations of premenopausal women are warranted.

6.5 POST-MENOPAUSAL SYMPTOMS

The reported incidence of hot flushes, one of the most common symptoms of the menopause, varies markedly between different countries, with high levels in Europe (from 70-80% of postmenopausal women), intermediate levels in Malaysia (57%),

000444

FAXED & MAILED

Dr Linda Kahl
Office of Pre-Market Approvals
U.S.F.D.A. # HFS 206
200 C St SW
Washington DC 20204

~~R. E. Jones~~
R. D. H.
WILKINGAREI
NEW ZEALAND
APRIL 29 1998
FAX 64. 9 - 4340567

Dear Dr Kahl

GRN # 000001

I have today airmailed to you a number of published research reports to support my previous correspondence.

Since Archer Daniels Midland has used a historic definition of "toxicity" to equate with the LD-50 standard for rat baits, and has ignored chronic toxicity, hormonal toxicity, and carcinogenicity, I have submitted slightly different material from that listed in my first communication of 3/25/98

This is what is coming:

000445

1. Dietary Estrogens: Probable Cause of Liver Disease and Infertility in Captive Cheetahs - Setchell et al.
2. Causes of Adverse Responses to Soybean Milk Replacers in Young Calves - Gardiner et al
3. Roasted Soybeans and an Estrogenic Growth Promoter Affect the Thyroid Status of Beef Steers - Rumary et al 000445
4. Anti Thyroid Isoflavones from Soybean: Isolation Characterization and Mechanism of Action - Qui et
5. Effects on the Thyroid Gland of Soybean Administered Experimentally in Healthy Subjects - Ishizuki et.
6. Breast and Soy-Formula Feedings in Early Infancy and the Prevalence of Autoimmune Thyroid Disease in Children - Fort et al

non presence of environmental estrogen
in children - Fort et al
Hale et al - Environmental

2

- 9) Phytoestrogens in Soy Based Infant Foods:
Concentration, Daily Intake and Possible Biological
Effects - Irvine et al
- 10) Herbal Medicines, Phytoestrogens and Toxicity:
Risk Benefit Considerations - Sheehan
- 11) Dietary Genistein Exerts Estrogenic upon the Uterus,
Mammary Gland, and the Hypothalamic/Pituitary
Axis in Rats - Santell et al

In each of the enclosed research reports, I have placed arrows alongside other reports in the reference lists. I wish those other reports to be regarded as an integral part of this submission.

Your namesake, Dr Stanislaw Kahl, a co-author of paper # 3 above, seems to be a scientist whom you should consult.

Sincerely

~~Richard J. Jones~~

000446

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Computer Technology Services, Inc.

000447-
000450

To: Dr. Linda Kahl, Office of Premarket Approval,
Center for Food Safety, Food and Drug Administration,
200 C Street, S.W. (HFS - 206) Washington D.C.
20204

At For. 001 - 202 - 418 - 3131.

From: ~~Valerie Jones~~ at For 64 - 9 - 434 - 0567.

Re GRAS GRN 000001 (A.O.M)

Date: April 29th. 1998.

Dear Dr. Kahl,

000451

I completed the following very late last night. Please excuse the writing but since most of it is exact quotes from paper mailed, the original text will be clearer. Please note however, that AOM's assertion that "clover disease" was caused by coumestrol rather than isoflavones in clover is false. Also the effects were more severe than just a reduction in fertility.

Thank you for your continuing communications.

Yours sincerely,

~~Valerie Jones~~

To: Dr. Linda Kahl, Office of Pre-market Approval, Center for Food Safety, Food and Drug Administration, 200 C Street, S.W. (HFS-206) Washington D.C. 20204.

At: Facsimile 001-202-418 3131

From: ~~David Jones~~, Rural Delivery 4, Whangarei, N.Z.

At: Facsimile 64-9-434 0567.

Date: Tuesday, April 28th, 1998.

Re: GRAS Notice # GRN 000001 (ADM)

Dear Dr. Kahl,

Today I mailed you copies of a number of published scientific papers from my study files. Because I realize ~~that~~ some misrepresentations may have been made to you about my convictions and affiliation, I have chosen to provide entire documents so that they can be independently assessed, rather than just provide quotes which can actually mislead as to the impact and message of the entire paper.

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The ADM submission #000001 contains many mistakes of fact. The following documents have been mailed to you to illustrate just a few. It would take a "basket," rather than a "package" to provide documentation to illustrate all of the mistakes.

The papers I have selected are as follows, with reasons given for inclusion in the mailed "package"

1. "Geistin (an Isoflavone Glucoside) and its Aglycone Geistin from Soybeans"; Walter 1961. (Front page only). This shows that the ability to extract isoflavones from soy is approx

40 plus years old and is not a newly developed technique.

2. One page from a "data base" showing that genistein (from studies of the 1950's) when added as 0.2% of diet elicited estrogenic effects. (I apologize for poor quality of reproduction as the information forced from data base, arrived here like that)

3. "Effect of Genistein on Reproduction of the Mouse."

Carter, Matrone and Smot. (This is a classic paper and often quoted) Diet 1, was control with genistein removed, Diet 2, had 0.02% added back, Diet 3 made up of commercial soy bean meal, with approx 0.01% genistein.

Summary Both commercial soybean oil meal and isolated genistein significantly lowered the age at which the vaginals of immature mice opened. The principal effects on reproduction of 0.02% genistein in the diet was a decrease in the number of litters born, whereas litter size was not affected. The commercial soybean oil meal (80% of diet) was a decrease in the number of litters.

4. "Effect of Genistein on Growth and Development of the Male Mouse." Carter, ^{Smot}Matrone, Smot. 000453

Results Ten mice died, 4 receiving highest level of genistein, two receiving third and four were scattered among other levels. Males on highest level of genistein lost weight. Genistein also had depressing effect on testes weight.

No spermatozoa present in the testes of groups receiving two higher levels of genistein. The higher levels of genistein appeared to be lethal. "It appears from these results that genistein, in relation to its estrogenic activity, has a greater depressing effect than does stilbestrol."

5. "Metabolism of Estrogenic Isoflavones in Domestic

Animals" Lundh. Soc. for Exp. Bio and Med., 1995

This is referenced in A.O.M. presentation. It does NOT state "infertility effects are not general to all animals" as submitted by A.O.M. It does discuss reasons for differences between sensitivity between pigs, sheep and cattle. In cattle, daily intakes may reach up to 50-100g ^{estrogenic phytoestrogens} per day which may result in temporary infertility in cattle. The paper discusses detoxification mechanisms of the rumen, and more serious effects in sheep because, it is suggested, cattle have fewer estrogen receptors in uterus. (2 to 4 times higher in sheep than cattle. Pigs are more sensitive than either.

P.30. Glucuronidation is the major detoxification system for potentially toxic endogenous and exogenous substances including phytoestrogens. Note that for cattle and sheep the phytoestrogen in this red clover silage were Formononetin, daidzein and genistein found mainly as conjugates in the blood (about 5% of total amount as unconjugated substances in the plasma.

6. "Hormone Studies in Ewes Grazing on ~~Estrogenic~~ (Yarloop clover pasture During the Reproductive Cycle" Obst, Seemak. 1975 Aust. J. Biol Sci 28 279-90.

"Clover Disease" described as a "syndrome of aberrant 000454 reproductive function" signs are "ewe infertility, dystocia, uterine prolapse resulting in high ewe and lamb mortality."

Study investigates "the possibility that the clover acts by causing disturbances in the endocrinology of reproduction."

"The mean content of the isoflavones formononetin, genistein and biochanin A in Yarloop clover during July and August 1970 was 1.2, 2.5 and 0.54% respectively of dry leaf weight." The result was that "performance of ewes grazing Yarloop is associated with marked disturbances in reproductive

endocrine function."

- 7. "Toxicants of Plant Origin" Edited by Peter Cheek, 1989.
(Chapter 2; Phytoestrogens by H.R. Adams.)

Isoflavones (Table 2) listed as: — Formononetin, Genistein, Biochanin A. plus a discussion of isoflavones which were "very low in the absence of infection." "Plant estrogens have less affinity for the receptor than steroidal estrogens and the ranking is similar across species of animals." "In every case that has been examined, phytoestrogens produce changes in the reproductive tract which are qualitatively the same as those produced by endogenous estradiol. Both steroidal and plant estrogens affect the ovary and mammary gland.

- Exogenous estrogen causes dysplasia of the ovarian granulosa cells, thus impeding the maturation of the ovarian follicles." Note p. 36 comment on DES | estradiol | coumestrol. "Sheep grazed on highly estrogenic pastures consisting of relatively pure stands of subterranean clover or red clover containing high concentrations (0.8%) of the isoflavone formononetin may suffer marked clinical signs. Ewes may be affected by severe 000455 infertility accompanied by gross pathological changes in the uterus, including hydrops uteri, pyometra, cystic hyperplastic endometritis, and massive adhesions resulting from metritis." "The infertility results from abnormal function of the pituitary/ovarian axis. The reproductive problem frequently resembles the abnormalities associated with cystic ovaries, including prolonged but irregular estrus, nymphomania, or even the development of masculine sexual characteristics".
- 8. "Organizational and Activational Effects of Phytoestrogens on the Reproductive Tract of the Ewe" Adams. Soc. Exp. Biol Med 1995
"Isoflavone compounds including genistein, Biochanin A and formononetin

Occur in subterranean clover or red clover. Genistein and Biochanin A are usually broken down by microbial activity in the rumen of the sheep while formononetin is converted to the isoflavone equol, which is rapidly absorbed and is responsible for most of the estrogenic activity in ruminants."

"Temporary infertility results from actions of estrogen that are similar to the organizational effects of estrogen in most species of mammals. The permanent infertility results from changes to the cervix which are analogous to the organizational effects of estrogen reported in other species treated during organogenesis."

9. "Phytoestrogen Content and Estrogenic Effect of Legume Fodder", Saloniemi, Wähälä, Nykinen-Kurki, Karela and Saastomoinen Soc. for Exper. Bio and Med. 1995.

"All red clover studied contained estrogenic isoflavones, especially formononetin and Biochanin A. The phytoestrogen content varied from 1.0% to 2.5% of dry matter."

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"Interest in phytoestrogens has generally been aroused by their adverse properties. They may, however, also be beneficial by increasing the growth of animals and the milk yield of cows." "Biochanin A and genistein, which in monogastric animals have an estrogenic effect, are broken down in the rumen of ruminants. daidzein and formononetin, are converted by ruminal microbes into the active equol." The chart on p. 15 shows phytoestrogen content of red clover. That content consists of

Daidzein, Genistein, Formononetin, Biochanin-A.

"Red clover has the highest phytoestrogen content of Finnish legume fodder plants, varying from 1% to 2.5% of dry matter. The biological effect on the uterus of immature rats is large."

10. "Clinical Changes in Ovariectomized Ewes Exposed to Phytoestrogens and 17 β -Estradiol Implants",
Nwanna, Lundh, Madej, Fredriksson, and Björnå. Soc. for Exp. Bio. Med. 1987.

"Genistein and other isoflavones daidzein, biochanin A, and formononetin were isolated from clover and proved to be the cause of the disorders." "Mammary glands were noticeably more voluminous, and palpation revealed the presence of irregular, solid lumps of various sizes towards the end of the feeding period and long afterwards. The udder gradually changed from ^{pale to} pink, ~~and~~ then to red with increasing edema." "In this experiment, the irregular solid lumps of tissue found during clinical examinations looked like developing tumors.

Genistein, but not formononetin, and the estrogenic metabolite of formononetin called equol, can stimulate the growth of estrogen-dependent breast cancer cells ⁰⁰⁰⁴⁵⁷ in vitro. The estrogenic activity of biochanin-A and genistein in ruminants is limited to the first few days of exposure when the unadapted rumen microbes cannot convert them to their non estrogenic metabolites p-coumyl phenol and phenolic acid. formononetin... considered to be the overall cause of clinical changes observed."

11. "The Effects of Plant Oestrogens on Animal Reproduction"
Donald A. Shaw, Endeavour 1976.

Note that this paper is very commonly cited as an authority.

"The isoflavones genistein, biochanin A and formononetin occur in variable concentrations in the leaves of many clover species"

"The reason why formononetin is oestrogenically more active than genistein in the sheep was found to lie in the digestion of plant constituents in the modified digestive system of the sheep." "It has been shown that the degradation of biochanin A and genistein to inactive phenols in the rumen of the sheep becomes ^{increasingly} more efficient over a period of about five days." "There appears to be a shorter adaptive period in the cow and the detoxification and excretion of the absorbed isoflavones and metabolites are more efficient." "Physiologically, clover-induced infertility is brought about by a combination of factors, including interference with spermatozoan transport through the genital tract of the ewe, abnormal transport of ova and interference with implantation. There is also evidence that the neuro-endocrine centres in the brain controlling the reproductive cycle of the ewe are suppressed." "However, the techniques of identification and quantitative assessment of oestrogens in plants should allow a quicker screening for possible oestrogenic compounds when they are suspected of reducing fertility in other animals, or having a contraceptive action in women." "From the wider ~~point of~~ viewpoint of evolution it is interesting that compounds have evolved in plants that not only give the plant some protection from foliar pathogens, but also reduces the fertility of animals ingesting the plant!"

12. "Toxicants of Plant Origin" Cheeke - 1989, Introduction.

"Plant products have been used in folk medicine from ancient times as aphrodisiacs, aids in childbirth, abortifacients and promoters of fertility. Both increases and decreases in fertility in animals have also been attributed to specific compounds in plants." "Clinical effects

8.

"It became apparent that plant estrogens could reduce the fertility of ewes in the absence of obvious clinical signs."

If ewes are exposed to estrogenic pasture for more than two seasons they may suffer a decline in fertility from which they never recover. -- However permanent subfertility usually occurs in the absence of obvious clinical signs.

13. "Effects of Plant Estrogens in Ruminants"

M.F. McDonald. Proc. of the Nutr. Soc. of NZ. 1995 Vol 20.

"Fortunately management plans can be devised to avoid feeding oestrogenic herbage at critical times to breeding livestock." "The isoflavones are mainly found in the clovers" "The effects of ingesting oestrogenic compounds

in the ruminant should be anticipated with a variety of animal responses. These include anabolic and lactation responses as well as effects on reproduction. A spurt in mammary growth and udder secretion is often noticed in immature sheep. " "Embryo mortality - Growing

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ewes on oestrogenic clover may cause oviductal hyperplasia and uterine oedema with a likely effect on the developing embryos." "Feeding Red clover for 8 days before and during the first cycle of mating resulted in higher service times than in control animals. The effects on conception persisted for at least 3 weeks after removal from red clover, but an earlier recovery of oovulation rate occurred.

14. Pages from recent "Dairy Exporter" NZ. I believe that

the reason that say based formula sales dropped in N.Z. when it became apparent that they contained phytoestrogens was because New Zealanders were from farming stock (pastoral) and so both benefits and risks of phytoestrogen exposure from plants ~~was~~ commonly understood. (ie. Nothing to do with the imagined "lobby groups". !!!)

Every issue of this magazine, provided to farmers through New Zealand, contains comments on the effects of phytoestrogens because an understanding of them is basic to good animal husbandry. The copies include a) An advertisement for a new strain of clover with lower oestrogen levels to "give fewer fertility problems with breeding stock"

b) An advertisement showing proportion of clover to rye grass seed for pasture. Note that proportion of clover to total feed is rather low, normally.

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c) Information on effects of various levels of clover in pasture and relation to milk yield (increases yield) But cautions exist e.g. "Phyto-oestrogens in the new cultivars are 65% lower than in the older cultivars, greatly reducing the risk of problems with animal production"

d) Letter to the editor, showing level of interest in and understanding of estrogen exposure within the N.Z. community. "Estrogens are involved in Thyroid function, which suggests a toxic estrogen is likely to be a Thyroid poison. It is time to find out. Goitre has been known to induce serious

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reproductive failure and failure of fetal development."

16. Letter to the Editor of *ATDC* in *Pediatric Forum*. Kerla Montague-Brown. "The authors found a positive statistical association between premature menarche and consumption of various meat products and soy-based formula, and a maternal history of ovarian cysts."

16. Second page of "Short paper" Frances Patterson. (Front page faced previously) Effects (estrogenic) were noted when soy was 5% of diet. Later studies done when soy was 10% of diet. Diet gives DES equivalents.

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17. "Phytoestrogens: Toxicology and regulatory recommendations" Robertson. *Proc. of the Nutr. Soc. of NZ*. 1995 Vol 20.

"It must be stressed that beneficial and adverse effects are not mutually exclusive." "Whatever the implications for fertility in women, it is clear that addition of quite modest amount of soy protein to a complex diet had physiological effects, which lasted for several months after soy was removed from the diet." "The main effects are those due to possible alteration of endocrine dependent regulation. These might include carcinogenic, reproductive and developmental outcomes, including effects on sexual maturation and behaviour, which would not necessarily be readily apparent either in time or in appearance."

18. "Dietary Intervention Study to Assess Estrogenicity of Dietary Soy among Postmenopausal Women" Baird, Umbach, Condeelis.

Hughes, Setchell, Weinberg, Harvey, Wilcox and McLachlan.

"nor did sex hormone binding globulin increase" "Phyto-estrogens do not bind well to SHBG."

Note that these results have been repeated in many studies. The claim of ADH that phytoestrogen administration increases SHBG is not due to be substantiated. I'll follow these notes with a page from the "Final Report to MAFF" which also states this.

19. Journal of Pharmaceutical Sciences "Potential Use of Plants as sources of new antitumor agents" (Parts I & II, 1975. Note that soy was included in these considerations (because of isoflavone content.)

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20. "The Case for Expanded Phytoestrogen Research"

Sheehan, Report of introduction to 2nd Internat. Phytoestrogen Conference. 1995 Proc. Soc. for Exp. Bio & Med. 1995.

"A sequence of findings in experimental animals demonstrated that phytoestrogens possess the same wide range of biological activities previously found with traditional estrogens." "As with any biologically active chemical, it is crucial to define adverse versus beneficial effects of the phytoestrogens."

Thank you for considering the above!

Yours,

~~Harvey~~

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Institute for Environment
and Health

IEH assessment on

PHYTOESTROGENS IN THE HUMAN DIET

FINAL REPORT TO THE
MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

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November 1997

The Institute for Environment and Health (IEH) was established by the Medical Research Council at the University of Leicester in 1993. The Institute is partly funded by the various UK government departments and agencies by way of specific research and consultancy contracts.

This literature review¹ has been prepared by IEH for the Ministry of Agriculture, Fisheries and Food. The principal focus of this document is on the potential beneficial effects of phytoestrogens on adults. Potential detrimental effects on adults and the influence on other life stages were specifically excluded from consideration. It also contains an assessment of the factors influencing the phytoestrogen content of food and the relative potencies of the various phytoestrogens. The assessment incorporates the output of a workshop held in Leicester in March 1997 which was chaired by Professor Lewis Smith, IEH. The Institute gratefully acknowledges the contribution of all those who attended the workshop and provided material for inclusion in the assessment but assumes no endorsement from these scientists for the conclusions and recommendations contained herein.

The Ministry of Agriculture, Fisheries and Food has provided funding for this project but has not conducted the research or written this report. The views expressed here do not necessarily represent those of any government department or agency.

Prepared by
Dr Charles Hamfrey and Mr Philip Holmes, IEH

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¹ Literature search to November 96, supplemented by additional papers to June 97.

producing a number of effects which have been associated with a reduced breast cancer risk. In premenopausal women, soya has been shown to increase the length of the menstrual cycle and/or delay menstruation, and to reduce the levels of LH, FSH and progesterone at various stages of the cycle. The reported effects of soya on blood levels of 17 β -oestradiol have not been consistent, one study reporting a reduced level throughout the cycle (Lu *et al.*, 1996), another reporting an increase in the follicular phase only (Casidy *et al.*, 1994) and another finding no change in levels (Baird *et al.*, 1995). Based on the studies reviewed, the evidence for changes in levels of the adrenal androgen DHEAS is conflicting and further investigations would be required to clarify these opposing findings. Plasma levels have been shown to vary with energy intake, for example, a recent study in premenopausal women found that for each additional MJ (239kcal) consumed, levels of DHEAS decreased by 5.1% (Dorgan *et al.*, 1996). To ensure that energy intake does not confound intervention studies using soy products, it would seem sensible to ensure that all diets investigated are isocaloric.

Both lignans and isoflavones have been reported to increase the levels of plasma SHBG, which may decrease the blood levels of biologically active sex hormones and thus influence cancer risk. Again, however, epidemiological studies are conflicting; levels were lower in postmenopausal women with breast cancer than in vegetarians or omnivores (Adlercreutz *et al.*, 1989 and 1992), although an earlier study found no difference between postmenopausal breast cancer patients and controls (Bruning *et al.*, 1983). In more informative controlled trials, in which lignans (as linseed) or soya (as textured vegetable protein and miso) was added to the diets of premenopausal women, a small, but significant, decrease in the level of SHBG was seen only in women consuming the linseed supplement. From these studies, isoflavones do not appear to have any effect on SHBG levels.

Although preliminary, the potentially important finding of Petrakis *et al.* (1996) that soya consumption may have an oestrogenic effect by increasing the incidence of hyperplastic epithelial cells in the nipple aspirate fluid of pre- and postmenopausal women constituting a risk factor for breast cancer, should be the subject of further investigation.

Overall, these studies show that phytoestrogens are biologically active in women and can affect the levels of sex hormones and potentially therefore contribute to a reduced breast cancer risk. The effects produced have not always been consistent between studies, although this may relate to the use of different doses, types of product used, study design or the generally small numbers of women studied. In order to elucidate the potential beneficial effects of phytoestrogens in breast cancer risk reduction, further controlled studies in larger populations of premenopausal women are warranted.

6.5 POST-MENOPAUSAL SYMPTOMS

The reported incidence of hot flushes, one of the most common symptoms of menopause, varies markedly between different countries, with high levels in Europe (from 80% of postmenopausal women), intermediate levels in Malaysia (57%),

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High levels in China (18%) and Singapore (14%) (Knight & Eden, 1995 and 1996). As a result of dietary differences between these countries, particularly the constant variation in consumption of soya products, several studies have investigated the possible role of these products in modulating these symptoms. It is of interest to note that many plants of the family Leguminosae, which contain high levels of isoflavones and their glucosides, have long been used to treat menopausal symptoms such as hot flushes, sweating and depression by Chinese herbal doctors (Lien & Lien, 1996). Examples include alfalfa, dandelion, red clover and liquorice.

Wilson *et al* (1990) showed that consumption by 25 postmenopausal women of a diet supplemented with soya flour (45g/day), red clover sprouts (10g dry seed/day), and linseed (25g/day), each for two weeks, had no effect on LH or FSH levels when analysed after each individual two week supplement, but had a marginal cumulative effect on FSH levels over the six week study. No control group was included in this study. However, oestrogenic effects were observed when measured as cytological maturation of the vaginal epithelium. This was not confirmed in subsequent studies by Murkies *et al* (1995) and Baird *et al* (1995). In the former study, postmenopausal women with more than 14 hot flushes per week consumed a diet supplemented with either soya flour (45g/day, n=25) or wheat flour (45g/day, n=22) for 12 weeks. No effect on vaginal cell maturation was seen in women consuming either supplement, although hot flushes were significantly reduced at six weeks in the women consuming soya flour and by 40% and 25% at the end of the study in women consuming the soya flour or wheat flour, respectively. A subjective assessment of menopausal symptoms also showed significant reductions in both groups by the end of the study. Urinary levels of daidzein, equol and enterolactone were significantly higher at the end of the study in the soya flour group but not in the wheat flour group (although wheat flour also contains phytoestrogens). As a result of the decrease in flush frequency over the study period, a placebo effect could not be discounted. In the study of Baird *et al* (1995), groups of postmenopausal women consumed either a normal diet (control, n=25) or a diet supplemented with soya foods (equivalent to 165mg isoflavones/day, n=25) for four weeks. Despite an average 105-fold increase in urinary excretion of isoflavone phytoestrogens in the soya diet group and an average two-fold increase in the control group (which was not significant), no significant difference in vaginal maturation index was noted between the two groups. In addition to these observations, there were no significant differences in the levels of FSH, LH and SHBG between the two groups or comparing levels before and after the dietary intervention. A slight increase in serum oestradiol levels was noted in both groups during the study, but neither was significant. The use of a different sampling technique in this study to the previous one may have falsely lowered the estimate of vaginal maturation (Knight & Baird, 1996). However, the lack of measurement of biological dose in this study makes it difficult to determine whether the lack of effect was due to the low dose of isoflavones or to the lack of measurement of biological dose.

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BEST ORIGINAL COPY

As a result of Knight & Eden (1996), research is being undertaken throughout the world to assess the effectiveness of phytoestrogens in treating symptoms of the menopause. The current evidence suggests that there may be some beneficial effects



Dr Linda Kahl
Office of Pre-Market Approvals
U. S. F. - D. A. H HFS 206
200 C St SW
Washington DC 20204

~~R. D. H. JAMES~~
R. D. H.
WILKINGAREI
NEW ZEALAND
APRIL 29 1998
FAX 64. 9 - 4340567

Dear Dr Kahl

GRN # 000470

I have today airmailed to you a number of published research reports to support my previous correspondence.

Since Archer Daniels Midland has used a historic definition of "toxicity" to equate with the LD-50 standard for rat baits, and has ignored chronic toxicity, hormonal toxicity, and carcinogenicity, I have submitted slightly different material from that listed in my first communication of 3/25/98

This is what is coming:

1. Dietary Estrogens: Probable Cause of Liver Disease and Infertility in Captive Cheetahs - Schell et al.
2. Causes of Adverse Responses to Soybean Milk Replacers in Young Calves - Gardner et al
3. Roasted Soybeans and an Estrogenic Growth Promoter Affect the Thyroid Status of Beef Steers - Rumsey et al
4. Anti Thyroid Isoflavones from Soybean: Isolation Characterisation and Mechanism of Action - Dix et al
5. Effects on the Thyroid Gland of Soybean Administered Experimentally in Healthy Subjects - Ishizuki et al
6. Breast and Soy-Formula Feedings in Early Infancy and the Prevalence of Autoimmune Thyroid Disease in Children - Fort et al
7. Premature Thelarche in Puerto Rico - Freni-Titulaer et al.
8. Dietary Estrogens Stimulate Human Breast Cells to Enter the Cell Cycle - Dees et al

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- 9) Phytoestrogens in Soy Based Infant Foods:
Concentration, Daily Intake and Possible Biological
Effects — Irvine et al
- 10) Herbal Medicines, Phytoestrogens and Toxicity:
Risk Benefit Considerations — Sheehan
- 11) Dietary Genistein Exerts Estrogenic upon the Uterus
Mammary Gland, and the Hypothalamic/Pituitary
Axis in Rats — Santell et al

In each of the enclosed research reports, I
have placed arrows alongside other reports in
the reference lists. I wish those other reports
to be regarded as an integral part of this
submission.

Your namesake, Dr Stanislaw Kahl, a
co-author of paper # 3 above, seems to be a
scientist whom you should consult.

Sincerely

~~Richard J. Evans~~

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Dietary Estrogens—A Probable Cause of Infertility and Liver Disease in Captive Cheetahs

K. D. R. SETCHELL, S. J. GOSSELIN, M. B. WELSH, J. O. JOHNSTON, W. F. BALISTRERI, L. W. KRAMER, B. L. DRESSER, and M. J. TARR
Clinical Mass Spectrometry Laboratories, Department of Pediatric Gastroenterology and Nutrition, Children's Hospital Medical Center, Cincinnati; Department of Pathology/Toxicology and Department of Enzyme Chemistry, Merrell-Dow Research Institute, Cincinnati; Cincinnati Zoo; Department of Obstetrics/Gynecology, University of Cincinnati, Cincinnati, Ohio; Kings Island Wild Animal Habitat, Kings Island, Ohio; and Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio

The cheetah in the wild is "racing towards extinction" mostly due to habitat destruction. Its survival will probably depend on accelerated captive breeding. At this time, however, reproductive failure and liver disease threaten the future of the captive cheetah population. Histopathological evaluation of more than 100 cheetah livers identified veno-occlusive disease as the main hepatic lesion responsible for liver disease in this species. Analysis of the commercial feline diet by high-performance liquid chromatography and gas-liquid chromatography-mass spectrometry revealed large amounts of two phytoestrogens identified as daidzein and genistein. These compounds were found to be derived from a soybean product that was a component of the cheetah diet, and their concentrations both ranged from 8 to 35 $\mu\text{g/g}$ diet. The adult cheetah consequently consumes ~50 mg/day of these weak estrogens. When extracts of the diet were tested for estrogenicity using a bioassay, a dose-related increase in uterine weight was observed. In 4 cheetahs studied,

withdrawal of this feline diet by substitution with a chicken diet resulted in an improvement in conventional liver function tests and a normalization in the appearance of hepatic mitochondria. We conclude that the relatively high concentrations of phytoestrogens from soybean protein present in the commercial diet fed to captive cheetahs in North American zoos may be one of the major factors in the decline of fertility and in the etiology of liver disease in this species. The survival of the captive cheetah population could depend upon a simple change of diet, excluding exogenous estrogen.

Cheetah populations are diminishing in the wild as a result of poaching and habitat destruction (1). Another factor that may be contributing to their decline is the lack of genetic variation within the species (2). To quote cheetah researcher Randall Eaton, the cheetah is "racing towards extinction" (3). The survival of the cheetah, as a species, will probably depend on accelerated captive breeding. At this time, however, reproductive failure, as well as shortened life spans, threaten the future of the captive cheetah population. The situation has become alarming for this already endangered species. North American zoos cannot maintain their cheetah populations because deaths have outnumbered births during the last few years. In 1985, North American zoos reported 29 deaths and only 18 births of which 7 died before reaching adulthood (4).

The average life span of the captive cheetah in North American zoos is 8.9 yr (5), a much shorter

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Address requests for reprints to: Kenneth D. R. Setchell, Ph.D., Children's Hospital Medical Center, Division of Pediatric Gastroenterology and Nutrition, Elland and Bethesda Avenues, Cincinnati, Ohio 45229-2899.

The authors acknowledge the technical assistance of C. L. Wright, L. C. Meyer, D. E. Loudy, and W. A. Rogers. They also thank the following zoological institutions for their contributions to this project: Baltimore, Baton Rouge, Cleveland, Cheyenne Mountain, Cleveland, Columbus, Detroit, Wayne, Gladys Porter, Henry Doorly, Houston, Kansas City, Louisville, National Zoological, St. Louis, San Antonio, San Diego, Toledo, Toronto, Wolf Run Ranch, Wildlife Safari, Detroit Cheetah Research and Breeding Centre, South Africa, and manufacturers of commercial feline diet, Animal Science, Omaha, Nebraska. © 1987 by the American Gastroenterological Association

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Abbreviations used in this paper: GLC, gas-liquid chromatography; HPLC, high-pressure liquid chromatography; MS, mass spectrometry.

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bioassays for uterotrophic activity were performed using immature mice and ovariectomized rats (11).

The dietary extracts were solubilized in 0.85 ml of warm ethanol and 3.0 ml olive oil. Four immature mice (21 days old, body weight ~10 g) per treatment group were injected subcutaneously for 2 days with the first extract, representing the equivalent of 20 and 40 g of the original feline diet per mouse per day. A further 6 mice per group were injected with olive oil and increasing amounts of estradiol-17 β (0.1–1.0 μ g/kg · day). At necropsy, uteri were removed, uterine fluid if present was expressed, and uteri were weighed to the nearest 0.1 mg.

Immature female Sprague-Dawley rats (60–80 g) were bilaterally ovariectomized at day 30 of age. On day 34, 2 rats per group were treated subcutaneously for 3 days with the second extracts (equivalent to 8 g of the original feline diet per rat per day) of commercial feline diet and extracts of raw horse meat. For controls, olive oil and increasing amounts of estradiol-17 β (0.01–3.0 μ g/kg · day) were injected into 6 rats per group.

Results

Liver Pathology and Infertility in Captive Cheetahs

The livers of more than 100 cheetahs obtained from zoos throughout North America were evaluated by light microscopy. The main hepatic lesion (Gosselin S, personal communication) was a vascular lesion that was characterized by partial or total occlusion of the centrilobular and sublobular hepatic veins with loosely arranged to dense fibrous connective tissue (Figure 1). There was slight to severe perivenular fibrosis with sometimes bridging of adjacent central veins. The surrounding sinusoids were usually congested and occasionally there was extensive central hemorrhagic congestion. The parenchymal lesion varied from minor loss of liver cells to focal areas of degeneration and necrosis. The incidence of this hepatic vascular lesion, called venoocclusive disease of the liver, was ~60% in the adult captive cheetah population.

This is in contrast with what has been observed in a population of captive cheetahs at the DeWildt Cheetah Research and Breeding Centre in South Africa. No clinical signs of liver failure have been seen in that population (Meltzer D, Ebedes H, personal communications) and there was no histopathological evidence of venoocclusive disease in the liver tissues available for this study ($n = 7$). Furthermore, the DeWildt Cheetah Breeding Centre has been quite successful in breeding cheetahs. Two hundred thirty cubs have been born in less than 10 yr from an initial population of only 10 females and 19 males (12,13).

One major difference between the North American and DeWildt captive cheetah populations is the diet.



Figure 1. Liver pathology in a captive cheetah: presence of spindle-shaped cells and fibrillar material in the lumen of the central vein (thick arrow). Perivenular fibrosis (thin arrow). Masson trichrome, $\times 100$.

Over the last 10 yr, the vast majority of the animals in North American zoos have been fed a commercially prepared feline diet consisting primarily of horse meat. To a lesser extent animals may alternatively be fed horse, beef, deer, chicken, or rabbit meat, supplemented with vitamins and minerals. At the DeWildt Research Centre, cheetahs are fed whole carcasses of beef, chicken, and occasionally hoofed zoo animals.

Based on this observation, the decision was made to subject four Cincinnati Zoo cheetahs (1 male, 3 females) to a change in diet. The animals were consuming ~1 kg/day of commercially prepared feline diet (Nebraska Brand Feline Diet) before being switched to a diet consisting of chicken meat supplemented with vitamins and minerals.

While consuming the commercial feline diet, the mean prothrombin time and mean partial thromboplastin time were 7.4 ± 0.3 and 13.1 ± 0.6 s, respectively. There was a significant change noted in each animal after 3 mo on the chicken diet (prothrombin time = 8.9 ± 0.7 s, partial thromboplastin time = 22.5 ± 6.6 s; $p < 0.05$, Student's *t*-test). None of the animals showed any obvious abnormalities when evaluated by several hepatic biochemical tests while ingesting the commercial diet; however, with the dietary change, alanine aminotransferase, aspartate aminotransferase, γ -glutamyltransferase, and total bilirubin decreased in all 4 cheetahs, but as a group this was not statistically significant. At the ultrastructural level, the most obvious change was an alteration in shape and an increase in size of the mitochondria of the liver cells in the cheetahs fed the commercial diet, which normalized during the period of the chicken diet (Figure 2).

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time span than is seen for other exotic cats kept in captivity. It has been known since the 1960s that one of the major causes of death in captive cheetahs is liver disease of unknown etiology (6).

This report will identify the main hepatic lesion responsible for liver failure and illustrate how liver disease can be associated with infertility in captive cheetahs. It will also provide evidence for the presence of high concentrations of phytoestrogens in the commercial feline diet, and from studies involving dietary manipulation indicate their possible role in the etiology of liver dysfunction and reproductive failure of cheetahs kept in captivity.

Methods

Subjects

Liver tissue from 103 cheetahs from 23 North American zoos were evaluated by light microscopy and histochemistry.

Four adult cheetahs (1 male, 3 females) were studied before and after a modification of their diet. The normal daily diet of these cheetahs, which consisted of a commercially prepared feline diet (Nebraska Brand Feline Diet; Animal Spectrum Inc., Lincoln, Neb.; main ingredients include horse meat, horse meat by-products, soybean product, bone meal, liver, fish meal, and a variety of vitamins and trace minerals) was changed to one consisting of chicken meat supplemented with vitamins and minerals.

Blood samples for hematologic (including coagulation times) and blood chemistry evaluations were obtained before the change in diet and after 3 mo on the chicken diet. Liver function tests performed included serum total protein, albumin, γ -glutamyltransferase, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, and direct bilirubin. Other blood measurements included triglycerides, cholesterol, lactic dehydrogenase, glucose, creatinine, urea nitrogen, uric acid, sodium, potassium, chloride, phosphorus, and calcium. Liver biopsy specimens were obtained and evaluated by light and electron microscopy before and after the dietary change.

Chemical Analysis of the Diet

Analysis of the cheetah diet was carried out using high-pressure liquid chromatography (HPLC) and capillary column gas-liquid chromatography-mass spectrometry (GLC-MS) (7-9). Specifically, a search was made for possible estrogenic compounds of animal or plant origin. The commercial feline diet (20 g), the chicken diet (20 g), and samples of horse meat (20 g), textured soy (5 g), and soy flakes (5 g) were individually homogenized and refluxed in 80% ethanol (250 ml) for 2 h to extract all steroids, polar steroid conjugates, and similarly related compounds. The organic extracts were cooled and centrifuged, and the supernatant was removed. The ethanol was evaporated using a rotary evaporator and the lipids were extracted from the remaining aqueous extract by partition-

ing twice into hexane (4 vol). The aqueous extract was taken to dryness. Sequential hydrolysis of polar conjugates was carried out. As plant estrogens exist mainly as glycoside conjugates, these were hydrolyzed using a β -glucosidase enzyme (Sigma Chemical Co., St. Louis, Mo) in 0.1 M acetate buffer (pH 5.0) overnight at 37°C. The hydrolysate was passed through a cartridge of reverse phase octadecylsilane bonded silica (Bond Elut-C₁₈; Analytichem International, Harbor City, Calif.) to extract all steroids and related compounds. After washing the cartridge with water, these compounds were recovered by elution with methanol (5 ml). After evaporation of the methanol a second hydrolysis was performed using combined β -glucuronidase and sulfatase preparation (H-Lix pomatia; Sigma, 0.2 ml) in 0.5 M acetate buffer (pH 4.20 ml) for 24 h at 37°C. The hydrolysate was again passed through a Bond Elut-C₁₈ cartridge to extract the hydrolyzed products, which were recovered by elution with methanol (5 ml) and analyzed by HPLC and GLC-MS.

High-Pressure Liquid Chromatography Analysis

A small volume (10 μ l) of the extracts was subjected to a recently developed HPLC technique for the analysis of dietary estrogens (10). This method allows the simultaneous detection of the principal classical estrogens, plant estrogens, and equine estrogens. High-pressure liquid chromatography analysis was carried out using Varian 5000 liquid chromatograph (Varian Associates Palo Alto, Calif.) equipped with a UV-100 detector set 280-nm wavelength to specifically detect phenolic or estrogenic compounds. Chromatography was achieved using a 25-cm ODS-Hypersil (5 μ m) column with a solvent system of 0.1 M ammonium acetate/acetonitrile (63:3 vol/vol).

Gas-Liquid Chromatography-Mass Spectrometry

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Gas-liquid chromatography-mass spectrometry was performed on a Finnigan 4635 quadrupole instrument (Finnigan MAT, San Jose, Calif.) interfaced with a Sup-Incos data system. The gas chromatograph housed a 25-DB-1 fused silica capillary column and was equipped with an all glass solid injection system. The carrier gas was helium (flow rate 2 ml/min) and the column temperature was maintained at 265°C. Mass spectrometry was performed using electron impact ionization (70 eV) and spectra were recorded over the mass range 50-800 daltons by repetitive scanning (2 s/cycle) of the GLC effluent.

Bioassay for Estrogenic Activity

Two extracts of the feline diet were prepared. The first extract was completely hydrolyzed using the β -glucosidase, β -glucuronidase, and sulfatase enzyme preparations as described above and contained estrogenic compounds of plant and animal origin (10). The second extract excluded the β -glucosidase hydrolysis step and therefore contained only estrogens of mammalian origin. Separation

estrogenic effects at the levels tested in these bioassays. On the other hand, when the fully hydrolyzed extracts (β -glucosidase, β -glucuronidase, and sulfatase) of the commercial diet were injected into immature mice at concentrations of 20 and 40 g of the original diet per mouse per day, increases in uterine weight above controls of 16% and 79%, respectively, were observed. In the same model, a 17% increase in uterine weight was observed with 0.1 μ g estradiol-17 β /kg body wt. The estrogenic response observed from the fully hydrolyzed extract must therefore be due to the relatively large amounts

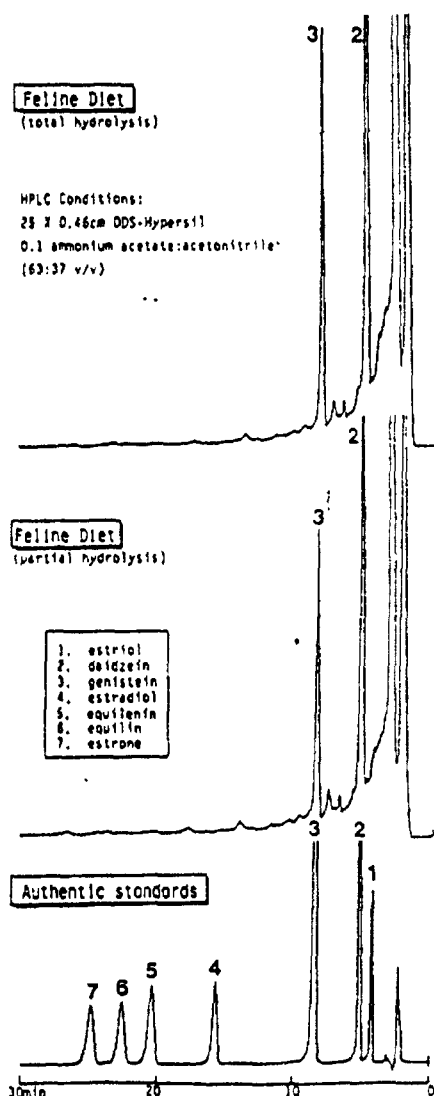


Figure 3. High-pressure liquid chromatography of (a) the extract of commercial feline diet (33.3 mg) fully hydrolyzed, (b) the extract of commercial feline diet (33.3 mg) in which the plant estrogens only were hydrolyzed, and (c) a mixture of authentic standards of the classical estrogens (estrone, estradiol, and estriol), the equine estrogens (equilin and equilenin), and the phytoestrogens (daidzein and genistein).

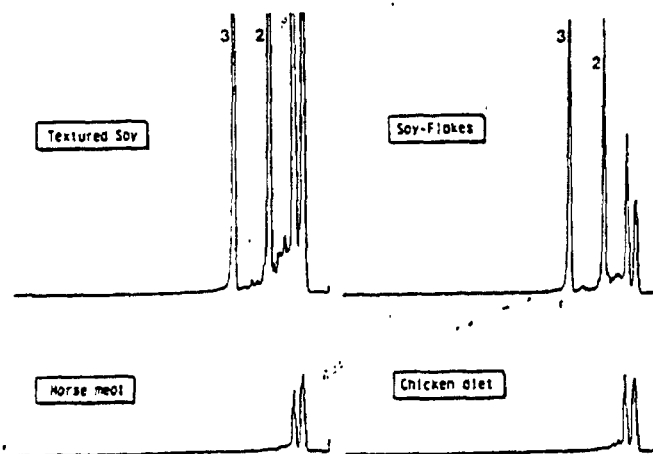


Figure 4. High-pressure liquid chromatography of the fully hydrolyzed extracts of textured soy, soy flakes, raw horse meat, and chicken diet. High-pressure liquid chromatography conditions as described in text and shown in Figure 3.

of the phytoestrogens daidzein and genistein present in the commercial diet.

Discussion

The causes of liver disease and reproductive failure in cheetahs kept in North American zoos are probably multifactorial; however, estrogens of plant origin that we have identified in the commercial diet fed to captive cheetahs may play a major role. The removal of these estrogens by a change of diet was associated with an improvement in coagulation time, liver function, and hepatic mitochondrial alteration, demonstrating some relationship between dietary estrogen and liver dysfunction. Venocclusive disease, found in ~60% of the adult captive cheetah population, could be the result of the direct effect of phytoestrogens on the vascular wall or changes in blood coagulation with secondary liver involvement, as has been suggested for other estrogens (15-18). Estrogens also exhibit cholestatic effects (19), and thus bile retention, with preferential shunting of substances across the basolateral membrane of the hepatocyte into the vascular compartment, may also play a role in the etiology.

Because it is classified as an endangered species, the cheetah cannot be readily subjected to experimentation. It will be difficult, therefore, to definitively prove a direct relationship between prolonged estrogen intake and the induction of hepatic venocclusive disease; however, the link between the intake of dietary estrogens and reproductive failure could be more successfully demonstrated.

High-pressure liquid chromatography and GLC-

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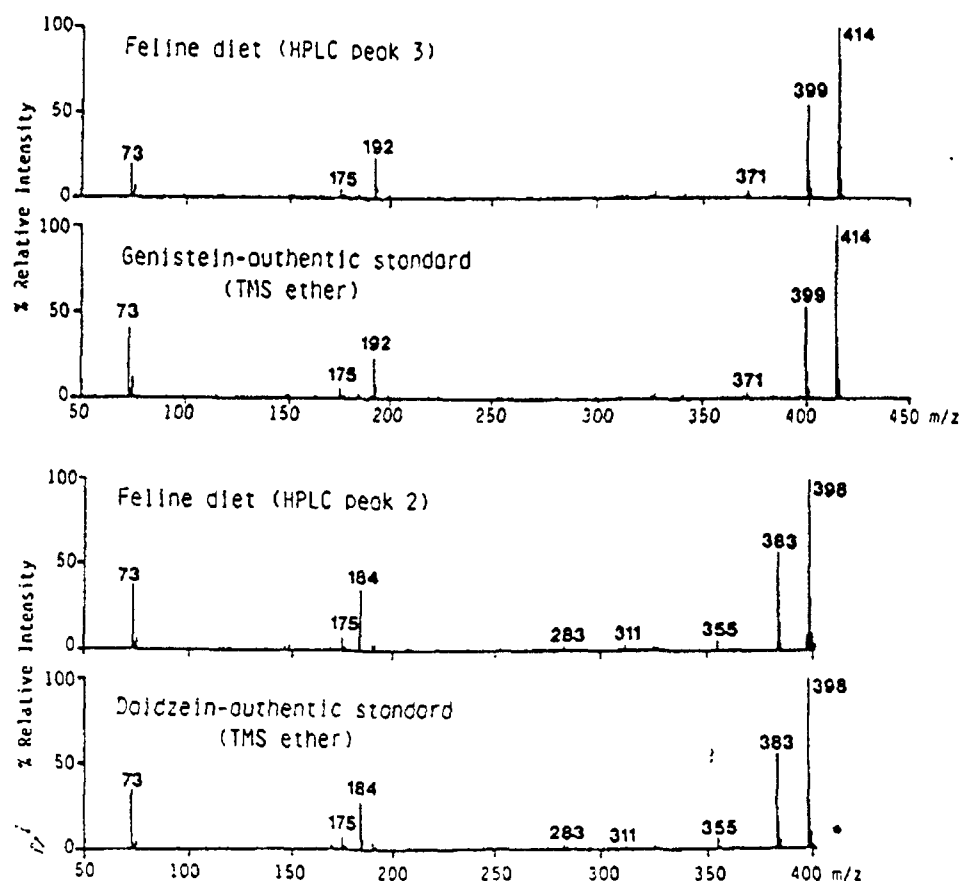


Figure 5. Comparisons of the mass spectra (70 eV) of the trimethylsilyl ether derivatives of the two major estrogens isolated from the commercial feline diet by HPLC with those of the authentic compounds daidzein and genistein.

MS analysis of the commercial diet fed to cheetahs revealed large quantities of two isoflavones, daidzein and genistein; however, the levels of equine estrogens, estradiol, estrone, and estriol were too low to be detected by these relatively sensitive techniques. These phytoestrogens were not present in the chicken diet or horse meat (Figure 4) and therefore were derived from the added soy protein present in the commercially prepared feline diet. Daidzein and genistein have been previously demonstrated to be the major phytoestrogens in soybean, first in 1931 by Walz (20) and more recently by others (21–25), including ourselves (8,10). Quantitatively our data indicates that on a daily basis the cheetah ingests ~50 mg of these phytoestrogens. Although they are weakly estrogenic (26–29), ingestion of this large amount of phytoestrogen is equivalent to exposing the animal to 50 µg of estradiol-17β per day. When dietary extracts containing these plant estrogens were tested, a dose-related estrogenic response was found in a uterotrophic bioassay.

The best known example of the effects of ingesting phytoestrogens was seen in the serious decline in lambing rates in Australia due to sheep grazing on

Trifolium subterraneum, an abundant species of clover rich in the isoflavone formononetin (30). Prolonged grazing resulted in permanent infertility after three seasons (31); the syndrome is known as "clover disease." Cystic endometrium is a common finding in these infertile ewes and it is sometimes associated with fibrosis in the muscle layer of the uterus (30,32).

Only 9%–12% of the sexually mature female cheetahs in North American zoos have been producing live cubs during the last 5 yr as compared with 60%–80% found in the DeWildt Cheetah Research Centre (5,13). Cheetah infertility could be attributed to the effects of exogenous estrogens in suppressing the hypothalamic-pituitary-gonadal axis, which appears to be overridden in a few cases by gonadotrophin-releasing hormone treatment (33). Additionally, fertility could be further affected by the lesions we observed in the uteri of these animals (Gosselin S, personal communication). The uterine lesions characterized by cystic endometrium, myometrial fibrosis, and endometrial fibrosis could potentially interfere with the normal implantation and nutrition of the newly fertilized egg, if conception

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occurs. The reversibility of some of these uterine lesions is questionable, possibly rendering several female cheetahs permanently sterile.

The first recognition that plants contained substances capable of inducing estrus in animals was documented 60 yr ago (34). In 1975, more than 300 plants were reported to have estrogenic activity (35). Although the mammalian estrogens estrone, estradiol-17 β , and estriol have been shown to occur in only a few plants, the isoflavones, coumestans, and the resorcylic acid lactones are the most common classes of phytoestrogens found in many plants regularly consumed by humans and animals. The isoflavones, which include daidzein and genistein, in particular, bear a striking resemblance in structure to the steroidal estrogens and to the potent and synthetic steroid diethylstilbestrol (Figure 6). Daidzein, genistein, and formononetin all have weak estrogenic activity relative to estradiol-17 β of the order of 0.002 to 0.001 (26-29). Although the absolute levels of these compounds in plants are low, it should be emphasized that consumption of several foodstuffs, particularly the legumes and forage plants, by humans and animals is high, and the net effects of long-term exposure to dietary estrogens should not be considered as insignificant (9,36,37).

Soybean is an increasingly important commercial source of protein. Uterotrophic effects have been reported in mice fed commercially pelleted diet containing soya meal (38,39). Previous studies have shown that in humans and rats, the phytoestrogens present in soy are efficiently metabolized by intestinal microflora and converted to the nonsteroidal estrogen equol (8,9,36). Furthermore, when most adults are fed meals consisting of soy protein, equol is excreted in the urine (8,36) at levels far in excess of the endogenous steroidal estrogens (9). Daidzein and O-desmethylanaglesin, a minor metabolite, have also been identified in human urine (8,13,40). Equol (Figure 6), which is formed from formononetin in the gastrointestinal tract of the sheep, is a weak estrogen and was the active agent incriminated in the infertility of "clover disease" (41-43).

The metabolism of daidzein and genistein in cheetahs is unknown. It is conceivable that as for the sheep, rat, and human, these isoflavones may be converted to equol, or activated to other more potent estrogens. Equol and daidzein are excreted by humans and rats primarily as the glucuronide conjugates (7,8). A clue as to why the cheetah may be sensitive to these dietary estrogens may lie in the fact that hepatic conjugation of many xenobiotics and phenolic compounds, an important pathway for their inactivation and excretion, is generally poor in the cat species (44). The "free" or biologically active

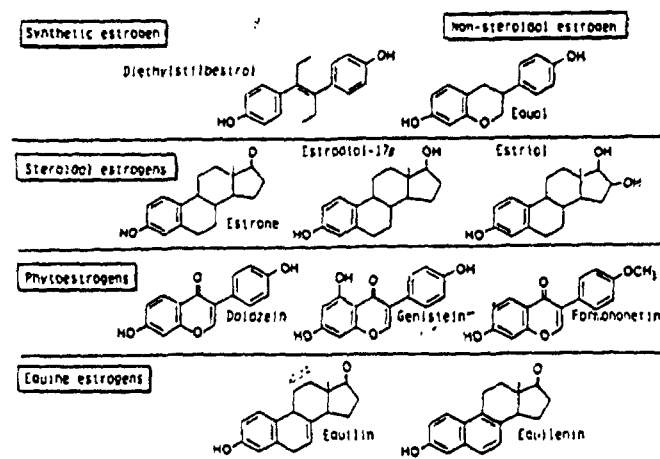


Figure 6. Comparisons of the chemical structures of the potent synthetic estrogen diethylstilbestrol with the steroids estrone, estradiol, and estriol; equine estrogens; the phytoestrogens daidzein, genistein, and formononetin; and the nonsteroidal estrogen equol.

unconjugated isoflavones may therefore be less efficiently inactivated and excreted by the cheetah.

Diethylstilbestrol (45-49), a potent estrogen, was withdrawn from agricultural use in the western world because of its adverse effects in humans and animals. Animal tissue consumed by humans is strictly monitored for contamination by this synthetic estrogen and for its abusive use as an anabolic agent. Despite concerns over the deleterious effects of diethylstilbestrol and other anabolic agents contaminating meats consumed by humans (50-52), it is apparent that the contribution of naturally occurring plant estrogens to the diet is rarely considered. This is surprising particularly as the level of phytoestrogens in foods is substantially higher than estrogen levels in animal tissues. Interestingly, it has been claimed that soy may be as beneficial as diethylstilbestrol as a growth promoter in animals (24,53), and our observations in cheetahs further support more serious consideration of the potential implications of dietary estrogens in humans (9,36).

Further studies are needed to assess the deleterious effects of dietary estrogens on cheetah reproduction as well as in the human population. Cheetahs have always been difficult to breed in captivity, but the additional insult of a diet rich in estrogens may well be one of the major factors in the decline of fertility in cheetahs kept in North American zoos. Our findings suggest that simple dietary manipulation, by excluding, in particular, foods rich in estrogens, may potentially offer a means of reversing the trend of liver dysfunction and infertility in the species, and possibly of resurrecting an active breeding program for this now endangered species.

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R. 1. 3

Causes of Adverse Responses to Soybean Milk Replacers in Young Calves¹

R. W. GARDNER
Department of Animal Science
and Benson Agriculture and Food Institute

M. G. SHUPE
Department of Animal Science

W. BRIMHALL
Department of Animal Science

D. J. WEBER
Botany Department
Brigham Young University
Provo, UT 84602

ABSTRACT

Sixty Holstein bull calves were randomly assigned to one of three treatment groups following an initial 3-d colostrum feeding period. They were fed either whole cows' milk or ethanol-extracted or hexane-extracted soy flour in milk replacers to 6 wk of age. These products were used to identify possible causative factors associated with adverse responses to soybean in milk repl. Average weight gains to 6 wk of age were 13.8, 7.3, and 2.8 kg and mortality was 0/20, 4/20, and 9/20 for calves fed milk, ethanol-extracted soy, and hexane-extracted soy, respectively. Heart rates (beats/min) were increased by the soy flours: 99.1 (ethanol extracted) and 116.3 (hexane extracted) versus 87.6 (milk). There was also an increased respiratory rate (breaths/min) with 67.6 and 61.1 versus 41.6 for the same treatment groups. Intradermal wheal growths verified an allergic sensitivity to the soybean products. Serum prostaglandin F_{2α} was 22% higher in the serum of calves fed the hexane-extracted soy milk replacer than in the serum of calves fed milk. Phenolic compounds in the soybean flour were implicated as possible

causative factors in the adverse responses to the soybean milk replacers. Ethanol extraction of the soy flour was more effective than hexane extraction in removing phenolic compounds (2.19 vs. 1.00% phenolics).

(Key words: soybean milk replacers, phenolics, prostaglandins)

INTRODUCTION

There have been reports of gastrointestinal allergy, or adverse responses, in calves fed milk replacers containing soybean flour (15, 16, 18). The assumption has been that the proteins of soybean, particularly glycinin and B-conglycinin, are probably the active agents (18). However, use of isolated soy protein in milk replacers causes fewer side effects than soy flour or concentrate, which suggests that the proteins in soy may not be the principal causative agents in these responses (13), particularly since the product is over 90% protein (19). Gardner et al. (4) hypothesized that phenolic (aromatic) compounds in soybean and other foodstuffs may be responsible for undesirable side effects following their ingestion. Pharmacological and toxicological effects of these compounds were observed in human subjects consistent with immunotoxic reactions. Immunoglobulins E and A were less than normal in these patients, but prostaglandin F_{2α} was elevated (4).

Extraction of soybean flour with ethanol using correct temperatures reportedly eliminated digestive disturbances in calves by denaturing

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antigenic proteins (18). Another possible effect of ethanol extraction is removal of toxic phenolic compounds, which are soluble in alcohol but insoluble in the hexane often used in extraction processes. These phenolic agents activate synthesis of prostaglandins and leukotrienes from arachidonic acid via stimulation of adrenergic receptors or as enzyme cofactors (6, 7, 8, 9, 17, 21). Several phenolic compounds have been identified and quantified in soy flakes (14).

Availability of precursor long-chain unsaturated fatty acids (C_{18-20}) in feedstuffs is also a factor affecting synthesis of eicosanoids (7, 8). Jenkins et al. (5) reported that unsaturated fatty acids in milk replacers fed to calves resulted in severe scours and inferior performance. In addition, cyclooxygenases and lipoxygenases, which catalyze the oxygenation of unsaturated fatty acids to form significant amounts of prostaglandins, thromboxanes, and leukotrienes have been identified in soybeans or soybean products (1, 8).

In a pilot study, calves were fed whole milk to which phenyl (benzyl) isothiocyanate was added daily to duplicate the quantity (90 mg) consumed by calves fed a soybean milk replacer. Calves exhibited the same pattern of diarrhea and illness as calves fed a soybean milk replacer. Total gains from 3 d to 4 wk of age averaged .7 kg, whereas calves fed whole milk gained 8.0 kg and had normal bowel function. Positive intradermal wheals developed in these 3-d-old calves when they were injected with phenyl isothiocyanate. This test was made to determine if this phenolic compound would cause an intradermal reaction such as Freedman et al. (3) observed upon injection of chlorogenic acid, another phenolic compound. They identified chlorogenic acid as the constituent of oranges responsible for intradermal reactions in humans allergic to oranges.

The objective of this study was to test the hypothesis that phenolic compounds in soybean milk replacers cause detrimental effects in calves by increasing production of prostaglandins through activation of cyclooxygenase activity. A second objective was to determine if ethanol extraction of soybean removes, or substantially reduces, the concentrations of toxic phenolic compounds and thus results in improved animal performance.

MATERIALS AND METHODS

Experimental Procedures

Sixty Holstein bull calves were randomly assigned at 3 d of age to three treatment groups following an initial colostrum feeding period. Treatments were: 1) whole cows' milk (M), which was discarded milk from the dairy; 2) an ethanol-extracted commercial soy flour protein source in a milk replacer (ES); or 3) a hexane-extracted soy flour protein source in a replacer (HS), to 6 wk of age. Composition values of the milk replacers appear in Table 1. The soy flour was mildly heat treated to inactivate trypsin inhibitors.

The calves were fed 1.8 l of whole milk (or milk replacer dry matter equivalent) at each of two feedings daily. Water was available for ad libitum consumption. A calf starter (Table 2) was available to all calves commencing at 1 wk of age. Calves were housed in hutches and bedded on straw. They were weighed when assigned to treatments and weekly thereafter. Records were made of diarrhea and other abnormalities. Electrolyte solutions were administered to treat diarrhea under the supervision of a veterinarian. Antibiotics were also prescribed by a veterinarian.

Wheal skin tests were made by intradermal injection of .05 cc of milk or milk replacer solutions in the flank area after the calves had been on treatment for 2 wk and again at 5 or 6 wk of age. Wheal measurements were averages of the shortest and longest diameters in millimeters. All calves were tested for sensitivity to milk and the two soy milk replacers. An initial wheal approximately 2 mm in diameter was formed by the injection. Measurements were made at the time of injection and 20 min later.

Heart action (beats/min) and respiration (breaths/min) were measured at 2-wk intervals. These measurements were made to determine possible vasoconstrictor and bronchoconstrictor action of phenolic compounds and eicosanoids associated with experimental rations. Body temperatures were also monitored.

Blood samples were drawn at 2 and 5 or 6 wk of age. The serum was analyzed for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) using radioimmunoassay (20). A commercial kit (Seragen Co., Boston, MA) with $PGF_{2\alpha}$ standard was used. The serum was stored at $-20^{\circ}C$ until the assay could

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TABLE 1. Ingredients and compositions of milk replacers.¹

Ingredients	Hexane-extracted soy	Ethanol-extracted soy
	(%)	
Whey	49.1	54.0
Whey protein concentrate	4.0	0
Nonfat dry milk	1.3	1.3
Soy isolate	5.5	0
Soy protein concentrate	0	18.5
Eggt. soy flour	15.0	0
Fat blend 7/40	23.7	24.8
Basic premix	.5	.5
Additional premix (Ca + Se)	.7	.7
DL-Methionine	1	.1
Brewers' yeast	1	.1
Protein, min. %	21.0	21.0
Fat, min. %	15.0	15.0
Fiber, max. %	.6	.6
H ₂ O, max. %	3.5	3.5
Vitamin A, min. IU/kg	66,000	66,000
Vitamin D ₃ , min. IU/kg	22,000	22,000
Vitamin E, min.	44	44
Ca, min. %	.77	.77
P, min. %	.42	.63

¹Milk replacers provided by National Vitamin Products, Minneapolis, MN, and soybean components by Central Soya, Fort Wayne, IN. Composition values are feed tag guaranteed minimums.

be completed. Siliconized glass tubes were used in the assay to avoid binding of the labeled antibodies to the glass surface. The assay involved adding the radioactively labeled antibodies specific for PGF_{2α} in the serum, after which the excess labeled antibodies were absorbed onto charcoal and the bound antibody solution was counted in a liquid scintillation

TABLE 2. Ingredients and composition of calf starter.¹

Ingredient	% Ration (as fed)
Chopped alfalfa	20.0
Roller barley	20.5
Meat and bone meal	4.0
Molasses	5.0
Trace-mineralized salt	1
Dicalcium phosphate	1
Supplement ²	.1
Dry mass, %	86.0
Crude protein, %	13.6
Digestible protein, %	9.5
Digestible energy, kcal/kg	2.7

¹Supplement added 22 mg nicothylic acid, 7500 IU vitamin A, 2510 IU vitamin D₃, and 7 IU α-tocopherol acetate/kg of starter.

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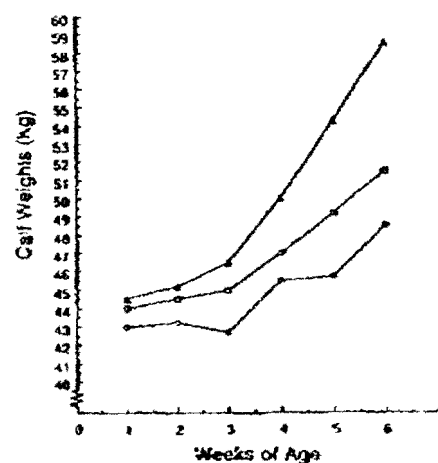


Figure 1. Comparative average weight gains of calves fed milk (Δ), ethanol-extracted soy flour (□), and hexane-extracted (○) soy flour.

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TABLE 3. Treatment effects on weight gains, physiological responses, and mortality

Treatments	Average weight gains (6 wk)		Respiratory rates		Heart rates		Mortality		Age at death	
	(kg)		(insp/min)		(Beats/min)		No.	%	(d)	
	\bar{X}	SE	\bar{X}	SE	\bar{X}	SE			Avg	Range

Milk	13.8	1.3 ^a	41.6	2.6 ^a	37.6	6.5 ^a	1/20 ^b	0	0	
Ethanol-extracted soy	7.3	1.0 ^b	67.7	4.2 ^b	99.1	4.4 ^b	1/20 ^b	11	16.28	
Hexane-extracted soy	2.8	1.0 ^c	61.6	4.3 ^b	115.3	5.0 ^b	9/20 ^c	12	19.18	

^{a,b,c}Values without common superscript letters differ ($P < 0.01$) between treatments.^bNumber dead/total number calves.

spectrometer to determine the amount of labeled antibody binding to the $\text{PGF}_{2\alpha}$ in the serum. The radioactive counts were converted to concentration by reading from a standard curve. The university veterinarian and a state veterinary pathologist conducted post-mortem examination of calves.

Milk and replacers were analyzed for total phenols using the Prussian Blue Method (2). Unsaturated fatty acids in the milk and milk substitutes were analyzed by gas chromatography-mass spectrometry (11). They were identified by comparisons of their spectra with known spectra in the mass spectra computer library. Comparative amounts of these fatty acids were ascertained by electronic integration of the area of the fatty acid peaks. Relative values were then calculated.

RESULTS AND DISCUSSION

Growth Response

The average weight gain of calves fed the whole cows' milk was approximately double that of calves fed the replacer containing I.S. (Table 3, Figure 1). Gains of the calves fed the

I.S. was only 20% of those fed M and 38% of those fed the E.S.

Diarrhea was common in calves fed the soy products. Using a coding system of 1 for normal through 4 for very watery diarrhea, the average value was 4. Color of the feces ranged from yellow to grey to black. Mortality on the soy replacers were substantial (Table 3) and were preceded by severe diarrhea. Degenerative changes and inflammation in the midsections of the small intestine were noted upon post-mortem examinations. Edema of lamina propria on tips of villi was a common observation also. These observations correspond with others making like examinations of the small intestines of calves fed soy milk replacers (15). Accumulation of gas in the abomasum and rumen was observed in several of the calves. The veterinarian's diagnoses were malnutrition and enteritis in the calves posted.

Serum $\text{PGF}_{2\alpha}$ concentration was increased by 27% when the hexane extracted soy product was substituted for milk (Table 4). That difference was erased by extracting the soy with ethanol. A limitation in comparisons of $\text{PGF}_{2\alpha}$ was imposed by the disproportionate number of death losses, thus excluding a number of obser-

TABLE 4. Weight gain from intradermal challenge, and serum levels of prostaglandin $\text{E}_{2\alpha}$, $\text{PGF}_{2\alpha}$

Treatments	Wheat growth from							
	Milk		Ethanol-extracted soy		Hexane-extracted soy		PGF _{2α}	
			(mm)				(g/100 ml)	
	\bar{X}	SE	\bar{X}	SE	\bar{X}	SE	\bar{X}	SE
Milk	1	2	1.1	2	1.7	5	760	68
Ethanol-extracted soy	1.0	3	2.9	3	1.7	2	565	55
Hexane-extracted soy	1.5	3	3.2	3	2.6	5	929	53

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various. $\text{PGF}_{2\alpha}$ was the only prostaglandin, or eicosanoid, examined. Prostaglandin E_2 (PGE_2) has been identified as being released in large amounts (6, 8); thus, further studies of other prostaglandins, thromboxanes, and leukotrienes are needed from tissue, blood, and stool samples. Gastrointestinal cramps and diarrhea were among the first side effects to be noted when prostaglandins were employed in gynecological practice (12). However, in addition to these effects on motility, the prostaglandins also influence water and electrolyte transport across the intestinal mucosa, thus enhancing the diarrhea (12). Prostaglandin E_1 (PGE_1), PGE_2 , and $\text{PGF}_{2\alpha}$ inhibit reabsorption of water and electrolytes in the small intestine, leading to enteropooling (12). Prostaglandins and leukotrienes are also mediators of inflammation seen in the bowels (6).

Extracts of the soy flour with ethanol reduced the quantity of phenolic compounds to less than half of that extracted with hexane (2.18 vs. 1.00%). The concentrations in whole milk and soy protein concentrate were .62 and 2.58%, respectively. Analysis of the unsaturated fatty acids also disclosed high concentrations in the soy products with relative quantities of 6 (M), 12 (ES), 15 (HS), and 95 (soy protein concentrate) calculated from the electronic integration of the areas of the fatty acid peaks.

Tachycardia resulted ($P < .01$) when calves consumed the soy replacers (Table 3). Even larger differences ($P < .01$) were observed in effects on respiration (Table 3). Tachycardia and bronchoconstriction are syndromes that have been identified as pharmacological effects of phenolic compounds and eicosanoids (12, 17), particularly since several phenols potentiate adrenaline toxicity (17).

Wheat growths from intradermal injections (Table 4) verified allergic reactions to the soybean products, if one accepts Muller's (10) hypothesis from work with humans that a growth of 2 mm or more is evidence of a sensitivity to the substance injected. Body temperatures were not affected ($P > .05$) by treatments 38.8 (M), 39.2 (ES), and 39.2 (HS).

The results of this experiment suggest that phenolic compounds and unsaturated fatty acids in soybean flour may adversely affect calves by increased production of prostaglandins. Additional studies are needed to measure changes in other eicosanoids (i.e., prostaglandins other

than $\text{PGF}_{2\alpha}$, thromboxanes, and leukotrienes) and to assess their effect on physiological responses.

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Roasted Soybeans and an Estrogenic Growth Promoter Affect the Thyroid Status of Beef Steers^{1,2,3}

Theron S. Rumsey,⁴ Theodore H. Elsasser and Stanislaw Kahl*

Growth Biology Laboratory, Livestock and Poultry Sciences Institute, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705-2350 and *Department of Animal Sciences, University of Maryland, College Park, MD 20742

ABSTRACT We investigated the interactive effects of a roasted soybean (RSB)-supplemented diet and an estrogenic ear implant [Synovex-S[®] (SYN), 20 mg estradiol benzoate + 200 mg progesterone] in young beef steers on measures of thyroid status before and after challenge injections of thyrotropin-releasing hormone (TRH) + growth hormone-releasing hormone (GHRH). Twenty steers (body weight 255 ± 5 kg) were assigned to the following treatments: 1) no SYN and a soybean meal-supplemented diet, 2) no SYN and a RSB-supplemented diet, 3) plus SYN and soybean meal, and 4) plus SYN and RSB. Steers were individually fed 1.13 MJ metabolizable energy/kg metabolic body wt daily of an 18% protein diet. After a 5-wk growth period, all steers were challenged (intravenous injection) over a 3-wk period with three dose levels of a combination of TRH + GHRH (0.1 + 0.01, 1.0 + 0.1 and 2.5 + 0.25 $\mu\text{g/kg}$ body wt). There were no dose by SYN or RSB interactions. Across dose levels, values for baseline plasma thyroid-stimulating hormone (TSH) were 0.37, 0.35, 0.61 and 0.33 $\mu\text{g/L}$ for treatments 1, 2, 3 and 4, respectively (SYN, $P < 0.07$; RSB, $P < 0.01$; SYN \times RSB, $P < 0.03$, SEM 0.06). Net areas under the response curve for TSH were 66.4, 51.3, 91.4 and 64.4 ($\mu\text{g/L} \times \text{min}$), respectively (RSB, $P < 0.08$; SEM 12.0). Similar treatment effects and/or numerical differences after challenge were noted for thyroxine (T_4) but not triiodothyronine (T_3). Baseline (2.22 vs. 2.00 $\mu\text{g/L}$, $P < 0.02$) and peak (3.07 vs. 2.03 $\mu\text{g/L}$, $P < 0.03$) T_3 concentrations were less for steers fed RSB than for steers fed soybean meal. This study indicates that in young growing beef steers, SYN increases TSH release from the adenohypophysis and that the primary effect of RSB is reduced plasma T_4 , possibly through an effect on peripheral T_4 deiodination. J. Nutr. 127: 352-358, 1997.

KEY WORDS: • beef cattle • estrogens • thyroid hormones • thyroid-stimulating hormone • soybeans

The use of oilseeds is an important option for increasing the energy density of ruminant diets in addition to supplying dietary protein to support high rates of production. However, animals may use certain oils or fatty acids with different efficiencies than are currently documented (Su and Jones 1993). Feeding whole soybeans to lactating dairy cows reduces milk protein production (Casper and Schingoethe 1989). Rumsey et al. (1996) recently reported that feeding roasted soybean (RSB)⁵ reduced weight gain of young beef steers. Although several studies suggest that certain oils or dietary fatty acids

affect the thyroid status of ruminants (Kahl et al. 1991, Lough et al. 1993 and 1994, Romo 1995, Norton et al. 1987 and 1988) primarily through changes in peripheral deiodination of the inactive hormone thyroxine (T_4) to the active hormone triiodothyronine (T_3), little is known of the effects oil supplements may have on the hypothalamic release of thyroid-stimulating hormone (TSH). In the research reported by Rumsey et al. (1996), the ability of thyrotropin-releasing hormone (TRH) plus growth hormone-releasing hormone (GHRH) to release growth hormone (GH) was shown to be reduced in young steers fed RSB.

Estrogenic growth promoters are widely used in growing and finishing beef production systems. The estrogenic growth promoter Synovex-S[®] (SYN, ear implant containing 20 mg estradiol benzoate + 200 mg progesterone) affects the thyroid status in beef steers (Kahl et al. 1978), probably via the T_4 deiodinase mechanism (Rumsey et al. 1985b). Limited reports suggest that estrogens may increase TSH release in sheep (Davis et al. 1978) and rats (DeLean and Labrie 1977). Trenkle (1969) suggested that estrogens increase the sensitivity of the thyroid gland to TSH. Thus an interaction may exist between the feeding of oilseeds and the use of an estrogenic growth promoter with regard to effects on the thyroid axis.

This study was undertaken to determine the influence of feeding a diet supplemented with RSB compared with soybean meal on the ability of TRH and GHRH to stimulate TSH release in young growing beef steers either implanted or not

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⁴ To whom correspondence should be addressed.

⁵ Abbreviations used: GH, growth hormone; GHRH, growth hormone-releasing hormone; RSB, roasted soybeans; SYN, Synovex-S ear implant containing 20 mg estradiol benzoate and 200 mg progesterone; TSH, thyroid-stimulating hormone; TRH, thyrotropin-releasing hormone; T_4 , thyroxine; T_3 , triiodothyronine. Treatment abbreviations: -SYN -RSB, no SYN implant and soybean meal-supplemented diet (no dietary RSB); -SYN -RSB, no SYN implant and RSB-supplemented diet; +SYN -RSB, SYN implant and no dietary RSB; +SYN -RSB, SYN implant and RSB-supplemented diet.

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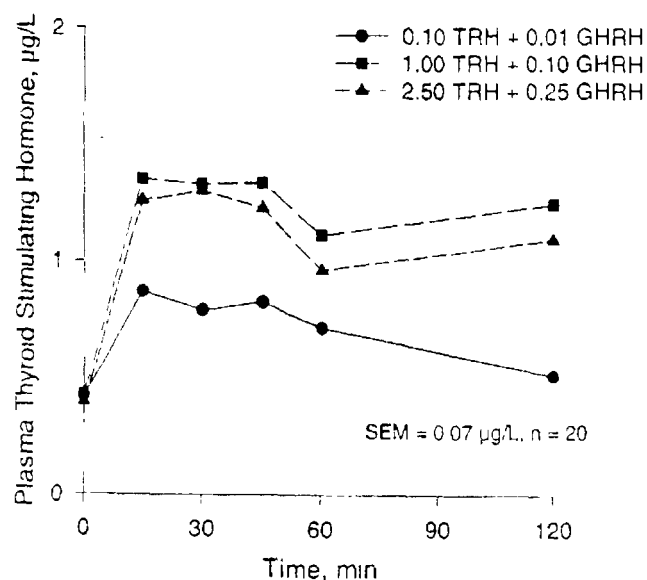


FIGURE 1. Thyroid-stimulating hormone response curves averaged across treatments, showing the response patterns following challenge injections to three dose levels of a combination of thyrotropin-releasing hormone (TRH) + growth hormone-releasing hormone (GHRH) in young growing beef steers (0.1 + 0.01, 1.0 + 0.1 and 2.5 + 0.25 µg/kg body wt).

implanted with SYN. We used the combination of hypothalamic releasing hormones (TRH + GHRH) to test, in the same steers, the responsiveness of the pituitary gland to release both GH (Rumsey et al. 1996) and TSH.

MATERIALS AND METHODS

The animals, diets, experimental protocol and design were described in detail earlier (Rumsey et al. 1996). Briefly, 20 young growing beef steers were used in a 2 × 2 factorial arrangement of treatments. The treatments were a 30.70 silage-concentrate diet supplemented with either soybean meal (+RSB, control diet) or red sorghum (+RSB) and with steers either not implanted (−SYN) or implanted with SYN (or implants (+SYN, 20 mg estradiol benzate + 200 mg progesterone; Syntex Animal Health, Des Moines, IA). The RSB were prepared commercially by heating soybeans to 127°C for 10 min. The animal protocol for the research in this report was approved by the Beltsville Agricultural Research Center Institutional Animal Care and Use Committee.

Steers were individually fed either −RSB or +RSB diets during the study to gain an estimated 1.3 kg/d. After adaptation to pens and the −RSB diet, steers were switched to their assigned diets, implanted or assigned, and fed for 9 wk. The initial 5-wk period was for recording weight gain (Rumsey et al. 1996). During wk 7, 8 and 9, multiple hormone challenges were conducted on each steer to measure TSH, T_4 and T_3 responsiveness. This was followed by an interim feeding period of 3 wk with steers continuing on their respective diets. Steers were then implanted as per original implant assignment, continued on their respective diets for an additional 5 wk for recording weight gain, and then killed in the Beltsville abattoir as per Rumsey et al. (1996). The liver, kidney and pituitary of each steer were collected and weighed. The pituitary and duplicate 10-g samples of liver and kidney were sealed in plastic bags, frozen in liquid nitrogen and stored at −80°C until analyzed for 5 α -dehydroxycorticosterone activity (Kobayashi et al. 1995).

All steers were challenged with three levels of TRH+GHRH (0.1 + 0.01, 1.0 + 0.1, 2.5 + 0.25 µg/kg body wt) over the 3-wk challenge period as described (Rumsey et al. 1996). For each challenge, blood samples were obtained at −10, 0, 5, 10, 15, 20, 30, 45, 60, 120, 240 and 360 min after challenge for T_4 and T_3 analysis and at −10, 0, 15, 30, 45, 60 and 120 min for TSH analysis. Thyroxine-

stimulating hormone concentration in plasma was determined by radioimmunoassay (Elisasser et al. 1992), with inter- and intra-assay CV of 12.6% and 12.6%, respectively. T_4 and T_3 concentrations were determined by radioimmunoassay as reported by Kobil et al. (1992), with inter- and intra-assay CV of 4.8% and 6.3% for T_4 and 5.2% and 7.8% for T_3 , respectively. The hormone response curves were evaluated for baseline concentration before challenge, area under the curve, peak response (visual evaluation of plasma concentrations), and time from challenge to peak.

Data were analyzed using the GLM procedure of SAS (1988). Response curve data were analyzed as a 2 × 2 × 3 factorial (RSB, SYN, RSB, dose level) and then interaction as non-effects. Differences were considered significant at $P \leq 0.05$ and to show a trend at $0.05 < P \leq 0.10$. Where appropriate, differences between individual means were evaluated using the Duncan's new multiple range test protected with a significance F test ($P = 0.05$).

RESULTS AND DISCUSSION

The dose response curves for plasma TSH concentrations and subsequent changes in plasma T_4 and T_3 concentrations are shown in Figures 1, 2 and 3, respectively. For each dose level, the data points represent the average across treatments. These curves are presented as a visual description of the thyroid axis responses to the TRH+GHRH challenges used in this study. Compared with the response to the low dose, the response in plasma TSH concentration was markedly greater for the middle and high doses of TRH+GHRH. However, the response in plasma TSH concentration was similar for the middle and high doses of TRH+GHRH. This indicates that the challenge doses used in the present study were able to test the potential of the pituitary gland to respond to hypothalamic stimulation.

The degree of TSH responsiveness to TRH+GHRH challenge was evaluated among treatments by comparing indicators of response curve characteristics. These indicators were the baseline (zero time) plasma concentration, the number of peaks (one or two), the time lapse between challenge times,

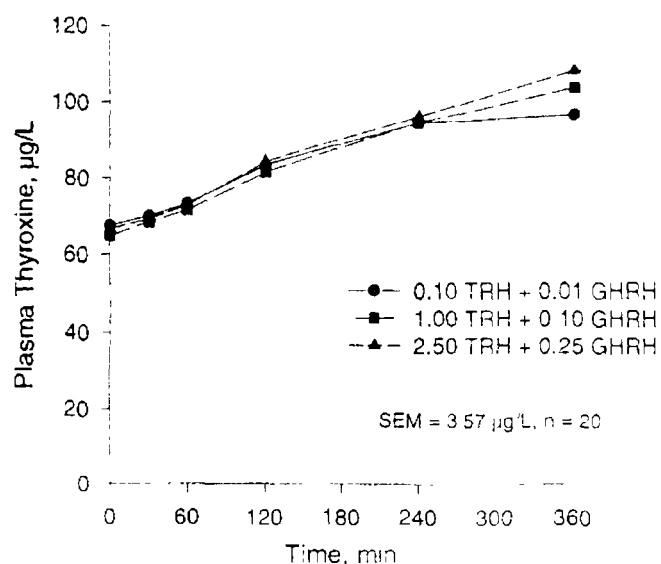


FIGURE 2. Thyroxine response curves averaged across treatments, showing the response patterns following the challenge injections to three dose levels of a combination of thyrotropin-releasing hormone (TRH) + growth hormone-releasing hormone (GHRH) in young growing beef steers (0.1 + 0.01, 1.0 + 0.1 and 2.5 + 0.25 µg/kg body wt).

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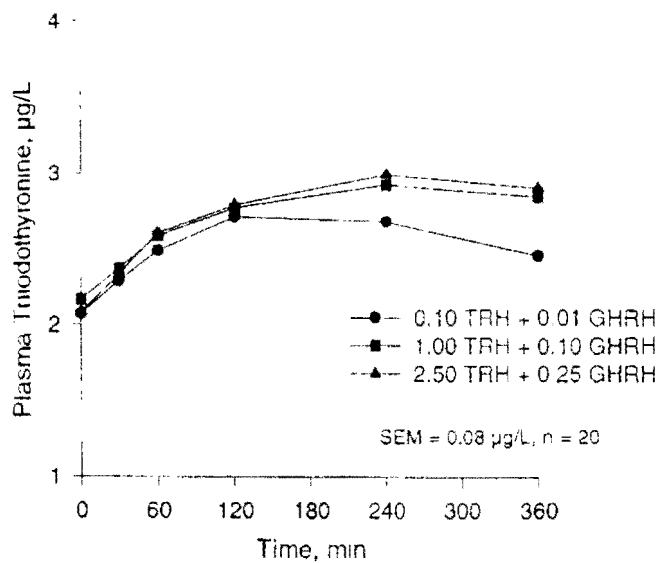


FIGURE 3 Triiodothyronine response curves averaged across treatments, showing the response patterns following the challenge injections to three dose levels of a combination of thyrotropin-releasing hormone (TRH) + growth hormone-releasing hormone (GHRH) in young growing beef steers ($0.1 + 0.01$, $1.0 + 0.1$ and $2.5 + 0.25$ µg/kg body wt).

tion and peaks, the peak concentrations, and the net area under the response curves (total area minus baseline). The evaluation of the T_4 and T_3 response curves was the same as for TSH except only single response peaks were observed. In general, if baseline and peak concentrations vary in a similar manner across treatments and net area across treatments is not different, then the response was considered to suggest a sustained long-term effect of treatment. If baseline concentration is not different across treatments and peak concentrations and net area under the response curve vary in a similar manner across treatments, then an effect of treatment on the immediate sensitivity to hormone challenge is suggested. If baseline and peak concentrations and net area all differ among treatments, then both a sustained treatment effect and an immediate sensitivity are involved. A change in lapsed time between challenge injection and peak is interpreted to be positively related to sensitivity to challenge.

Response of thyroid-stimulating hormone following thyrotropin-releasing hormone plus growth hormone-releasing hormone challenge. The primary effect of the hormonal challenge protocol on thyroid axis hormones in this study would be on TSH release and subsequent plasma concentrations. For TSH (Fig. 1), the response to all doses occurred rapidly, with a somewhat sustained response from 15 through 45 min. For the lowest dose, the response then returned to baseline at 120 min. For the two higher doses, the plasma concentrations of TSH began to return to baseline from 45 to 60 min but then were elevated at 120 min. Apparently all challenge dose levels stimulated the immediate release of bound, readily available TSH, but only the two higher doses stimulated a longer-term synthesis of TSH. This is consistent with current understanding of the synthesis and release of TSH by the adenohypophysis (Eschobar del Rey et al. 1962).

The response curve indicators for TSH for individual challenge dose levels averaged across the SYN and RSB treatments are shown in Table 1. There were no dose level \times treatment interactions. Baseline TSH concentration was not different

across dose level. This result is consistent with the design because dose level would not be expected to affect prechallenge concentrations.

The number of TSH response peaks following hormonal challenge shifted from one peak toward two peaks, which reflects the fact that most steers at the two higher challenge doses displayed the initial increase in TSH, followed by a small reduction and then another increase, presumably a latent stimulation of TSH synthesis. Because of this effect on the number of TSH response peaks, statistical analysis for number of peaks included only data for the middle and high dose levels. There was no significant difference between the middle and high dose levels in the number of TSH response peaks. Similarly, statistical analysis compared only the middle and high dose levels for peak 2 concentration and minutes to peak 2. There was no significant difference between the middle and high dose levels for peak 2 concentration and only a trend ($P < 0.10$) toward a greater lapse time between challenge injection and peak 2 for the high dose compared with the middle dose.

The overall effects of challenge dose level were to increase plasma concentration of TSH for peak 1 ($P < 0.03$) and to increase net area under the response curve ($P < 0.01$). In both cases, the responses to the middle and high doses were similar to each other but different ($P < 0.05$) from the response to the low dose level. As expected, these response curve changes are consistent with those expected for an immediate sensitivity to hormone challenge.

The effects of SYN and RSB treatments averaged across dose level on the TSH response curve indicators are summarized in Table 2. Across SYN treatments, TSH baseline concentration ($P < 0.01$), concentrations of TSH for peak 1 ($P < 0.03$) and peak 2 ($P < 0.05$), and minutes to peak 2 ($P < 0.02$) were lower for RSB-supplemented steers than for steers fed soybean meal following hormonal challenge, and area under the TSH response curve tended to be lower ($P < 0.08$) for the RSB-supplemented steers. These effects of RSB on the TSH response curve are consistent with a sustained long-term effect of RSB and possibly a reduced immediate sensitivity to hormonal challenge.

The overall effect of SYN was a trend for baseline plasma concentration ($P < 0.07$), peak 1 concentration ($P < 0.06$) and peak 2 concentration ($P < 0.07$) of TSH to be greater for SYN-implanted steers than for steers not implanted. These effects of SYN are consistent with a sustained long-term effect of SYN.

The main effects of SYN on the baseline and peak 2 concentrations of TSH were primarily a result of a greater concentration for the +SYN-RSB treatment compared with the other treatments. The SYN \times RSB interaction was significant for the baseline concentration of TSH ($P < 0.03$), and the data showed a trend ($P < 0.07$) for a similar interaction for peak 2 concentrations. As with baseline and peak 2 concentrations, the highest numerical value for the net area under the response curve was observed for the +SYN-RSB-treated steers compared with the other treatment groups, but statistical significance was not obtained for the main effect of SYN or for the SYN \times RSB interaction. This may have been a function of the relatively high error variation obtained for this indicator. In general, these interactions would suggest that the sustained long-term effect of RSB on plasma TSH limits the release and/or production of TSH under conditions in which long-term TSH release is enhanced, such as in SYN-implanted steers.

The overall enhancement of TSH status and the sensitivity to challenge by SYN in growing steers is consistent with the overall physiological effect of increased metabolic rate reported

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TABLE 1

Plasma thyroid-stimulating hormone (TSH), thyroxine (T_4) and triiodothyronine (T_3) responses to three dose levels of a venous injection challenge of TRH + GHRH in young growing beef steers¹

	Dose level ²			SEM ³	P value ⁴
	0.1 + 0.01	1.0 + 0.10	2.5 + 0.25		
TSH indicators					
Baseline, $\mu\text{g/L}$	0.42	0.43	0.39	0.052	NS
Number of peaks ⁵	1.0	1.8	1.9	0.09	NS*
Peak 1, $\mu\text{g/L}$	1.01 ^a	1.52 ^b	1.42 ^b	0.137	<0.03
Peak 2, $\mu\text{g/L}$	— ⁶	1.27	1.09	0.135	NS*
Time to peak 1, min	24	27	27	2.9	NS
Time to peak 2, min	—	101	113	5.3	<0.10*
Area, $(\mu\text{g/L}) \times \text{min}$	32.3 ^a	91.2 ^b	81.8 ^b	10.36	<0.01
T ₄ indicators					
Baseline, $\mu\text{g/L}$	67.3	64.6	66.7	2.64	NS
Peak, $\mu\text{g/L}$	98.8	103.5	108.5	3.25	NS
Time to peak, min	318 ^a	360 ^b	360 ^b	8.1	<0.01
Area, $(\mu\text{g/L}) \times \text{min}$	6843 ^a	7897 ^b	8057 ^b	360.7	<0.03
T ₃ indicators					
Baseline, $\mu\text{g/L}$	2.09	2.16	2.09	0.074	NS
Peak, $\mu\text{g/L}$	2.75 ^a	3.0 ^{a,b}	3.09 ^b	0.090	<0.03
Time to peak, min	162 ^a	246 ^b	264 ^b	17.0	<0.01
Area, $(\mu\text{g/L}) \times \text{min}$	155.9 ^a	214.3 ^{a,b}	254.5 ^b	15.30	<0.01

¹ Values are means. Within a row, values with different letter superscripts differ ($P < 0.05$).

² All steers were challenged (by intravenous injection) with three dose levels of a combination of thyrotropin-releasing hormone (TRH) + growth hormone-releasing hormone (GHRH), 0.1 + 0.01, 1.0 + 0.1 and 2.5 + 0.25 $\mu\text{g/kg}$ body wt. Additionally, in a factorial arrangement of treatments, the steers were either implanted or not with a Synovex-S (SYN) ear implant contained 20 mg estradiol benzoate + 200 mg progesterone (Syntex Animal Health, Des Moines, IA) and fed either supplemental soybean meal or roasted soybeans commercially roasted at 127°C for 10 min. There were no treatment \times dose interactions, thus data are presented as means across treatments. Treatment means are presented in Table 2.

³ SEM = common standard error of the mean taken from the ANOVA ($n = 20$).

⁴ Summary of statistical results from treatment effects and interactions. Values for $P > 0.05$ to $P = 0.10$ are considered a trend. NS = not significant ($P > 0.10$). * Analysis was based on a model that included only the middle and high dose levels.

⁵ All challenge dose levels stimulated the immediate release of bound, readily available TSH, the two high doses more frequently stimulated a longer-term synthesis of TSH, creating a second elevation or peak concentration.

⁶ A second peak was not detected for the low dose level.

⁷ Area under the response curve with baseline subtracted.

earlier (Rumsey and Hammond 1990, Rumsey et al. 1990). Possibly the increased TSH status seen here and the increased GH status reported recently (Rumsey et al. 1996) in SYN-implanted steers are related to the ability of estrogens to enhance the level of cAMP (Dickson 1984), which in turn is associated with release of hypothalamic hormones. Our results are consistent with other reports that indicate estrogens increase the release and sensitivity to TRH in sheep (Davis et al. 1975) and rats (Delean and Lubrie 1977). However, SYN did not affect plasma TSH concentrations in beef steers in a study reported by Kahl et al. (1992). In that study, the steers were about 600 kg heavier, suggesting a possible age effect.

The general depression in response by RSB in young growing beef steers may reflect a similar effect on cell membranes and hormone release as suggested in the recent study of GH (Rumsey et al. 1996). Specific fatty acids may affect the fluidity of cell membranes and reduce the affinity to receptor sites (Klausner et al. 1990, Renaud et al. 1985). A specific explanation for the observed interaction between SYN and RSB is not apparent at this time.

Response of thyroxine following thyrotropin-releasing hormone plus growth hormone-releasing hormone challenge. The general pattern of the response curves obtained for T_4 is consistent with that for TSH. The plasma T_4 response curves (Fig. 2) would suggest an attenuated stimulation of T_4 production until after 60 min and a similar initial stimulation to all challenge doses even though the initial TSH response appeared different for the lowest dose. The plasma T_4 concentra-

tions increased with time after the challenge injection, but the response to the lowest challenge dose seemed to be diminishing at 360 min, which is consistent with less TSH response. The response to TRH+GHRH challenge appeared less and slower for T_4 than for TSH, which would be consistent with a secondary or indirect effect of TRH on T_4 release.

The response curve indicators for T_4 for individual challenge dose levels averaged across the SYN and RSB treatments are shown in Table 1. There were no dose level \times treatment interactions. Challenge dose increased the elapsed time between challenge injection and peak T_4 concentration ($P < 0.01$) and the net area under the response curve ($P < 0.05$). In both cases, the responses to the middle and high doses were similar to each other but different ($P < 0.05$) from that for the low dose level. Similarly, peak response concentration was numerically increased with dose level, but the differences were not significant. These changes are generally consistent with the effects of dose level on TSH response.

Apparent sensitivity of the thyroid gland to TSH decreased with increasing challenge dose level. Considering the ratio of the net area under the response curve for T_4 and TSH as an indicator of sensitivity, that is, the units of T_4 produced per unit of TSH, the respective average ratios were 1, 100, 130 and 150 for the increasing challenge dose levels. This suggests that the lowest level of TSH was the most effective in stimulating T_4 production, and this is consistent with a tightly controlled and sensitive metabolic regulating system.

The effects of SYN and RSB treatments averaged across

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TABLE 2

Plasma thyroid-stimulating hormone (TSH), thyroxine (T_4) and triiodothyronine (T_3) responses to venous injection challenge of T_4 + GHRH in beef steers either implanted (- SYN) or not (- SYN) with Synovex-S² and fed soybean meal (- RSB) or roasted soybean (- RSB) supplemented diets^{1,2}

	- SYN		+ SYN		SEM ³	P < value ⁴		
	- RSB	+ RSB	- RSB	+ RSB		RSB	SYN	RSB × SYN
TSH indicators								
Baseline, $\mu\text{g/L}$	0.37 ^a	0.35 ^a	0.61 ^b	0.33 ^a	0.06	<0.01	<0.07	<0.03
Number of peaks ⁵	1.5	1.5	1.5	1.6	0.1	NS	NS	NS
Peak 1, $\mu\text{g/L}$	1.23	1.09	1.75	1.18	0.16	<0.03	<0.06	NS
Peak 2, $\mu\text{g/L}$	1.39	0.97	1.71	1.01	0.19	<0.05	<0.07	<0.10
Time to peak 1, min	29.0	23.0	27.0	25.0	3.4	NS	NS	NS
Time to peak 2, min	112.5	94.0	120.0	101.3	7.4	0.02	NS	NS*
Area ⁶ ($\mu\text{g/L}$) × min	66.4	51.3	91.4	64.4	12.0	0.08	NS	NS
T ₄ indicators								
Baseline, $\mu\text{g/L}$	66.6 ^a	64.0 ^a	75.2 ^b	59.0 ^a	3.04	<0.01	NS	<0.03
Peak, $\mu\text{g/L}$	103.6 ^a	98.8 ^a	115.9 ^b	96.1 ^a	3.75	<0.01	NS	<0.05
Time to peak, min	352	344	344	344	9.4	NS	NS	NS
Area, ($\mu\text{g/L}$) × min	7651	6663	8347	7735	416.6	<0.06	<0.04	NS
T ₃ indicators								
Baseline, $\mu\text{g/L}$	2.24	1.91	2.19	2.09	0.09	<0.01	NS	NS
Peak, $\mu\text{g/L}$	3.06	2.80	3.07	2.86	0.10	<0.03	NS	NS
Time to peak, min	224	208	248	216	19.68	NS	NS	NS
Area ($\mu\text{g/L}$) × min	211.1	233.0	219.5	199.9	17.7	NS	NS	NS

¹ Values are means. Within a row, values with different letter superscripts differ ($P < 0.05$).

² Synovex-S (SYN) implant contained 20 mg estradiol benzoate + 200 mg progesterone (Syntex Animal Health, Des Moines, IA). Roasted soybeans were commercially roasted at 127°C for 10 min. All steers were challenged (by intravenous injection) with three dose levels of a combination of thyrotropin-releasing hormone (TRH) + growth hormone-releasing hormone (GHRH), 0.1 + 0.01, 1.0 + 0.1 and 2.5 + 0.25 $\mu\text{g/kg}$ body wt. There were no dose × treatment interactions; thus data are presented as means across dose levels. Dose level means are presented in Table 1.

³ SEM = common standard error of the mean taken from the ANOVA ($n = 15$).

⁴ Summary of statistical results from treatment effects and their interactions. Values for $P > 0.05$ to $P = 0.10$ are considered a trend. NS = not significant ($P > 0.10$). * Because a second peak was not detected for the low dose level, analysis was based on a model that included only middle and high dose levels ($n = 10$).

⁵ All challenge dose levels stimulated the immediate release of bound, readily available TSH; the two high doses more frequently stimulate longer-term synthesis of TSH, creating a second elevation or peak concentration.

⁶ Area under the response curve with baseline subtracted.

Dose level on the T_4 response curve indicators are summarized in Table 2. Across SYN treatments, T_4 baseline concentration and concentration of peak T_4 were lower ($P < 0.01$) for RSB-supplemented steers than for steers fed soybean meal following hormonal challenge, and area under the T_4 response curve tended to be lower ($P < 0.06$) for the RSB-supplemented steers. This general effect of RSB on T_4 is consistent with the effect of RSB on TSH. Across RSB treatments, area under the T_4 response curve was greater ($P < 0.04$) for the SYN-implanted steers compared with those not implanted. As with TSH, the SYN × RSB interaction was significant for baseline T_4 concentration ($P < 0.03$) and peak T_4 concentration ($P < 0.05$), primarily as a result of the greater concentrations for the -SYN-RSB steers compared with the other treatment groups. As with TSH, the T_4 results indicate that SYN increases the responsiveness of TSH plasma concentrations to TRH-GHRH challenge and that feeding RSB in place of soybean meal reduced this responsiveness, particularly in SYN-treated steers. In general, these results reflect the changes in circulating concentrations of TSH.

Increased plasma concentrations of T_4 have been reported in previous studies with beef steers treated with SYN (Kahl et al. 1978), although this effect of SYN has not been found to be consistent. Decreased 5'-deiodinase activity increases circulating concentrations of T_4 (Escobar del Rey et al. 1962; Rumsey et al. 1985a). Although SYN has been shown to decrease deiodinase in beef steers in vivo, this did not occur

when beef liver tissue was treated with SYN in vitro (Rumsey et al. 1985b). This suggested an indirect effect of SYN: circulating concentrations of T_4 that could be explained by the stimulatory effect of SYN on TSH observed in the current study.

Response of triiodothyronine following thyrotropin-releasing hormone plus growth hormone-releasing hormone challenge. The response curve indicators for T_3 averaged across SYN and RSB treatments are shown in Table 1. There were no dose level × treatment interactions. Challenge dose increased the peak plasma concentration ($P < 0.03$), the lapse time between challenge injection and peak ($P < 0.01$) and net area under the response curve ($P < 0.01$). For peak concentration and the time lapse between challenge injection and peak, the responses to the middle and high dose levels were similar to each other and greater ($P < 0.05$) than the response to the low dose level. For area under the T_3 response curve, the response to the high dose level was greater ($P < 0.05$) than the response to the low dose level, with the response to the middle dose being intermediate and not different from that of either the low or high dose level. These dose responses are from response curves that showed a quicker response to TRH-GHRH challenge (Fig. 3) than was observed for T_4 . The response curves for T_4 indicated the response was maximal at 240 min even though T_4 continued to increase during the time. After 120 min, the T_4 response seemed to be less for the lowest challenge dose than for the other doses. The T_3 responses

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curves may reflect T_3 release from the pituitary via type II deiodinase in response to TRH+GHRH challenge and autoregulation of plasma T_3 concentrations via feedback on deiodinase (Spira and Gordon 1986, Visser 1988).

The effects of SYN and RSB treatments averaged across dose level on the T_3 response curve indicators are summarized in Table 2. Across SYN treatments, both baseline ($P < 0.01$) and peak ($P < 0.03$) T_3 concentrations were lower for the RSB-supplemented steers than for the steers supplemented with soybean meal. There were no other significant treatment effects found for T_3 concentration. Unlike the effect of treatments on TSH and T_4 , the +SYN-RSB treatment did not result in the highest baseline or peak concentration and net area was not affected by treatment. Thus, in general, the results suggest that feeding RSB compared with soybean meal has a sustained long-term effect of reducing plasma T_3 concentration but does not measurably affect the sensitivity of T_3 plasma concentrations to a TRH+GHRH challenge. These results are reasonable because T_3 is the third step in the cascade from the effects of TRH, the result of metabolism of T_4 via deiodinase, and under feedback regulation.

The long-term effects of RSB on circulating T_3 are consistent with the general reduction in concentrations of TSH and T_4 in RSB-fed steers and possibly reflective of a shift in thyroid status because of the TSH and T_4 changes. However, decreased basal plasma concentration of T_3 could also suggest decreased 5'-deiodination of T_4 in extrathyroidal tissues. In the current study, we evaluated 5'-deiodinase activities in some extrathyroidal tissues. Although different in type, 5'-deiodinase activities tended to be decreased both in liver (type I) and pituitary gland (type II) in RSB-supplemented steers compared with soybean meal-supplemented steers at time of killing. Activity of type II 5'-deiodinase, which is responsible for the generation of T_3 involved mainly in the local regulation of GH and TSH synthesis (Nunez 1988), tended to decrease in RSB-supplemented steers regardless of SYN implantation (main effect, 0.79 and 0.62 pmol $I \cdot h^{-1} \cdot mg \text{ protein}^{-1}$ for -RSB and +RSB, respectively; SEM 0.07, $P < 0.10$). Activity of type I 5'-deiodinase in liver, which is responsible for most of the circulating T_3 was affected by RSB \times SYN interaction ($P < 0.05$); RSB supplementation decreased activity in nonimplanted steers (4.35 vs. 3.19 nmol $I \cdot h^{-1} \cdot mg \text{ protein}^{-1}$, SEM 0.41, $P = 0.07$) but was without effect in SYN-implanted animals. This is also consistent with the differences in T_3 concentration seen in this study. Although this reduced deiodinase activity is in contrast to in vitro studies of Norton et al. (1987 and 1988), Kahl et al. (1993 and 1994) and Su and Jones (1993), Romo (1995) recently reported a depression in vitro 5'-monodeiodinase activity in the liver of 6-mo-old calves by linoleic acid, which is a predominant fatty acid in whole soybeans. More research is needed in this area. Norton et al. (1987) reported that fatty acid effects on 5'-monodeiodinase were concentration dependant, and the influence of saturation by the ruminal environment needs to be considered.

In conclusion, this study evaluated the effect of two current practices used in beef production (feeding RSB and the use of estrogenic ear implants) on the thyroid axis of young growing beef steers. The use of the estrogenic growth-promoter Synovex-S increased the release of TSH and the subsequent release of T_4 . Feeding RSB reversed this effect. Thus the substrates necessary for enhancing the thyroid status of beef steers were increased by estrogen and decreased by feeding a RSB supplement. If this enhancement is needed for improved growth, then the results explain why growth was depressed in the RSB-

supplemented steers and enhanced in steers given an estrogen ear implant in our recent study (Rumsey et al. 1996).

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Anti-Thyroid Isoflavones from Soybean ISOLATION, CHARACTERIZATION, AND MECHANISMS OF ACTION

Rao L. Divi, Hebron C. Chang and Daniel R. Doerge*
NATIONAL CENTER FOR TOXICOLOGICAL RESEARCH, JEFFERSON, AR 72079, U.S.A.

ABSTRACT. The soybean has been implicated in diet-induced goiter by many studies. The extensive consumption of soy products in infant formulas and in vegetarian diets makes it essential to define the goitrogenic potential. In this report, it was observed that an acidic methanolic extract of soybeans contains compounds that inhibit thyroid peroxidase (TPO)-catalyzed reactions essential to thyroid hormone synthesis. Analysis of the soybean extract using HPLC, UV-VIS spectrophotometry, and LC-MS led to identification of the isoflavones genistein and daidzein as major components by direct comparison with authentic standard reference isoflavones. HPLC fractionation and enzymatic assay of the soybean extract showed that the components responsible for inhibition of TPO-catalyzed reactions coeluted with daidzein and genistein. In the presence of iodide ion, genistein and daidzein blocked TPO-catalyzed tyrosine iodination by acting as alternate substrates, yielding mono-, di-, and triiodoisoflavones. Genistein also inhibited thyroxine synthesis using iodinated casein or human goiter thyroglobulin as substrates for the coupling reaction. Incubation of either isoflavone with TPO in the presence of H_2O_2 caused irreversible inactivation of the enzyme; however, the presence of iodide ion in the incubations completely abolished the inactivation. The IC_{50} values for inhibition of TPO-catalyzed reactions by genistein and daidzein were ca. $1-10\ \mu M$, concentrations that approach the total isoflavone levels (ca. $1\ \mu M$) previously measured in plasma from humans consuming soy products. Because inhibition of thyroid hormone synthesis can induce goiter and thyroid neoplasia in rodents, delineation of anti-thyroid mechanisms for soy isoflavones may be important for extrapolating goitrogenic hazards identified in chronic rodent bioassays to humans consuming soy products. *BIOLOGICAL PHARMACOLOGY* 54:10 1087-1096, 1997.
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KEY WORDS. thyroid peroxidase; soybean; isoflavonoid; inhibitor; goitrogen; mechanism

The soybean and its products have been considered goitrogenic in humans and animals. Goiter and hypothyroidism were reported in infants receiving soy-containing formula [1-4], and such feedings in early life have also been associated with the development of autoimmune thyroid disorders [5]. Several investigators have reported induction of goiter in iodine-deficient rats maintained on a soybean diet [6-11]. Furthermore, Kimura *et al.* [9] reported the induction of thyroid carcinoma in rats fed an iodine-deficient diet containing 40% defatted soybean. Konijn *et al.* [10] showed that the anti-thyroid activity present in acidic acetone soybean extracts is water soluble, is dialyzable, and is not precipitated by either ammonium sulfate or trichloroacetic acid. The active ingredient was characterized partially by these workers as a small molecular compound of non-peptide origin, since it was not destroyed by either digestion with pancreatin or by boiling for 2 hr.

The function of the thyroid is synthesis of thyroid hormones, and TPO¹ catalyzes iodination of tyrosyl residues on Tg and the subsequent coupling of iodotyrosyl residues required for iodothyronine hormone formation.

Inhibition of TPO-catalyzed reactions results in decreased levels of circulating thyroid hormones, which lead to increased secretion of TSH by the anterior pituitary. The increased levels of TSH provide a growth stimulus to the thyroid, and it has been proposed that a prolonged stimulus can select for clones of follicular cells with the potential for transformation [12]. This mechanism predicts that any compound that inhibits TPO-mediated thyroid hormone synthesis is a potential thyroid carcinogen.

The widespread use of soy products in infant food formulas and the significant consumption of soy products by people consuming a vegetarian diet require a closer evaluation and examination of the anti-thyroid activity of the soybean. This is important because of the current promotion of soy-based products as health foods possessing putative beneficial estrogenic and anti-carcinogenic properties. For example, genistein, but not daidzein, inhibits tyrosine kinase activity, and this property has been explored for

*Corresponding author. Tel. (501) 543-7943, FAX (501) 543-7720,
E-mail: DDOERGE@NCTR.FDA.GOV
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¹Abbreviations: APCI, atmospheric pressure chemical ionization; CcP, cytochrome c peroxidase; CID, collision-induced dissociation; DIT, 3,5-diiodotyrosine; LPO, laccoperoxidase; MIT, 3-iodotyrosine; PEG, polyethylene glycol; rT₃, 3,3',5'-triiodothyronine; T₄, thyroxine; T₃, 3,5,3'-triiodothyronine; Tg, thyroglobulin; TPO, thyroid peroxidase; TSH, thyroid stimulating hormone; and MMS, 2-(N-morpholino)ethanesulfonic acid.

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potential anti-cancer potential [13]. Information in the scientific literature regarding the chemical nature of the active anti-thyroid component(s) from soybean, as well as the mechanism of action, is far from complete. In the present study, we report the chromatographic separation of the active anti-thyroid compounds of soybean, the elucidation of chemical structures, and the mechanisms for inhibition of TPO-catalyzed reactions.

MATERIALS AND METHODS

Reagents

Genistein, genistin, glucose, and glucose oxidase were obtained from the Sigma Chemical Co. (St. Louis, MO) and used as obtained. Daidzein was a gift from Dr. K. D. R. Setchell. TPO used in the present study was purified from porcine thyroid glands and quantified spectrophotometrically as previously described [14]. Human goiter Tg was a gift from Dr. Alvin Taurog, University of Texas Southwestern Medical School. The isoflavones were dissolved in either ethanol or DMSO that had been purified by distillation. A constant concentration of ethanol or DMSO (5%), which did not affect enzyme activity, was maintained in incubation mixtures.

Preparation of Soybean Extracts

Whole soybeans, advertised as not treated with herbicides or fungicides, were obtained from a local health food store and ground to a fine powder. The powdered sample (5 g) was extracted by stirring with 250 mL of acidic methanol (12 N HCl/CH₃OH, 10/90, v/v) with heating at reflux for 4 hr. The mixture was centrifuged at 20,000g for 20 min, the supernatant evaporated *in vacuo*, and the residue dissolved in 10 mL of 95% ethanol.

Liquid Chromatography

A fraction of the soybean extract was diluted 100-fold with methanol, and a 25- μ L aliquot was injected into a reversed-phase HPLC column (NovaPak C18, Waters Associates, Milford, MA) using a GPM quaternary gradient pump (Dionex, Sunnyvale, CA). The column was eluted using a solvent system consisting of 30% solvent A and linearly increasing to 100% A in 20 min at a flow rate of 1.5 mL/min [A = acetonitrile, B = acetic acid/acetonitrile:water (0.5/50/94.5 by vol)]. The peaks were monitored using a Spectra Focus forward-scanning optical detector (SpectraPhysics, San Jose, CA) at 260 nm and also by obtaining UV spectra between 200 and 350 nm. The same HPLC system was used to separate iodinated isoflavones. Elution was carried out with a mobile phase gradient starting with 50% A and increasing linearly to 100% A in 20 min at a flow rate of 1.5 mL/min. Peaks were detected using 275 nm absorbance.

For determination of TPO-inhibitory activity, individual identifiable peaks were collected after emerging from the

detector, and when peaks were not present, fractions of 1 min were collected across the entire elution profile. The collected fractions were dried *in vacuo* and dissolved in 100–200 μ L of ethanol. A 50- μ L aliquot was then added to the tyrosine iodination assay to determine inhibition of TPO activity (see below).

HPLC was used to monitor TPO-catalyzed iodination of tyrosine to MIT and DIT because the UV absorbance of the isoflavones interfered with the usual spectrophotometric assay [14]. The same NovaPak C18 HPLC cartridge was used with solvent system consisting of A = acetonitrile; B = 0.2% trifluoroacetic acid in 5% acetonitrile/water using a gradient of 10% A to 40% A in 20 min with a flow rate of 1.5 mL/min and UV detection at 232 nm. Retention times for tyrosine, MIT, and DIT were 2.5, 3.6, and 4.3 min, respectively.

Mass Spectrometry

MS experiments were performed using a VG Platform single quadrupole instrument (Micromass, Altonham, UK) equipped with an APCI interface. The total LC column effluent described above was delivered into the atmospheric pressure ion source (150°) through a heated nebulizer probe (500°) using nitrogen as the probe and bath gas (275 L/hr). Positive ions were acquired in full scan mode (m/z 100–600, 2.1 sec cycle time) in series with a UV detector set at 250 nm. Background-subtracted mass spectra were obtained by averaging spectra across the respective chromatographic peak and subtracting the average background immediately before and after this peak. At a low sampling cone-skimmer voltage (15 V), mass spectra for isoflavones and derivatives consisted predominately of the respective protonated molecule. At a higher voltage (50 V), in-source CID reactions produced numerous fragment ions. For on-line characterization of soy extracts, two separate scan functions were used to simultaneously obtain spectra at 15 and 50 V. The mass spectrometer was calibrated using a solution of PEGs [PEG 200 (25 μ g/mL), 300 (50 μ g/mL), 600 (75 μ g/mL), and 1000 (250 μ g/mL) obtained from Sigma Chemical Co.] in 50% acetonitrile in aqueous ammonium acetate (5 mM) over the range m/z 85–1200.

Inhibition of TPO-Catalyzed Reactions

Different amounts of soy extract, isolated HPLC fractions, or authentic isoflavones (2–80 μ M) were added to reaction mixtures to determine the concentration dependence of TPO inhibition. The solution was incubated for 4 min and the reaction stopped by injection onto the HPLC column. Tyrosine (100 μ M) and iodide (100 μ M) or guaiacol (2.5 mM) were incubated with TPO (10 nM) plus inhibitor, and the reaction was initiated by the addition of H₂O₂ (100 μ M) at 22 \pm 0.1°. HPLC was used to follow the concentration of MIT/DIT and isoflavone present in incubations containing TPO, the H₂O₂-generating system consisting of

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glucose (1.25 mM) plus glucose oxidase (10 nM), and genistein or daidzein.

Iodination of bovine casein (Sigma Chemical Co.) was carried out using Iodobeads (Pierce Chemical Co., Rockford, IL) using 10 beads in 5 mL MES buffer, pH 7.0, that contained 250 μ M iodide to generate I_2^- for 1 min. Then a 10-mL aliquot of casein solution (1.25 mg/mL) in the same buffer was added. After incubation for 10–15 min at room temperature, the solution was dialyzed overnight. The degree of iodination was estimated spectrophotometrically using the change in absorbance at 290 nm as a measure of DIT formation ($\Delta A = 0.92/\text{mole } I_2^-$, see Ref. 15). The content of MIT and DIT in iodinated casein was confirmed for one sample using HPLC analysis after proteolytic digestion of iodocasein; reasonable agreement with the spectrophotometric determination was seen (not shown).

Measurement of coupling, an *in vitro* assay of thyroid hormone synthesis, was carried out in the presence of TPO (20 nM) using chemically iodinated casein (1.25 mg/mL containing approximately 50–60 atom I/mol) as the source of iodotyrosines [16], various concentrations of isoflavone, and H_2O_2 (100 μ M) for 1 hr in 0.05 M MES buffer, pH 6.5, at $37 \pm 0.1^\circ$. Bovine mucosal alkaline phosphatase (10 units, Sigma Chemical Co.) was added to hydrolyze phosphate groups, including phosphoryrosines. This treatment increased yields of T_4 by approximately 25%. The reaction mixture was digested under a nitrogen atmosphere using pronase (250 μ g/mL final concentration) for 1 hr and then with leucine-aminopeptidase (50 μ g/mL) for 3 hr [17]. Thyroid hormones in the reaction mixture were extracted three times with ethyl acetate, and the extract was dried *in vacuo* and dissolved in a 100- μ L aliquot of starting HPLC solvent. Extraction efficiency for a standard addition of T_4 was determined to be 92–95%. Thyroid hormones were measured by HPLC using a Hamilton PRP-1 reversed phase column with a solvent system of A = acetonitrile, B = trifluoroacetic acid/acetonitrile/water (0.2:5:94.8, by vol) starting with 10% A in B and increasing linearly to 50% A in 20 min at a flow rate of 1.0 mL/min. The peaks were detected and iodothyronines quantified using 230 nm absorbance.

Measurement of TPO-catalyzed coupling was also carried out using human goiter Tg essentially as described by Turog *et al.* [18] except that the proteolysis and HPLC analysis described above were used for quantifying iodothyronines. Samples were analyzed in triplicate for at least four different concentrations of isoflavone bracketing the IC_{50} .

Inactivation of TPO by Isoflavones

TPO was inactivated by isoflavones by incubating enzyme (1.0 μ M) with 50 μ M daidzein or 50 μ M genistein and 200 μ M H_2O_2 at $25 \pm 0.1^\circ$ in 0.1 M MES buffer (pH 7.0). After 4 min, aliquots were withdrawn and diluted 1000 to 2000 fold, and the remaining tyrosine iodination activity was measured. The activity was not restored by treatment of

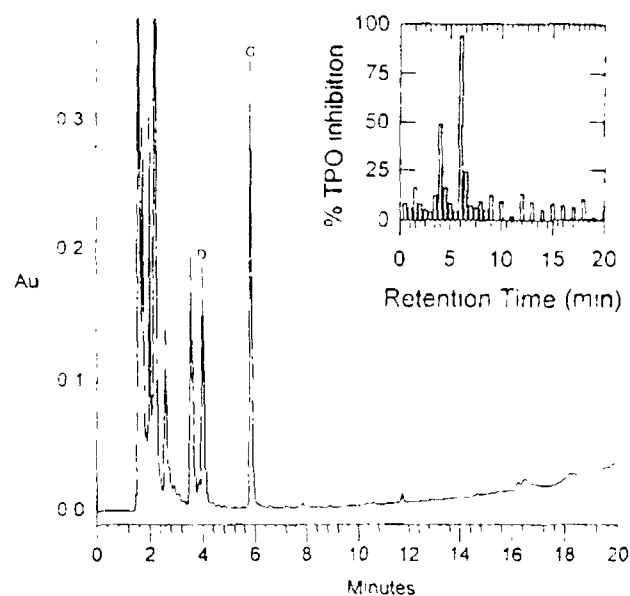


FIG. 1. HPLC fractionation of a soybean extract and inhibition of TPO-catalyzed tyrosine iodination. The soybean extract was fractionated using HPLC and UV detection (260 nm) as described in Materials and Methods, and the individually collected fractions were tested for inhibition of TPO activity (inset). The maximal TPO activity was 9 nmol MIT formed/min.

inactivated enzyme by centrifugal gel filtration or extensive dialysis as previously described [14]. In separate experiments to study its protective effect on inactivation, iodide (0.15 to 5.0 mM) was also included in the reaction mixture.

RESULTS

Characterization of Compounds Inhibiting TPO in Crude Soybean Extract

The presence of anti-thyroid components in soybean was investigated using a heated acidic methanol hydrolysis and extraction procedure. This procedure was selected to liberate the respective aglycones because glycoside conjugates are the predominant form in whole soybean [19–20]. It was determined that this procedure completely converted genistein and daidzein conjugates to the respective aglycones (data not shown). When the extract was fractionated using HPLC, two distinct peaks of UV absorbance (retention times 4.0 and 5.8 min; see Fig. 1) were found to contain most of the inhibitory activity (Fig. 1 inset). Peaks labeled D and G showed UV absorption maxima at 251 and 259 nm, respectively (data not shown). These chromatographic and spectral properties were identical to those observed from authentic standards of daidzein and genistein, and coelution of standards with the extract showed no evidence for inhomogeneity. The genistein and daidzein content based on HPLC analysis of the acidic methanol extract was determined to be 1.98 and 0.73 mg/g soybean, respectively, using external standard calibration.

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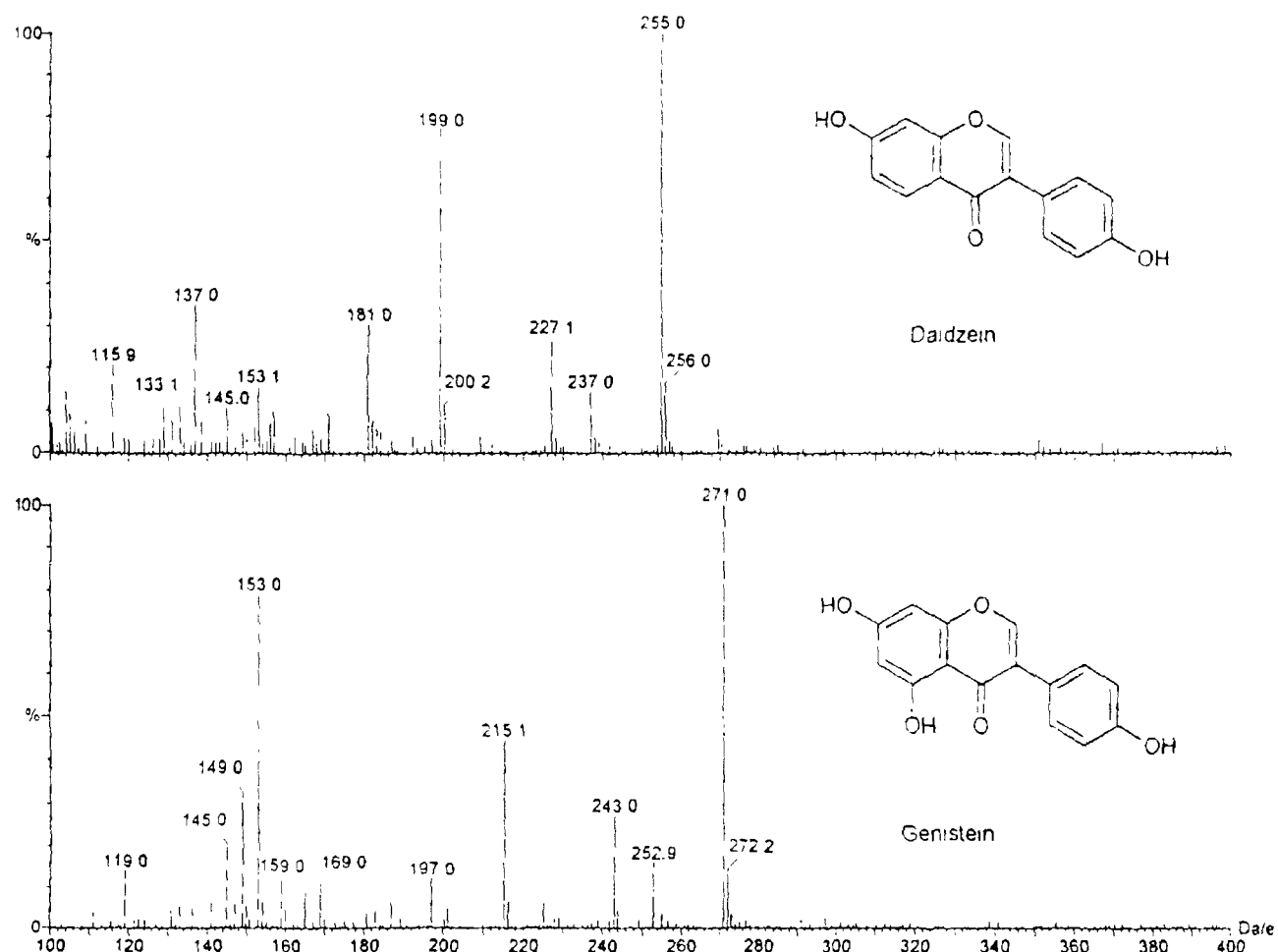


FIG. 2. On-line LC-APCI/MS analysis of genistein and daidzein from a soybean extract. Mass spectra were obtained following on-line LC separation of genistein ($M + H^+ = 271$ Da, bottom panel) and daidzein ($M + H^+ = 255$ Da, top panel) in the soybean extract as described in Materials and Methods. The spectra were obtained using a sampling cone-skimmer potential of 50 V.

These values are consistent with the total isoflavone content in soybean reported previously [9, 20].

The soybean extract was analyzed further by on-line APCI/MS under conditions that produced mass spectra containing protonated molecules ($M + H^+$) and fragment ions (see Fig. 2). Mass spectra from peaks D and G contained protonated molecules corresponding to the masses predicted for daidzein and genistein (mol wt. = 254 and 270, respectively). Diagnostic fragment ions were also observed. Not only were the observed protonated molecules and fragment ions identical to those produced from authentic standards (not shown), but they were also very similar to the CID spectra previously reported for genistein and daidzein using electrospray ionization with tandem mass spectrometry [21, 22]. Since the chromatographic, spectroscopic, and TPO inhibitory properties were found to be identical with those exhibited by authentic isoflavones, subsequent mechanistic studies were carried out with pure isoflavones.

Inhibition of TPO-Catalyzed Iodination and Coupling by Isoflavones

Genistein and daidzein were found to inhibit TPO-catalyzed iodination of tyrosine. The IC_{50} values for these reactions were estimated from concentration inhibition curves (not shown) to be 3.2 and 7.6 μ M, respectively. These values were similar to those reported previously for related flavonoids [14]. The glycoside genistin was approximately 10-fold less potent than the aglycone with an IC_{50} value of 38 μ M, and HPLC-UV analysis showed the commercial product to be devoid of the aglycone ($<0.1\%$). A 25- μ L aliquot of the crude extract (equivalent to 500 μ g of soybean powder) produced 50% inhibition of TPO-catalyzed tyrosine iodination activity (data not shown). It was possible to compare the inhibition of TPO activity by the crude extract with that predicted from the measured isoflavone content. The extract aliquot contained 0.99 μ g genistein and 0.37 μ g daidzein, and these amounts are predicted to produce approximately 67% inhibition of

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TABLE 1. Inhibition of TPO-catalyzed coupling in iodinated casein by genistein

Genistein (μM)	T_4 Residues (% control, average, $N = 2$)
0	100
1	93.8
2	57.0
4	32.7
8	23.8
20	26.3

TPO ($2.5 \mu\text{M}$) was incubated with chemically iodinated casein ($0.25 \mu\text{g/mL}$), alkaline phosphatase (various concentrations), H_2O_2 ($1.0 \mu\text{M}$) for 1 hr at $37 \pm 0.1^\circ\text{C}$. Duplicate 1-ml reaction mixtures were digested with proteases and the thyroid hormones were extracted and then quantified using HPLC as described in Miller, S. and Mieskes. The content of iodothyronines present in casein was below the detection limit ($0.026 \mu\text{g/mL}$ casein or 16 ng), and the amount of T_4 formed in the control incubations was $1.22 \mu\text{g/mg}$ casein (0.85 newly formed residues per molecule).

TPO-catalyzed iodination in the 10-mL incubation. This compares closely with the 50% inhibition of TPO-catalyzed tyrosine iodination observed from addition of the soybean extract. These results also provide evidence that no major additional anti-thyroid compound is present in the soybean extract.

Genistein was also tested for the ability to inhibit iodothyronine formation by using iodinated casein, a model substrate that permits measurement of TPO-catalyzed coupling [16]. In this reaction, casein-bound iodotyrosyl residues are oxidatively converted by TPO in the presence of H_2O_2 to protein-bound T_4 and rT_3 . The formation of prominent amounts of rT_3 , but not T_4 , is not observed *in vivo* or in model systems that use human goiter Tg as a coupling substrate [18, 23]. However, in all experiments, rT_3 formation mirrored T_4 formation, and inhibition by anti-thyroid chemicals was similar for both products. Bovine milk casein is a complex multimeric aggregate ($>300 \text{ kDa}$) consisting of subunits with an average monomer molecular mass of 23.3 kDa [24] and contains 3.3 tyrosyl residues/mol [25]. Before treatment with TPO, the amount of total iodothyronines detected by HPLC in hydrolyzed iodocasein was below the detection limit ($\text{T}_4 = 0.026 \mu\text{g/mg}$ casein or 16 ng on-column). In the absence of an inhibitor, TPO catalyzed the formation of approximately 1

$\mu\text{g T}_4/\text{mg}$ casein (approximately 0.85 residues or T_4 , 0.35 residues of rT_3 , and/or 0.17 residues of T_3 per mol of aggregated iodocasein, assuming a molecular mass of 312 kDa , see Ref. [24]) under the conditions described. Because the small amounts of T_3 observed were constant and unaffected by inhibitor concentration, we concluded that this resulted from artifactual deiodination of T_4 during sample preparation. Table 1 shows the concentration-dependent inhibition of T_4 synthesis in iodinated casein by genistein for duplicate experiments. Inhibition of rT_3 formation showed similar results (not shown). Using the combined data set for the two separate experiments, the IC_{50} value for genistein was approximately $3 \mu\text{M}$.

Additional experiments were performed using human goiter Tg to measure the formation of thyroid hormones and its inhibition by isoflavones in a simultaneous iodination/coupling procedure [18]. When using Tg as the substrate, T_4 was the predominant product (1.06 ± 0.03 newly formed residue/molecule Tg), and only trace amounts of T_3 and rT_3 were observed. These studies confirmed the inhibition of coupling alone by genistein described above for iodinated casein. Genistein, daidzein, and genistin inhibited the formation of T_4 in a concentration-dependent manner (see Table 2), and the IC_{50} values for genistein, daidzein, and genistin were approximately 2.0 , 8.8 , and $40.6 \mu\text{M}$, respectively. Genistein and daidzein also inhibited TPO-catalyzed oxidation of guaiacol. The IC_{50} values for these reactions were 0.7 and $12.4 \mu\text{M}$, respectively.

Characterization of Iodinated Isoflavones

Genistein and daidzein were potent inhibitors of TPO-catalyzed tyrosine iodination as described above. Genistein inhibition of TPO-catalyzed tyrosine iodination produced kinetics consistent with alternate substrate inhibition previously described for biochanin A [14]. In this mechanism (see Scheme 1), two-electron oxidation of iodide by TPO compound I produces an iodinating intermediate that is the equivalent of enzyme-bound hypoiodite [26]. Iodination of tyrosine by the enzymatic iodinating intermediate is blocked by isoflavones because of preferential iodination of

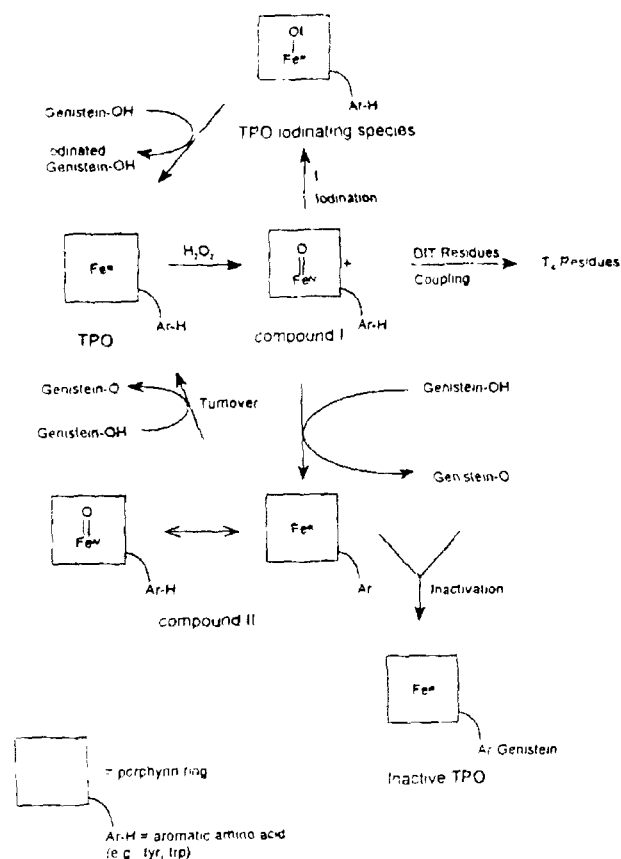
TABLE 2. Isoflavone inhibition of TPO-catalyzed coupling in human goiter Tg

Genistein (μM)	T_4 Residues (% control)	Daidzein (μM)	T_4 Residues (% control)	Genistin (μM)	T_4 Residues (% control)
0	100	0	100	0	100
1	52.4 ± 7.6	2.5	76.9 ± 6.5	10	98.5 ± 12.0
2.5	44.0 ± 8.0	5	58.5 ± 2.0	25	70.6 ± 1.8
5	19.8 ± 5.1	10	30.5 ± 4.8	50	46.9 ± 0.8
10	12.2 ± 2.4	20	12.2 ± 4.7	100	11.0 ± 0.7

TPO ($2.4 \mu\text{M}$) was incubated with KI ($0.02 \mu\text{M}$), 1.5 mM glucose, 5 mM Tris-glucose, and $5 \mu\text{M}$ Tg in various concentrations. The reactions for 1 hr at $37 \pm 0.1^\circ\text{C}$. Trisphosphate reaction mixtures were 0.2 M sodium phosphate, and the thyroid hormones were extracted and then quantified using HPLC as described in Miller, S. and Mieskes. The content of T_4 in thyroid tissue present in the Tg was below the detection limit (0.02 ng/mL casein or 1.6 ng) and the amount of T_4 formed in the control incubations was 1.06 ± 0.03 newly formed residue/molecule Tg.

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SCHEME 1. Proposed mechanisms for genistein inhibition of TPO-catalyzed reactions.

the flavonoid, presumably due to its greater reactivity with the electrophilic enzyme species.

Figure 3 shows the conversion of genistein to three products under such conditions. Analysis of reaction products using on-line LC-APCI/MS gave mass spectra consistent with derivatives containing either one, two, or three iodine atoms. The positive ion mass spectra are characterized by abundant protonated molecules (MH^+) as observed for the parent isoflavone (see Fig. 4). In addition, the iodination products show fragment ions corresponding to losses of successive iodine atoms ($-I^- + H^+$, $\Delta m = 126$ Da) including the parent isoflavone. For example, the triiodo-genistein spectrum consists of MH^+ (m/z 649) and fragment ions corresponding to the protonated diiodo (m/z 523) and moniodo derivative (m/z 397) as well as genistein (m/z 271). Similar results were obtained with daidzein where mass spectra contained MH^+ of m/z 381, 507, and 633, consistent with formation of mono-, di-, and triiodo-daidzein derivatives by TPO-mediated iodination (not shown). These results are similar to those obtained from LC-APCI/MS analysis of TPO-catalyzed iodination of biochanin A to mono- and diiodinated derivatives. In that study 1H -NMR was also used to determine that the resorcinol moiety was the site for mono- and diiodination [14]. By analogy, we propose that genistein is mono- and

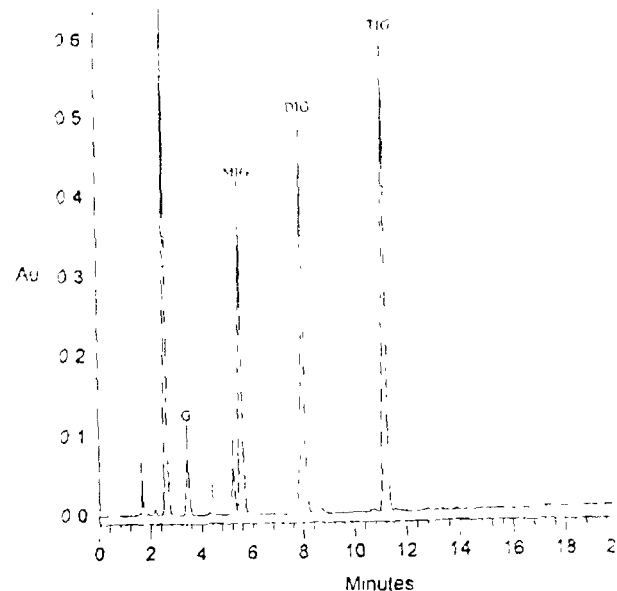


FIG. 3. TPO-catalyzed iodination of genistein. Genistein (2 μM) was incubated with TPO (50 nM) and iodide (100 μM) as described in Materials and Methods. The reaction was initiated by the addition of H_2O_2 (250 μM) and after 5 min the reaction mixture was analyzed using HPLC with UV 275 nm detector. Abbreviations: G, genistein; MIG, moniodo-genistein; DIC, diiodo-genistein; and TIG, triiodo-genistein.

diiodinated in the resorcinol ring (C6,8), and the third site for iodination is presumably the ortho position on the phenol ring (C3').

Inactivation of TPO by Isoflavones

In the presence of H_2O_2 , genistein and daidzein inactivated TPO by 60–70% (see Table 3). However, the glucoside genistin produced no loss of enzymatic activity under these conditions (data not shown). The inactivation was time dependent, increased with increasing concentrations of isoflavone or H_2O_2 , and was enhanced when the incubation was conducted under anaerobic conditions. The inactivation was concomitant with a 60–80% loss of Soret absorbance and a red shift of the absorbance maximum from 412 nm (native TPO) to 415 nm (genistein-inactivated TPO) or 419 nm (daidzein-inactivated TPO) (Fig. 5). The presence of at least 150 μM iodide in the incubation mixtures completely protected against genistein- or daidzein-mediated TPO inactivation (see Table 3).

DISCUSSION

The results presented here demonstrate that the aglycones genistein and daidzein are the compounds contained in a hydrolyzed extract of soybean that inhibit TPO-catalyzed reactions. The hydrolytic procedure was required to convert the predominately conjugated isoflavones present in soybean to aglycones as previously described [19, 22]. The

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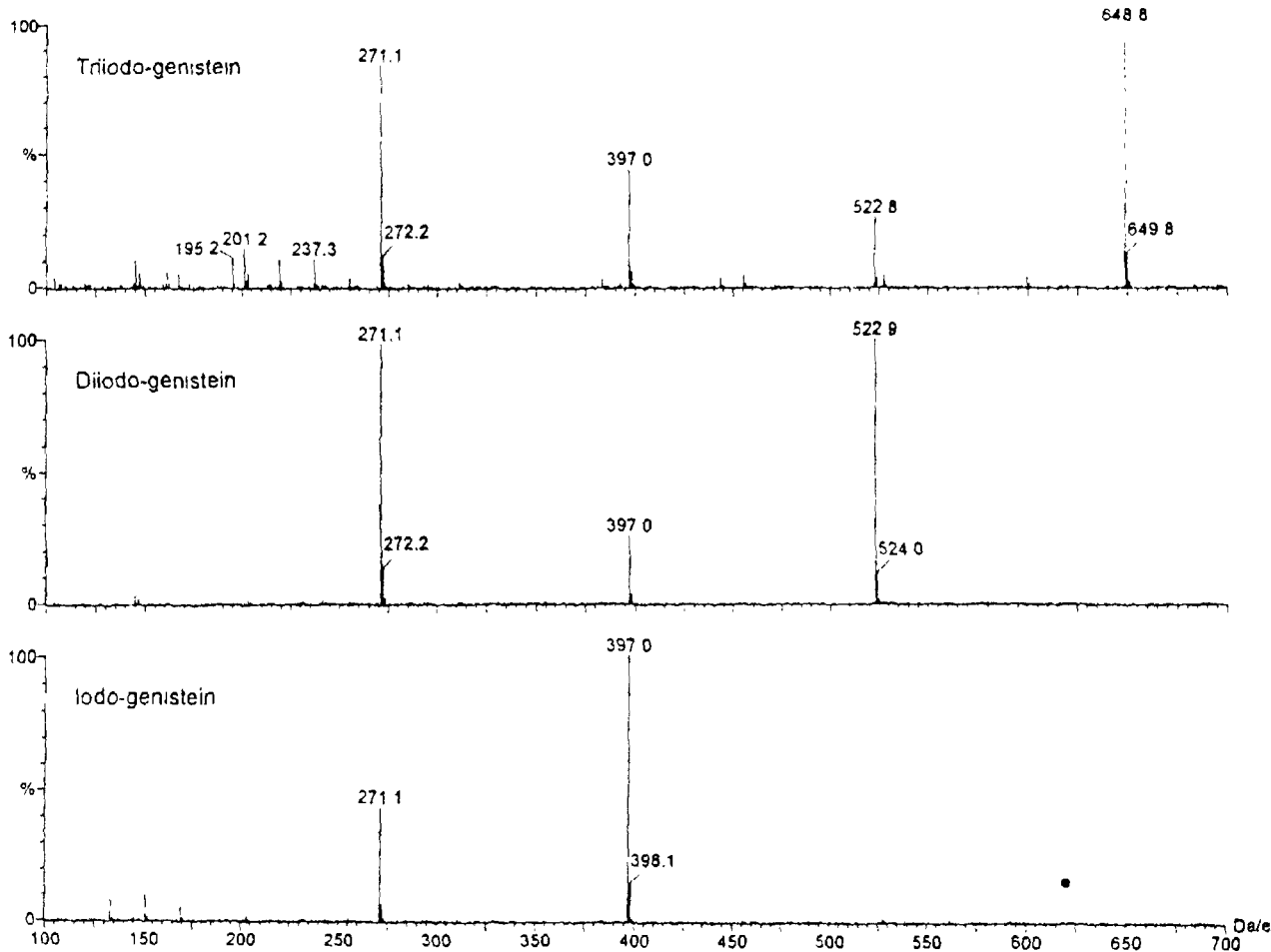


FIG. 4. Mass spectra of iodinated isoflavones. Incubations of TPO with genistein were conducted as described in Fig. 4 except LC-APCI/MS with a sampling cone-skimmer potential of 15 V was used to generate mass spectra of the genistein-derived products.

extract was fractionated by a reversed-phase HPLC separation (see Fig. 1), and the peaks containing inhibitory activity had chromatographic and spectroscopic properties (UV, APCI/MS) identical to authentic genistein and

TABLE 3. H₂O₂-dependent inactivation of TPO by isoflavones

Reaction conditions	Activity (% of control)
TPO	99 ± 3
TPO + H ₂ O ₂	96 ± 7
TPO + daidzein	97 ± 6
TPO + genistein	95 ± 5
TPO + daidzein + H ₂ O ₂	38 ± 9
TPO + genistein + H ₂ O ₂	29 ± 7
TPO + I ⁻	97 ± 5
TPO + daidzein + I ⁻ + H ₂ O ₂	94 ± 9
TPO + genistein + I ⁻ + H ₂ O ₂	95 ± 9

TPO (1.2 μM) was incubated with 50 μM I⁻ or 50 μM genistein, 150 μM H₂O₂, and 1.25 μM H₂O₂ in the combination indicated at 25 ± 0.1°C in 2.1 M MES buffer (pH 7.2). After 4 min, 1 μl of the reaction mixture was diluted 1000-fold into 20% MeOH and the absorbance at 410 nm was measured. The values are means ± SD (n = 3). Under these conditions, maximal TPO activity was 0.040 μM/min.

daidzein (see Fig. 2). All of the TPO inhibitory activity present in the extract was accounted for by the measured amounts of genistein and daidzein. Determining total isoflavones as the respective aglycone after hydrolysis can only give the maximum possible anti-thyroid potential of soy products because glucoside conjugates, which are the predominant forms in soybean, are weakly inhibitory. The mixture of glucoside conjugates and aglycones present in soybean have been shown to be bioavailable through identification of glucuronide and sulfate conjugates of isoflavones in plasma from humans consuming soy products [22]. This suggests that the conjugates are hydrolyzed during absorption from the gut as the aglycone. However, this does not give information about uptake into the thyroid, another critical factor for assessing goitrogenic potential that must be determined in future studies *in vivo*.

Genistein also inhibited TPO-catalyzed phenolic oxidations including guaiacol oxidation and coupling of diiodotyrosyl residues in casein and thyroglobulin to form iodothyronines (see Tables 1 and 2). These reactions proceed by phenoxyl radical intermediates, and the pres-

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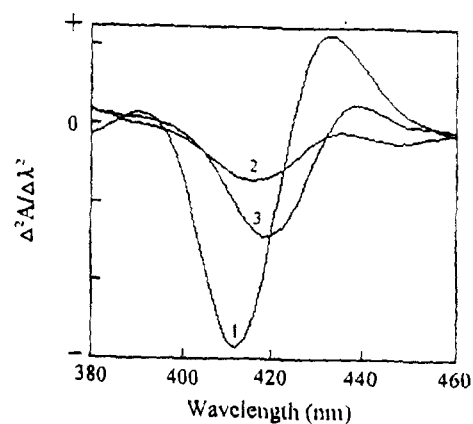


FIG. 5. Soret spectra for TPO and isoflavone-inactivated TPO. TPO (1 μ M) was incubated with genistein or daidzein (50 μ M) and H_2O_2 (200 μ M) as described in Materials and Methods. After a 4-min incubation, second-derivative visible spectra were recorded. The spectra are shown for native TPO (1), genistein-inactivated TPO (2), and daidzein-inactivated TPO (3).

ence of phenolic groups in the isoflavones would be predicted to react with oxidized enzyme species, Tg radical intermediates, or both, to block coupling [14, 18, 27]. The IC_{50} for inhibition of coupling in a simultaneous iodination/coupling assay using Tg was also similar to that observed in a coupling-only assay system using previously iodinated casein as the substrate. Because of the ability of the thyroid both to produce Tg and to concentrate iodide, it is likely that coupling *in vivo* occurs in a manner more comparable to the simultaneous assay. Furthermore, the IC_{50} values obtained for isoflavones were similar for inhibition of iodination and coupling. This suggests a primary interaction between isoflavone and TPO that affects coupling and iodination activity in a similar manner. Furthermore, the levels of total isoflavones observed in human plasma following consumption of soy foods (ca. 1 μ M, see Ref. 22) approach the concentrations required for inhibition of TPO-catalyzed reactions.

In the absence of iodide, genistein and daidzein caused time-dependent, irreversible inactivation of TPO concomitant to distinct changes in the visible spectrum of the heme prosthetic group (see Fig. 5). These observations are similar to those made for inactivation of TPO by other flavonoids (naringenin, quercetin, morin, and kaempferol; see Ref. 14) although the spectra of inactivated TPO have different long wavelength absorption maxima (428 nm for naringenin or kaempferol; 419 nm for daidzein; 415 nm for genistein). These data are consistent with the suicide inactivation mechanism for resorcinol and related flavonoids previously proposed for TPO, LPO, and CcP [14, 28]. Despite the dramatic changes in the heme visible spectrum, the results to date are consistent with enzyme inactivation through covalent binding to the polypeptide chain and not to the heme prosthetic group (see Scheme 1 and Ref. 29).

The inhibition of TPO-catalyzed iodination and coupling *in vitro* is consistent with the numerous reports of anti-thyroid effects in humans and animals from consumption of soy products, especially in cases of iodine deficiency. Many issues regarding the bioavailability of soy isoflavone conjugates and uptake into the thyroid remain unanswered. However, the demonstrated effects of the aglycones presented here, and the well-documented goitrogenic effects of soybeans in humans and animals, do provide a logical starting point from which possible anti-thyroid mechanisms can be examined. The different mechanisms reported for inhibition *in vitro* of the enzymatic reactions in thyroid hormone biosynthesis by isoflavones are useful for predicting potential anti-thyroid effects in animals and humans under several different dosing circumstances:

(A) In the normal case of iodine-sufficient individuals receiving intermittent or low doses of soy isoflavones, alternate substrate iodination would consume the inhibitory compounds after which Tg iodination and coupling reactions would resume unaffected. Since the normal thyroid contains significant amounts of iodide, its high substrate activity should prevent inactivation of TPO.

(B) In the case of iodine deficiency, low or intermittent doses of isoflavones could further deplete iodide levels by covalent incorporation of iodide into iodinated products. Also, enzymatic oxidation of the isoflavone would increase as the intrathyroidal iodide level decreased. Under these conditions, it is possible that inactivation of TPO could occur. This would produce a more long-lasting inhibition of hormone synthesis because enzymatic activity could be replaced only through *de novo* protein synthesis. However, either the alternate substrate inhibition or enzyme inactivation outcome is consistent with the anti-thyroid effects from soy observed in rodents maintained on an iodide-free diet [7, 9] and with the ability of added iodide to reverse the goitrogenic effect of a soybean diet in rats [7]. There are also reports of goiter and hypothyroidism in human infants receiving soy-based formulas [1–4] and evidence for elimination of such effects upon addition of iodide to the diet [3]. For this reason, it appears that iodide supplementation of formulas during manufacturing was implemented [1].

(C) In proposed rodent carcinogenicity bioassays, high doses of isoflavone will be administered chronically in a normal iodide-containing diet. Under these conditions, complete blockade of iodination and coupling is possible even with normal dietary iodide because alternate substrate inhibition would dominate. Since the body burden of isoflavone is replenished continually through feeding, the inhibition of thyroid hormone synthesis would persist throughout the lifetime of the animal. This hypothesis is consistent with observations of the hypothyroid condition that occurs in humans consuming principal foodstuffs (e.g. millet) that contain large amounts of anti-thyroid flavonoids [30, 31]. It is possible that these anti-thyroid effects could persist even if normal levels of iodide were present in the diet through universal iodination programs.

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Under either condition B or C, the inhibition of thyroid hormone synthesis would increase TSH levels and could eventually induce thyroid follicular hyperplasia and tumors [12, 32]. Tumor formation through this nongenotoxic, hormonal process results from the growth stimulus provided by TSH, which can provide the selective environment for a transformed phenotype [12]. The observation of metastatic thyroid tumors in iodine-deficient rats, but not in iodide-supplemented rats, receiving a defatted soybean diet [9] is consistent with this proposal. The role of TSH in mediating thyroid tumors is well-documented in rats, but the importance in humans is unclear [32].

Finally, other possible toxicological consequences from ingestion of soy isoflavones come from the demonstrated estrogen receptor binding activity of genistein and daidzein [33, 34]. Although anti-carcinogenic properties from soybean isoflavones have been suggested [13, 35], the dose-response relationships that may separate toxic and beneficial responses are not clear. A possible consequence of TPO-mediated isoflavone iodination is modification of such estrogenic receptor binding activity or changing the pharmacokinetics for elimination. For example, McCague *et al.* [36] reported increased estrogen receptor binding affinity as well as a decrease in metabolism for 4-iodo-tamoxifen relative to the parent compound. Further experimentation will be required to assess the importance of TPO-mediated iodination in the biological activity and excretion of isoflavones.

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Translation of:

The Effects on the Thyroid Gland of Soybeans Administered
Experimentally in Healthy Subjects

Y. Ishizuki, Y. Hirooka, Y. Murata, K. Togashi

(Nippon Naibunpi gakkai Zasshi, 67, 622-629, 1991)

Japan Communication Service
PO Box 3857
Wellington
Tel: +64 4 232 8434
Fax: +64 4 232 4308
Mobile: 021 631 051
E-mail: 100246.2672@Compuserve.com.

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The Effects on the Thyroid Gland of Soybeans Administered
Experimentally in Healthy Subjects

Y. Ishizuki, Y. Hirooka, Y. Murata, K. Togashi

(Nippon Naibunpi gakkai Zasshi, 67, 622-629, 1991)

Introduction

It is said that soy beans contain a goitrogenic substance ¹⁾ and that the administration of soy beans to experimental rats, even for a short period, lowers serum T_4 levels ²⁾ and suppresses the ^{131}I intake rate ³⁾. In the case of humans, there have been several cases in which the onset of goitres and hypothyroidism in infants fed with soy milk were reported ^{3) - 8)}. These writers reported that soy beans had slightly suppressed the thyroid function in chronic thyroiditis, and that they affected the thyroid gland in adults. ⁹⁾ However, there has been no systematic study on whether soy beans in the normal diet suppress the thyroid function or not.

In this study, soybeans were administered to normal healthy adults, and it was investigated whether they affected thyroid function in adults or not. The study also examined whether the effects of diet on the thyroid function can be ignored or not, when reading the values of various parameters.

Subjects and methods

The subjects were selected from healthy working adults who had never had thyroid disorders or goitres, had no serum antithyroid antibodies, and were not on medications which influenced TBG fluctuation. Eight males and forty six females, 54 in total, aged between 22 and 76, were divided into 5 groups. Group 1 was the short duration group. Seven males and thirteen females, 20 in total, aged between 22 and 60 were given soy beans for 1 month. The long duration (3 months) group was divided into 2 groups (Groups 2 and 3) by age. Group 2 comprised younger females aged between 22 and 39 (average 29), and Group 3 comprised an older male (1) and females (9) aged between 46 and 76 (average 61). Control groups comprising the same age distribution, average age and number of subjects as Groups 2 & 3 were selected as Groups 4 & 5. Hence, Group 4 comprised 7 subjects, and Group 5 comprised 10 subjects.

Vinegared soy beans were prepared by pickling roasted soy beans (Produce of Takayama) in rice vinegar. 30 g of this preserve was administered orally every day, twice a day. Soy bean curd, miso (soy bean paste), and seaweed were given as usual without any restriction.



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The administration of was continued for a year (sic.) from August 1989, the subjects who neglected daily intake of soy beans were excluded.

Examinations were carried out before administration, on the last day of administration, and on a morning more than 3 months after the cessation of soy beans. Examinations comprised an interview, palpation of the thyroid gland, and blood tests. Serum was stored at minus 80 deg. C, and the serums before and after the administration of soy beans were measured simultaneously. Those in whom goitre was detected were examined by ultrasonic scan. All symptoms during the period were recorded. Only sustained symptoms were selected, and the symptoms which were assumed to be caused by other causes were excluded. The symptoms which disappeared after the cessation of the administration of soy beans were considered to be the result of the administration of soy beans, and so recorded. 4 subjects missed the pre-administration examination. 2 subjects missed the post-administration examination.

Serum T₄, T₃, FT₃, gammaT₃, and TBG were measured by RIA, and TSH was measured by the high sensitivity method. NEFA was measured by the enzyme method. CPK, LDH, GOT, and GPT were measured by the UV method. The TRH test used a 500 micro g venous injection method. The TSH value after 30 minutes was treated as TSH, and T₃ was measured at the same time. Serum total iodine was measured by the alkali incineration method. The inorganic iodide value was calculated by subtracting the iodine value in the T₄ value measured by RIA from total iodine. The t test, the Mann-Whitney method, and the order addition test using Wilcoxon, the X² test, and the direct probability method were employed in the statistical analyses.

Results

1) Thyroid function of the short duration group

Serum T₃ values, and T₄ values of Group 1 showed a tendency to drop after soy bean intake over one month, but the drop was not significant. FT₄, FT₃, gammaT₃, TBG, the FT₄/T₄ ratio, and the FT₃/T₃ ratio showed no significant changes

Inorganic iodide values showed no difference before and after the treatment, but TSH levels significantly increased after soy bean intake. (P(U) < 0.01 Table 1)

2) Thyroid function of the long duration group

Serum T₄, T₃, FT₄, FT₃, Inorganic iodide, the FT₄/T₄ ratio, and the FT₃/T₃ ratio in Group 2 of the long duration (3 months) group showed no significant changes, while TSH levels clearly increased (P(U) < 0.01), though the rise was slight. Serum T₄, T₃, FT₄, FT₃, Inorganic iodide, the FT₄/T₄ ratio, and the FT₃/T₃ ratio in Group 3 showed no significant changes before and after soy bean intake, while TSH levels clearly increased (P < 0.05) although they stayed within the normal range. Inorganic iodide levels in the older group were higher than those in the younger group, but the inorganic iodide values when

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In the case of chronic thyroiditis where the reserve tends to decrease, the intake of soy beans for more than 5 months clearly induced a drop in serum thyroid hormones, and a rise in TSH ⁹⁾. However, the subjects in this test were healthy adults with an ample reserve. The duration of the test was as short as within three months. These factors were considered to have contributed to the result that the level of hormone reserve did not drop as much as to lower the concentration of serum thyroid hormones. In healthy adults, serum T₄ and T₃ correlated well, gamma T₃ and TBG did not fluctuate, and the thyroid hormone levels did not drop. These indicated that the effect of soy beans on the suppression of thyroid hormone synthesis and the slowing down of Tg hydrolysis in the follicles was weak/mild, and a longer duration of administration was required to cause hypothyroidism severe enough to be clinically noticed. In a report where soy beans were administered for 5 days ⁸⁾, 2 subjects out of 14 showed a drop in PBI ¹³⁾ despite the PBI remaining constant (sic.). The difference between the two indices was considered to be due to the loss of thyroid hormones in the faeces. There is an animal experiment ²⁾ where the addition of iodine to soy bean diet induced the disappearance of goitre which had occurred on a soy bean diet with iodine restriction. However, in the case of humans, goitre appeared with the normal intake of iodine, and a slight suppression of the thyroid function was detected. It was necessary to examine whether the drop in thyroid hormones and the rise in TSH in the elderly were caused by iodine or not. (Literal - Translator) The fact that the inorganic iodide levels in the older group were higher than those in the younger group indicated that the elderly had a higher intake of iodine than the young, and that soy beans did not inhibit the absorption of iodine. The higher intake of iodine in the older group was reflected on the high TSH levels before the administration of soy beans. Although the inorganic iodide levels after the administration of soy beans decreased compared with those before the administration, TSH increased, and Δ TSH was higher than that of the Control because of the intake of soy beans. However, the inorganic iodide levels did not correlate with TSH. This would indicate that the sporadic intake of a large amount of iodine in normal daily life is unlikely to affect the TSH fluctuation. There is a report in which older women showed a greater drop in serum T₃ and a greater rise in Δ TSH than young women, and it was concluded that this was due to lack of responsiveness of the thyroid gland caused by ageing. ¹⁰⁾ In the case of chronic thyroiditis, the majority ⁹⁾ of the subjects aged in a wide range showed a high Δ TSH due to the intake of soy beans, despite their inorganic iodine levels being in the normal range. The recovery of the thyroid function observed some period after the cessation of soy bean intake was not considered to be purely due to the age factor. Chronic thyroiditis shows a high sensitivity to iodine, and a high sensitivity to goitrogen in soy beans is also a possibility. However, the result of this experiment on healthy subjects indicates that soy beans may influence on the thyrotropic hormone from the pituitary gland, rather than the suppression of (thyroid) functions due to iodine intake. It also indicates that the elderly are more susceptible than the young. Although there is a report ¹¹⁾ which states soy beans encourage the elimination of thyroid hormones into faeces, thereby lowering the function, this is debatable as both peripheral thyroid hormone and TSH levels fluctuated within the normal range.

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Hypothyroidism was observed in half the subjects, and a rise in some enzymes in the blood were observed in both the elderly and the young, and only during the intake of soy beans. Therefore, it is hard to conclude that they were induced by muscular exertion. (Literal) Rather, it would indicate that the peripheral function of thyroid hormones was slightly and mildly suppressed by soy beans.

The normal range for hormone levels is wide. A value in the normal range of readings needs to be interpreted by taking clinical symptoms and the difference in sensitivity by age into account. In addition, the effects of dietary constituents such as soy beans and seaweed should be taken into consideration in order to give an accurate diagnosis of the thyroid function.

Summary:

The changes in the thyroid function were studied by administering 30 g of vinegared soybeans per day to 37 healthy subjects.

20 subjects were given soy beans for 1 month, and no change was detected in the peripheral thyroid hormone levels, while TSH was slightly raised ($P(U) < 0.01$). 17 subjects were administered soy beans for 3 months, and no change in the thyroid hormone levels was detected, but a clearly raised level of TSH was observed after the intake of soy beans, regardless of the age factor. ($P(U) < 0.01$ for the older group, ($P(U) < 0.01$ for the younger group). The TSH level in the older group (average age 61) rose significantly after the administration of TRH, compared with those in the Control group and in the younger group (average age 29). ($P(U) < 0.01$, $P(U) < 0.03$). In the older group, FT_4 and FT_3 levels rose, and Δ TSH and TSH levels dropped to those in the Control after the cessation of soy bean intake. No correlation was detected between serum inorganic iodide and TSH.

Diffuse goitre and hypothyroidism appeared in half the subjects after taking soybeans for 3 months, but they reduced and disappeared after the cessation of soy bean intake.

Serum CPK, NEFA, GOT, and GPT were unchanged by the administration of soy beans, while LDH alone rose. The LDH rise was more marked in the older group, but LDH levels dropped in all subjects after the cessation of soy bean intake.

The above results indicated that excessive intake of soy beans for a long duration might cause the enlargement of the thyroid gland, and slightly suppress thyroid function in healthy people.

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健康者甲状腺機能への大豆の効果 —大豆投与実験—

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石突甲状腺研究所 (株)

石 突 吉 持

愛知医科大学第四内科

広 岡 良 文

名古屋大学環境医学研究所

村 田 善 晴

SMI ブリストル (株) 北里バイオケミカルラボラトリー

富 樫 和 美

The Effects on the Thyroid Gland of Soybeans Administered Experimentally in Healthy Subjects

Ishizuki Thyroid Clinic

Yoshimochi ISHIZUKI

The Fourth Department of Internal Medicine, Aichi Medical University

Yoshifumi HIROOKA

The Research Institute of Environmental Medicine, Nagoya University

Yoshiharu MURATA

SMI - Bristol, Kitazato Biochemical Laboratories

Kazuyoshi TOGASHI

To elucidate whether soybeans would suppress the thyroid function in healthy adults, we selected 37 subjects who had never had goiters or serum antithyroid antibodies. They were given 30g of soybeans everyday and were divided into 3 groups subject to age and duration of soybean administration.

In group 1, 20 subjects were given soybeans for 1 month. Groups 2 and 3 were composed of 7 younger subjects (mean 29 y.o.) and 10 elder subjects (mean 61 y.o.) respectively, and the subjects belonging to these groups received soybeans for 3 months. The Wilcoxon-test and t-test were used in the statistical analyses. In all groups, the various parameters of serum thyroid hormones remained unchanged by taking soybeans, however TSH levels rose significantly although they stayed within normal ranges. The TSH response after TRH stimulation in group 3 revealed a more significant increase than that in

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Breast and Soy-Formula Feedings in Early Infancy and the Prevalence of Autoimmune Thyroid Disease in Children

P. Fort, MD, N. Moses, MNS, RD, M. Fasano, MS, RD, Terasia Goldberg, MS, RD, and F. Lifshitz, MD

Department of Pediatrics, North Shore University Hospital-Cornell University Medical College, Manhasset, New York

Key words: autoimmune thyroid disease, breast feedings, soy-containing formula

It has been suggested that feeding practices in infancy may affect the development of various autoimmune diseases later in life. Since thyroid alterations are among the most frequently encountered autoimmune conditions in children, we studied whether breast and soy-containing formula feedings in early life were associated with the subsequent development of autoimmune thyroid disease. A detailed history of feeding practices was obtained in 59 children with autoimmune thyroid disease, their 76 healthy siblings, and 54 healthy nonrelated control children. There was no difference in the frequency and duration of breast feeding in early life among the three groups of children. However, the frequency of feedings with soy-based milk formulas in early life was significantly higher in children with autoimmune thyroid disease (prevalence 31%) as compared with their siblings (prevalence 12%; $\chi^2 = 7.22$ with continuity factor; $p < 0.01$), and healthy nonrelated control children (prevalence 13%; $\chi^2 = 5.03$ with continuity factor; $p < 0.02$). Therefore, this retrospective analysis documents the association of soy formula feedings in infancy and autoimmune thyroid disease.

INTRODUCTION

It has been suggested that feeding practices in early infancy may affect the development of various autoimmune disorders later in life. For example, a link between the prevalence of insulin-dependent diabetes mellitus (IDDM) in children and breast feeding during infancy was suggested [1,2]. Although we were unable to confirm such an association in our population of children with IDDM, we did notice a higher incidence of antithyroid microsomal antibodies in children with IDDM who were formula-fed as compared to those who were breast-fed as infants [3]. In addition, IDDM children who were not breast-fed were more likely to receive soy-containing formulas in early infancy than nondiabetic control children [3].

Since thyroid alterations are among the most frequently encountered autoimmune conditions in children [4], we studied whether feeding practices in early life were associated with the subsequent development of autoimmune thyroid disease (ATD). Specifically, we attempted to assess the prevalence of breast feedings which may have a protective role in the development of

ATD later in life, and the type of milk formula feedings in early infancy which could be associated with a higher incidence of ATD later in life.

MATERIALS AND METHODS

The subjects of the study were 59 children, 40 females, and 19 males, with ATD who were being followed by the Division of Pediatric Endocrinology, Metabolism, and Nutrition at North Shore University Hospital-Cornell University Medical College, Manhasset, New York. Fifty-two children had autoimmune thyroiditis (Hashimoto's thyroiditis), with or without hypothyroidism, and seven had Graves' disease. The mean age of patients (\pm SD) at the time of evaluation was 14.7 ± 6.4 years. The mean age (\pm SD) at which the diagnosis of ATD was established was 9.6 ± 4.6 years. In all patients the diagnosis of ATD was made by the presence of goiter detected on physical examination and confirmed by laboratory assessment, which included the measurement of serum T₄, T₃RIA, TSH, antithyroid antibodies, and thyroid stimulating immunoglobulins, as

Address reprint requests to Pavel Fort, M.D., Department of Pediatrics, North Shore University Hospital, 300 Community Drive, Manhasset, New York 11030.

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other control children are shown in Table 1. Both the prevalence and duration of breast feeding among patients with ATD were similar to that seen in their siblings and in the healthy control children. Also, there was no difference between the age at which solid food was introduced into the diet among the three study groups (Table 1).

The mean age at which ATD was diagnosed was 10.0 ± 4.7 years in the breast-fed group as compared with 7.9 ± 4.0 years in the non-breast-fed group of patients, however, this difference was not statistically significant. Also, there were no significant differences in the initial values of serum T_4 , TSH, and titre of antithyroid antibodies (antithyroid globulin and microsomal) between those breast-fed and those not breast-fed.

The majority of children with ATD were fed cow's milk formula during infancy. However, 18 out of 59 children (31%) received soy-containing formula (Table 2). The prevalence of soy-containing formula feedings was significantly higher among patients with ATD than that found in their healthy siblings (prevalence 12%, $\chi^2 = 7.22$ with continuity factor; $p < 0.01$), and than that found in healthy nonrelated control children (prevalence 13%, $\chi^2 = 5.03$ with continuity factor; $p < 0.02$). There were no differences in the prevalence of soy-containing formula feedings between the siblings of patients with ATD and healthy nonrelated control children.

DISCUSSION

Our data show that the prevalence and duration of breast feedings during infancy in our population of children with ATD were the same as those found in their siblings who did not have any thyroid ailment, and were also similar to those of healthy age-matched children. Thus, in our studies we were unable to document any relationship between the history and duration of breast feeding and subsequent development of ATD. These findings conform with our previously reported observation that the incidence of other autoimmune conditions in children, such as IDDM, is not associated with breast feeding in infancy [3].

However, the so-called protective effect of human milk on the subsequent development of an autoimmune process, such as IDDM [1,2], may not be related to the human milk per se but rather to type of feeding given during the critical period of maturation of the immune system in early life. We observed a significantly higher prevalence of feedings with soy-containing formulas in early infancy in patients with ATD as compared with their healthy siblings and healthy nonrelated control

children. In fact, a child with ATD was two to three times more likely to have received soy-containing formula in early life than a child of similar age without any thyroid ailment. In contrast, the prevalence of feedings with soy-containing formula in infancy in the sibling and control groups was similar to that reported in the general pediatric population of the United States [5]. Thus, it appears that when breast milk was substituted by soy formula feedings in early life there would be an association with ATD whereas when cow's milk feedings were used, no such an association existed.

Although the precise reason for the introduction of soy-containing formulas in patients with ATD could not be determined by the retrospective study, it is generally agreed that such formulas are given to infants with gastrointestinal or other alterations while on cow's milk formula feedings. Thus, one could postulate at least two hypotheses, in terms of the possible association between soy-containing formula feedings in early infancy and subsequent development of ATD in children. On the one hand, one could theorize that in children with ATD there is a higher prevalence of gastrointestinal alterations and/or cow's milk formula intolerance during infancy than in the healthy population. Indeed, an increased frequency of antibodies to certain serotypes of intestinal pathogens, such as *Yersinia enterocolitica*, has been reported in patients with ATD [6]. Thus, cow's milk intolerance, which is frequently associated with gastrointestinal alterations in early life [7], could be a part of the long chain of events leading to subsequent development of an autoimmune process resulting in ATD. On the other hand, one could also postulate that in a genetically predisposed population of children for autoimmune diseases the soy-based infant milk formula may exert an adverse effect on the development of such conditions later in life. Indeed, it has been reported that soy-based formulas are highly immunogenic and may damage the intestinal barrier in infants with diarrhea [8]. A similar environmental effect on the development of an autoimmune process has been shown in IDDM, the development of which could be related, in certain geographical regions, to the intake of nitrosamines long before the development of β cell autoimmunity [9].

The cause of autoimmune thyroid diseases, such as Hashimoto's thyroiditis and Graves' disease, is believed to be multifactorial, involving a genetic predisposition to develop an autoimmune response which may be triggered by an environmental insult [10]. From our data it would appear that the soy protein could be one of such environmental triggering factors. A high prevalence of antithyroid antibodies in patients with IDDM who were fed soy-based formula during infancy was also found in previous studies [3].

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Table 1. The Prevalence of Breast Feedings in Early Infancy in Patients with Autoimmune Thyroid Disease (ATD), Their Healthy Siblings, and Other Healthy Children^a

	Patients with ATD	Healthy siblings	Healthy controls
Number of studied children	59	76	54
Number of children breast fed (%)	20 (34)	26 (34)	19 (35)
Duration of breast feeding (months)	5.2 ± 3.7	5.6 ± 3.2	8.7 ± 4.5
Solids started (months)	3.7 ± 3.3	3.0 ± 3.0	3.7 ± 2.8

^aData as mean ± SD.

Table 2. The Prevalence of Soy-Containing Milk Formula Feedings in Early Infancy in Children with Autoimmune Thyroid Disease (ATD), Their Healthy Siblings, and Other Healthy Children

	Patients with ATD	Healthy siblings	Healthy controls
Number of studied children [†]	59	76	54
Number of children fed soy-containing formula (%)	18 (31) ^{***}	9 (12)	7 (13)

[†]Includes breast-fed subjects.

*p < 0.01 vs healthy siblings; **p < 0.02 vs healthy controls.

roid scan and uptake with ¹²⁵I, when indi-

ne interview by a nutritionist was conducted on children with ATD being followed in our center. All of the children with ATD were asked the following questions: (1) history of breast feedings and/or formula in early life and its duration, (2) type of formula given, and (3) age at which solid foods were started. This information was obtained for all 59 children with ATD. Data were available on 76 of their siblings. Data were available on 54 of their healthy controls. All of whom were known to be free of thyroid illness.

The nutritionist obtained information on 27 of the healthy control children without apparent ATD. There were 15 females and 12 males with mean age 5.6 years. This comparative control group consisted of 27 friends of the patients with ATD

who were of the same age, lived in the same geographical area, and did not have, to the best of their knowledge, any thyroid illness. The remaining 27 children were being followed in our Pediatric Endocrine Ambulatory Center for familial and/or constitutional short stature; in these patients thyroid disease was ruled out by physical examination and measurement of thyroid function tests (T₄, TSH) and antithyroid antibodies.

The data were analyzed by 2 × 2 χ² tests to evaluate for the differences in feeding practices in infancy between the children with ATD as compared with their healthy siblings and healthy nonrelated control children. The threshold of significance was set at a p < 0.05 level.

RESULTS

The prevalence and duration of breast feeding in early infancy in patients with ATD, their healthy siblings, and

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In conclusion, although we were unable to document protective effects of human milk feeding in early life on the subsequent development of ATD in children, we find that this population of patients had a significantly later prevalence of a feeding history of soy protein-containing milk formulas in early infancy than healthy matched, both related and nonrelated, children. However, a more precise definition of the relationship between the ingestion of soy protein in infancy, heredity, and autoimmune disorders in a larger population of children should be pursued.

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Premature Thelarche in Puerto Rico

A Search for Environmental Factors

Lambertina W. Freni-Titulaer, MD, MSPH; José F. Cordero, MD, MPH;
Lillian Haddock, MD; Gloria Lebrón, MS; Ruth Martínez, PhD; James L. Mills, MD, MS

• Pediatric endocrinologists in Puerto Rico reported a threefold increase in the number of patients with premature thelarche seen between 1978 and 1981. A matched-pairs case-control study was conducted to evaluate associations with potential environmental exposures to substances with estrogenic activity, as well as with familial factors. Analysis was performed on 120 pairs, the case subjects of which were selected from those diagnosed between 1978 and 1982. In subjects 2 years of age or older at the onset of thelarche, no significant associations were found. In subjects with onset before 2 years of age, significant positive associations were found with a maternal history of ovarian cysts, consumption of soy-based formula, and consumption of various meat products. A statistically significant negative association was found with consumption of corn products. These statistical associations are probably not sufficient to explain the reported increase because in over 50% of the case subjects there was no exposure to any of the risk factors for which statistical associations were found. Exposure to other substances with possible estrogenic effect, such as waste products from pharmaceutical factories and pesticides, was also excluded as a possible cause. These findings suggest that better diagnosis and reporting, or conceivably the presence of entirely new, unsuspected factors, could account for the reported increase. (AJDC 1986;140:1263-1267)

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From the Center for Environmental Health, Centers for Disease Control, Atlanta (Drs Freni-Titulaer and Cordero); University of Puerto Rico, San Juan (Drs Haddock and Martínez, and Ms Lebrón); and National Institute of Child Health and Human Development, Bethesda, Md (Dr Mills).
Reprint requests to Division of Chronic Disease Control, Center for Environmental Health, Centers for Disease Control, 1600 Clifton Rd, Atlanta, GA 30333 (Dr Freni-Titulaer).

In 1982, pediatric endocrinologists in Puerto Rico reported an increase in the number of referrals for premature thelarche (isolated breast development occurring in girls before 8 years of age) seen in their practices from 1978 through 1981 compared with previous years.^{1,2} A survey among all pediatricians and pediatric endocrinologists on the island revealed a threefold increase in the number of premature thelarche cases seen in 1980 and in 1981 compared with 1978. No increase in other forms of early sexual development was observed in boys or girls at that time. In 1981, 3.7 cases were identified per 10 000 girls under 8 years of age.³ With no baseline data available on the rate of premature thelarche in the United States or Puerto Rico, it is not clear whether the increased number of cases represents improved diagnosis and reporting, changes in referral pattern, or an actual increase in incidence.

Little is known about the etiology of premature thelarche.⁴ Several commercial estrogens have been found to cause breast enlargement in humans; among them are diethylstilbestrol,⁵ ethinyl estradiol, and mestranol.⁶ Breast enlargement associated with consuming presumably contaminated milk and meat has been reported previously.^{7,8} In 1980, diethylstilbestrol was detected in baby food containing veal and poultry.⁹ In earlier reports,^{2,3,10,11} local poultry, meat, and milk contaminated with exogenous estrogens were considered causes of the reported increase of premature thelarche cases seen in Puerto Rico. These reports lacked adequate comparison groups, and no structured nutritional interviews were conducted. We conducted a matched-pairs case-control study designed to determine whether exogenous exposures or some familial or prenatal factors could explain the reported increase.

SUBJECTS AND METHODS

Definition of Case Subject and Sample Selection

A case subject was defined as a girl between the ages of 6 months and 8 years who had palpable breast tissue of at least 1.5 cm in diameter at the time of the diagnosis and who at that time did not have other evidence of early sexual development. Since palpable breast tissue is common at birth, we did not include those girls with breast enlargement at birth that disappeared before the age of 6 months. Age of onset was defined as the age in months when premature thelarche was first noted by the parents. For cases in which the breast enlargement was present at birth but disappeared before the age of 6 months (two case subjects), the date of recurrence was used as the age of onset.

Parents of the 552 subjects with premature thelarche diagnosed between 1978 and 1982 and reported in the 1982 survey among pediatric endocrinologists³ were sent a letter requesting permission for an interview and, if necessary, a review of the medical records. The resources available for this study permitted evaluation of 130 case subjects and 130 control subjects. A systematic sample of 130 case subjects was selected from the 397 children whose parents returned a signed consent form. The case subjects were representative of the total population of 552 with regard to pediatric endocrinologist, age of onset, year of diagnosis, and municipality of residence. Approximately half of the case subjects had been described by Sáenz de Rodríguez et al¹² and Pérez-Comas.³

For each case subject a matched control subject was selected, defined as a girl who had never had evidence of breast enlargement after the age of 6 months. Two criteria were used for matching on age. For case subjects who were less than 2 years old at the onset of premature thelarche, the control subject had to be at least 6 months of age and within 6 months of the case subject's age. For case subjects 2 years of age or older at the onset of premature thelarche, the control subject had to be at least 2 years old and within a year of the case subject's age. The younger case subjects were more closely matched on age to con-

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control for the rapid changes in feeding patterns that occur in infancy and for the unequal distribution of cases by age of onset. Mothers of case subjects were asked to identify a friend or an acquaintance with a female child the same age as the affected daughter to serve as a control subject. If the mother was unable to do so, a control subject was selected from the case subject's source of pediatric care.

The matching criteria could not be met for 21 pairs. Seventeen of these pairs were matched within nine months for case subjects with onset before the age of 2 years and within 18 months for case subjects with onset at or later than 2 years of age, and these pairs were used in the analysis; the other four pairs were dropped.

All parents of potential control subjects were asked if their daughters had ever had breast enlargement. Of the 175 potential control subjects contacted, 20 subjects were examined by the interviewer at the time of first contact either because the parent was uncertain about the presence of premature thelarche or because the parent thought that the girl had enlarged breasts. Fifteen of these control subjects were excluded because they had some palpable breast tissue at that time. This does not mean that these subjects had 1.5 cm of palpable breast tissue and would therefore fit the case definition. There were no instances in which a potential control subject had to be excluded because of a history of resolved premature thelarche.

Exposures

A 90-minute standardized home interview of the subject's mother was used for collecting exposure data. To minimize bias, different female interviewers administered the two-part interview. The first interviewer asked about family history of premature thelarche and, for the case subjects, the natural history of the condition, the diagnosis of ovarian cysts in the child, and the presence of other signs of early sexual development. This information was verified in the medical records. The second interviewer obtained information on various exposures of the child and histories of endocrine diseases, cysts, tumors, and ages at menarche of the mother and other family members. The mother was instructed not to reveal the status of her child (case or control subject) to the second interviewer.

A case subject was considered exposed if exposure occurred in the three-month period before onset of premature thelarche (or equivalent reference age for the control subject). When a case subject had continuous presence of breast enlargement from birth (22 case subjects), three months was

Exposure Category	Reason for Consideration
Meat, eggs, milk, water, dairy products Corn, rice	Possible contamination with estrogens or other anabolic substances Possible contamination with zearalenone, a mycotoxin with estrogenic activity*
Occupation of household members Creams, drugs, ointments	Possible exposure to dust contaminated with sex hormones Possible use of estrogen-containing medication by the child
Insecticides, pesticides	Some (eg, chlorophenothane [DDT], polychlorinated ketones, methoxychlor) known to have estrogenic activity† Possible predisposing factors
Maternal history of infertility and of threatened abortion, roentgenograms, sonograms, smoking, use of alcohol or sex hormones during index pregnancy Ovarian cysts, breast cysts, endocrine disorders in family	Possible genetic or familial predisposition

*Source: Pathe and Mirocha,¹² 1979.

†Source: Katzenellenbogen et al,¹³ 1979.

considered as the age of onset for the purpose of defining exposures; we assumed that breast enlargement would have disappeared by the age of 6 months if no post-natal exposure to estrogens had occurred. For milk the dates when each type was introduced and stopped were recorded. For other foods the age in months when each item was introduced was noted, and it was assumed that exposure continued.

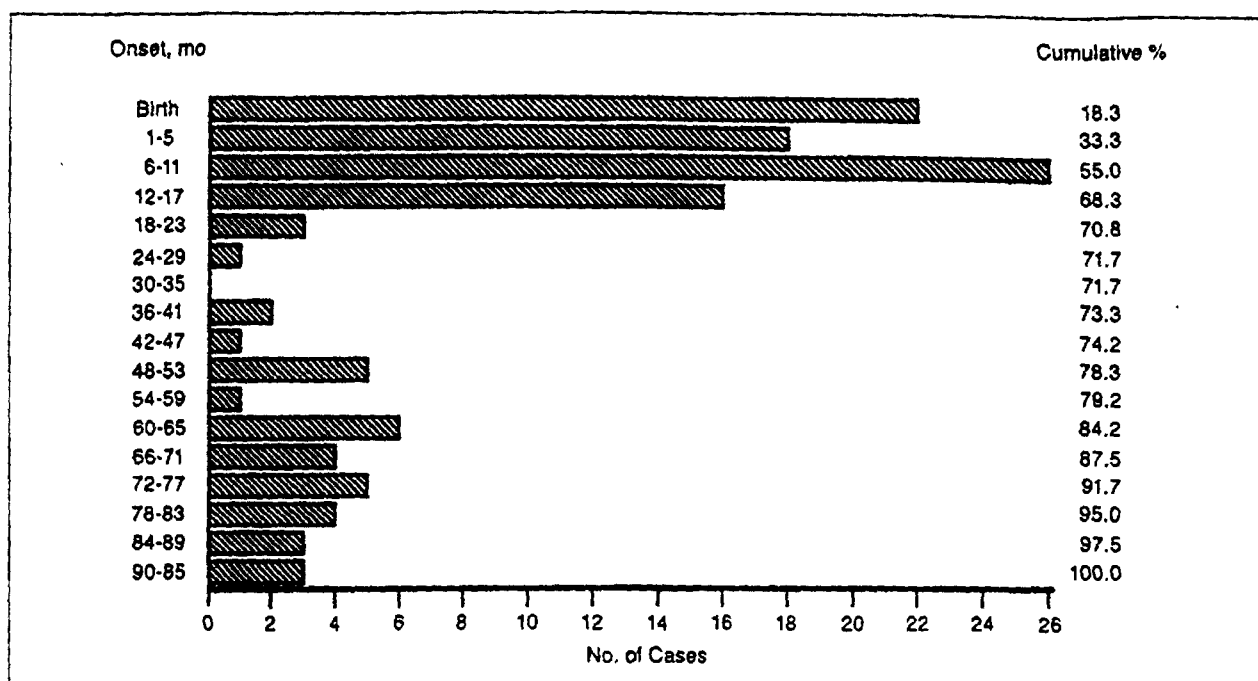
Many exposures were evaluated as possible risk factors for premature thelarche (Table 1). Food items were subdivided, where appropriate, into fresh, frozen, canned, baby food, and specific brands. Livers were considered separately because estrogens are known to concentrate in these organs. Because the majority of all oral contraceptives consumed in the United States are manufactured in Puerto Rico (L. M. Crawford, DVM, PhD, oral communication, 1982), occupation of household members in a pharmaceutical industry was considered a risk factor. In a pilot study of premature thelarche conducted by the Puerto Rico Department of Health, San Juan, and the Centers for Disease Control (CDC), Atlanta, an association was found with a maternal history of ovarian cysts (J.F.C., unpublished data, 1982). Therefore, family histories of ovarian cysts, breast cysts, and endocrine disorders were considered risk factors.

Statistical Methods

Analyses were performed separately for case subjects with onset before the age of 2 years, for case subjects with onset at or later than 2 years of age, and for all ages combined. Odds ratios (ORs) with exact 95% confidence intervals (CIs)¹⁴ were com-

puted by using a matched-pairs analysis.¹⁵ In such analysis only "discordant pairs," ie, those pairs for which exposure status in the case subject is different from exposure status in the control subject, are used. If exposure status of either the case or the control subject is unknown, the pair is not used in the analysis. The resulting OR is comparable to a relative risk obtained from a follow-up study. Categories in which the number of discordant pairs was fewer than six were not analyzed, since statistical significance at $\alpha = .05$ (two sided) cannot be attained with such small numbers. A multivariate analysis, namely, a stepwise conditional logistic regression, was used to control for possible confounding factors. Models were fitted for both age groups. Included in the initial models were variables that either showed statistically significant associations or had ORs that were smaller than 0.5 or larger than 2.0 in the univariate analyses, as well as interactions between exposures and familial factors. Variables that were overlapping, eg, *fresh chicken* and *any meat product*, were not used simultaneously in these models. Instead, two variables, *fresh chicken* and *meat other than fresh chicken*, were then used. Subsequently, those variables that were the least significant were removed one at a time until the models contained only those variables that were significant at the 5% level.

To exclude the possibility that an important association with an exposure was diluted because case subjects with very modest breast enlargement were included, we repeated the multivariate analysis using only the case subjects in which the diameter of the larger breast was at least 2.5 cm.



Number of cases by age of onset.

RESULTS

Of the 130 pairs in the sample, ten pairs were excluded from the analysis. Five pairs were excluded because the case subjects did not meet the criteria for inclusion in the study, four pairs were excluded because the control subjects did not meet the age criteria for matching, and one pair was excluded because the interview for the control subject was not completed. These exclusions resulted in a sample of 120 pairs. In these 120 pairs, 86 case subjects were matched with a friend or acquaintance, and 34 case subjects were matched with patients from pediatric practices. The distribution of age at onset revealed that 68% of the case subjects had onset before the age of 18 months (Figure).

In the univariate analysis, a significant positive association was found with consumption of soy-based formula, with an OR of 2.2 ($P = .050$). A significant negative association was found with consumption of any corn product for the category *less than 2 years old* only, with an OR of 0.2 ($P = .013$). No other food products were found to be statistically significantly associated with premature thelarche.

The strongest association was between a maternal history of ovarian cysts and premature thelarche. For all ages combined, the OR was 3.8 ($P = .002$); for case subjects with onset before 2 years of age, the OR was 8.0 ($P = .001$).

Three case subjects and none of the control subjects had a parent who worked in a pharmaceutical factory producing sex hormones during the reference period. This association was not statistically significant. No association was found between premature thelarche and occupation of a household member in any pharmaceutical factory.

In examining exposures to drugs and chemicals in the home, no statistically significant associations were found with oral contraceptives or with skin or vaginal creams. There was also no association with the use of insecticides, pesticides, or exterminator services in and around the house.

Examination of medical histories in the family revealed no significant associations with previous infertility problems of the mother, specific exposures during pregnancy with the index child, or a family history of endocrine diseases or breast cysts.

Significant and suggestive associations in the univariate analyses (defined as $P \leq .1$) were summarized (Table 2).

In the multivariate analyses, significant associations were found only for pairs in which the case subject had onset before the age of 2 years. Four associations were statistically significant. A history of ovarian cysts in the mother (OR = 6.8, $P = .017$), consumption of soy-based formula (OR = 2.7, $P = .029$), and consumption of fresh chicken (OR = 4.9, $P = .035$) were positively correlated with premature thelarche. Consumption of any corn product was negatively correlated (OR = 0.2, $P = .039$) (Table 3).

Consumption of various meat products was highly correlated. This finding made it impossible to determine which of these products was associated with premature thelarche. Replacement of *fresh chicken* in the final model with *consumption of any fresh meat* resulted in an OR of 5.2 ($P = .025$; 95% CI, 1.2 to 21.6), replacement with *any chicken* resulted in an OR of 4.0 ($P = .055$; 95% CI, 1.0 to 16.2), and replacement with *any meat* resulted in an OR of 5.4 ($P = .033$; 95% CI, 1.1 to 25.3).

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When the analysis was limited to the 9 pairs with onset before the age of 2 years and with breast sizes of at least 1.5 cm, consumption of soy-based formula (OR=3.5, $P=.025$), consumption of fresh chicken (OR=6.0, $P=.026$), and consumption of any corn product (OR=0.1, $P=.018$) remained significant. In this subgroup a history of ovarian cysts in the mother showed a positive association (OR=4.9), but this association was not statistically significant ($P=.065$).

COMMENT

Data for this study were collected after the condition had received a great deal of publicity through the local news media. In statements quoted in the press, pediatric endocrinologists suggested that fresh chicken and fresh milk were causally associated with premature thelarche. These physicians also advised that patients with premature thelarche begin diets without these substances. For both of these reasons, the association we found with consumption of fresh chicken may be due to selective recall. If this were the sole explanation for the found association, however, a positive association with consumption of fresh milk would be expected as well; yet, consumption of fresh milk was found to be almost significantly protective. An association with consumption of fresh chicken cannot be ruled out. It is questionable, however, whether this exposure caused the reported increase, as only 17 (20%) of 85 case subjects under 2 years of age had consumed local meat before the onset of premature thelarche. Therefore, consumption of local meat products cannot be the cause of 80% of these cases. If small children were exposed because the mother consumed contaminated meat products, as suggested by Sáenz de Rodríguez et al,¹⁴ we would expect most case subjects not exposed to local meat to have been exposed to breast milk. However, only 17 (25%) of these 68 case subjects were exposed to breast milk.

The negative association between consumption of any corn product and premature thelarche suggests general dietary differences between case subjects and control subjects rather than

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Table 2.—Results of Univariate Analysis: Summary of Significant and Suggestive Associations				
Exposure	Odds Ratio	95% Confidence Interval	Ratio of Discordant Pairs*	Two-Sided P Value
All pairs				
Maternal history of ovarian cysts	3.8†	1.5-11.5	23/6	.002
Soy-based formula	2.2†	1.0-5.2	22/10	.050
Any meat	3.7	1.0-20.5	11/3	.057
Fresh milk	0.5	0.2-1.0	11/23	.058
Age at onset <2 y				
Maternal history of ovarian cysts	8.0†	1.9-71.7	16/2	.001
Soy-based formula	2.2†	1.0-5.2	22/10	.050
Fresh meat	3.0	0.9-12.8	12/4	.077
Any chicken	3.3	0.9-18.8	10/3	.092
Any meat	3.3	0.9-18.8	10/3	.092
Any corn product	0.2†	0.0-0.8	3/14	.013
Fresh milk	0.4	0.1-1.0	7/17	.084
Age at onset ≥2 y				
Any yellow cheese	4.5	0.9-42.8	9/2	.065

*Refers to pairs for which exposure status in case subject is different from exposure status in control subject.

†The association is significant at the 5% level.

Table 3.—Results of Multivariate Analysis: All Case Subjects With Onset Before 2 Years of Age (N = 85 Pairs)			
Exposure	Odds Ratio	95% Confidence Interval	Two-Sided P Value
Soy-based formula	2.7	1.1-6.8	.029
Maternal ovarian cysts	6.8	1.4-33.0	.017
Fresh chicken	4.9	1.1-21.9	.035
Any corn consumption	0.2	0.0-0.9	.039

a direct protective effect of corn consumption. The multivariate analyses considered only differences in consumption of fresh chicken and soy-based formula, but not other dietary factors. Given the many associations that were analyzed, some positive and negative findings would be expected to show statistical significance by chance alone.

Most of the exposures that were considered in this study reflect possible vehicles of estrogens and not the estrogen per se. If certain products are contaminated sometimes, but not always, with estrogens, some associations possibly would not be detectable.

The association between maternal history of ovarian cysts and premature thelarche was also found in a pilot study (J.F.C., unpublished data, 1982). Study mothers were asked about any previous history of ovarian cysts but

were not asked when this diagnosis was made. There are many types of ovarian cysts, most of which are physiologic, transient, and of little clinical significance. A woman who has been examined frequently is more likely to have a cyst diagnosed at some time. The association may have been caused by a difference in the threshold for seeking medical consultation. If this were the sole explanation, however, an association with a maternal history of breast cysts might also be expected.

Because the majority of all oral contraceptives consumed in the United States are manufactured in Puerto Rico, exposures associated with this industry were also considered. Three case subjects and none of the control subjects had a family member who worked in this industry. This finding indicates that contamination of the home environment might be the cause

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of premature thelarche in these three case subjects, but not in the other 117 case subjects. If environmental contamination with waste products of the sex hormone-producing factories was the cause of increased incidence in premature thelarche, one would expect to see clustering of cases around these factories. No such clustering was seen; cases were diagnosed all over the island.¹³ Contamination of the entire aquifer with waste products can also be ruled out as a possible cause, since all pharmaceutical factories of the island are located in the north and since the aquifers of the northern and southern parts of the island are separated.¹⁴ In a separate study conducted by CDC, drinking water used by eight recently diagnosed case subjects and six control subjects was tested by rat uterine cytosol receptor assay. No estrogenic activity was detected (W. H. Hannon, PhD, unpublished data, 1984).

The case subjects in this study cannot be considered to be a random sample of all cases that occurred on the island in the specified period. They were selected from those who consulted a pediatric endocrinologist for the condition. Therefore, control subjects had to be selected from a population with equal access to pediatric endocrinologists. The only feasible way to do this was to choose a friend as a control subject. This method of control selection may have resulted in an overmatching of the control subjects to the case subjects. If overmatching occurred, it would not influence the estimated magnitude of possible associa-

tions, but the likelihood of finding these associations to be statistically significant would be reduced. Control selection does not, however, influence exposure among case subjects. More than 50% of the case subjects were not exposed to any of the possible risk factors identified in this study.

No incidence rates of premature thelarche are available for comparison. Therefore, whether the rates in Puerto Rico are comparable with or higher than those in other countries cannot be determined. This study focused on exposures that could explain the apparent threefold increased incidence in cases of premature thelarche in Puerto Rico in 1980 and 1981 compared with 1978. For this reported increase, these exposures should have a significant population attributable risk, ie, a measure of the proportion of cases to which a given exposure might contribute. On the basis of the population attributable risks, computed for case subjects with onset before the age of 2 years, maternal ovarian cysts could contribute to up to 18% of these cases, consumption of soy-based formula to up to 19%, and consumption of fresh chicken to up to 15%. Since one case subject has often been exposed to more than one possible risk factor, the population attributable risks for different exposures cannot be added together.

This study found positive statistical associations between premature thelarche and consumption of soy-based formula, various meat products, and a maternal history of ovarian cysts. A negative association was found with

consumption of corn. Although these relationships may have direct or indirect associations with the cause of premature thelarche in some of the cases, they cannot explain the premature thelarche in all case subjects with onset before 2 years of age, and they cannot explain any occurrences in case subjects with onset at or later than 2 years of age. This finding suggests that premature thelarche may not be a single condition but rather a clinical finding resulting from a variety of environmental and familial factors. Since all identified risk factors for premature thelarche were considered in this investigation, the reported increase could have been caused by better diagnosis and reporting or conceivably by entirely new, unsuspected factors.

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Dietary Estrogens Stimulate Human Breast Cells to Enter the Cell Cycle

Craig Dees,¹ James S. Foster,² Shamila Ahamed,² and Jay Wimalasena²

¹Risk Analysis Section, Health Sciences Research Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee; ²Department of Obstetrics and Gynecology, University of Tennessee Hospital, Knoxville, Tennessee

It has been suggested that dietary estrogens neutralize the effect of synthetic chemicals that mimic the effects of estrogen (i.e., xenoestrogens, environmental estrogens). Genistein, a dietary estrogen, inhibits the growth of breast cancer cells at high doses but additional studies have suggested that at low doses, genistein stimulates proliferation of breast cancer cells. Therefore, if dietary estrogens are estrogenic at low doses, one would predict that they stimulate estrogen-receptor positive breast cancer cells to enter the cell cycle. Genistein and the fungal toxin zearalenone were found to increase the activity of cyclin dependent kinase 2 (Cdk2) and cyclin D₁ synthesis and stimulate the hyperphosphorylation of the retinoblastoma susceptibility gene product pRb105 in MCF-7 cells. The steroidal antiestrogen ICI 162,780 suppressed dietary estrogen-mediated activation of Cdk2. Dietary estrogens not only failed to suppress DDT-induced Cdk2 activity, but were found to slightly increase enzyme activity. Both zearalenone and genistein were found to stimulate the expression of a luciferase reporter gene under the control of an estrogen response element in MVLN cells. Our findings are consistent with a conclusion that dietary estrogens at low concentrations do not act as antiestrogens, but act like DDT and estradiol to stimulate human breast cancer cells to enter the cell cycle. — *Environ Health Perspect* 105(Suppl 3):633–636 (1997)

Key words: antiestrogen, breast cancer, DDT, phytoestrogen, xenoestrogen

Estrogenic chemicals present in the human diet or environment have been called xenoestrogens or environmental estrogens. Recently it has been suggested that xenoestrogens may have a role in the etiology of breast cancer (1,2,3). Compounds that have been suggested to be xenoestrogens include pesticides, dyes, pollutants, plasticizers and food preservatives (2,4–7). For example, the pesticide DDT and the food colorant Red Dye No. 3 have been shown to bind to the estrogen receptor (ER) and to stimulate proliferation of ER-positive

breast cancer cells (2). At the molecular level, DDT and Red Dye No. 3 mimic the effects of estradiol by stimulating breast cancer cells to enter the cell cycle (2,8). Entry into the cell cycle requires cyclin D₁ synthesis, activation of cyclin-dependent kinase 2 (Cdk2), and retinoblastoma protein (pRb105) hyperphosphorylation (2,8). Phosphorylation of pRb105 by activated cyclin-dependent kinase can be detected as a migration shift using Western blot analyses after breast cancer cells are treated with estradiol, Red Dye No. 3, or DDT (8–11).

Whether xenoestrogens have a role in the etiology of human breast cancer remains controversial (1,12–16). It has been suggested that exposure to xenoestrogens cannot produce adverse effects on reproductive tissue because they are neutralized by estrogenic compounds derived from dietary sources (17). Therefore, if dietary estrogens have chemopreventive activity by antagonizing the effects of estrogen, one would predict that cellular processes associated with entry into the cell cycle (e.g., cyclin-dependent kinase activation, cyclin D₁ synthesis) would be blocked by dietary estrogens. Genistein has recently been shown to be a potent inhibitor of ER-positive breast cancer cell growth when added at high concentration (>10 μ M) (18,19). However, genistein at lower concentrations has also been shown to increase the growth of ER-positive cells (19,20).

In this study, we examined the effects of dietary estrogens on cyclin D₁ synthesis, Cdk2 activity, and pRb105 phosphorylation. By performing Cdk2 assays on MCF-7 breast cancer cells that were treated with low concentrations of genistein or zearalenone in addition to DDT, we also determined if genistein or zearalenone exhibited antiestrogenic activity. The ability of zearalenone and genistein to stimulate the expression of a reporter gene under the control of an estrogen response element in human breast cells stably transfected with this construct was also determined.

Materials and Methods

Chemicals

ICI 162,780 was a gift from Alan Wakeling, Zeneca Pharmaceuticals (Macclesfield, England). Dietary estrogens and estradiol were purchased from Sigma Chemicals (St. Louis, MO). Dietary estrogens and DDT were concentrated solutions in absolute ethanol. An equal amount of ethanol was added to all control cells.

Human Breast Cancer Cells

MCF-7 cells were maintained in Dulbecco's Modified Minimal essential medium/Hams F12 1:1 without phenol red (Sigma Chemicals). Both cell lines were incubated at 37°C in a 5% CO₂ atmosphere with 10% fetal bovine serum (FBS). Prior to studies on Cdk2 activation, MCF-7 cells were growth arrested by removal of serum and transfer into methionine-free medium for 72 hr before exposure. During dietary

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Address correspondence to Dr. C. Dees, PhotoGen L.L.C., 7327 Oak Ridge Highway, Suite A, Knoxville, TN 37931. Telephone: (423) 538-9975. Fax: (423) 538-9684. E-mail: genase@aol.com

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Abbreviations used: A/G, albumin-globulin; ATP, adenosine triphosphate; Cdk2, cyclin-dependent kinase 2; ER, estrogen receptor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; pRb105, retinoblastoma susceptibility gene product; t₀, time zero.

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estrogen exposure, breast cells were maintained in methionine-containing DMEM/F12 (phenol red- and PBS-free).

MVLN cells were provided by M.J. Duchesne, Montpellier, France. These cells have been stably transfected with a firefly-luciferase reporter gene that is under the control of an estrogen response element of the *Xenopus vitellogenin A2* gene and a thymidine kinase promoter of the Herpes simplex virus (21).

Estrogenicity Assay

The ability of zearalenone and genistein to stimulate the expression of luciferase production under the control of an estrogen response element was performed by depriving MVLN cells of estrogen. Estrogen deprivation in MVLN cells was accomplished by growing them in culture with phenol red-free DMEM/F12 medium containing 5% charcoal-stripped, delipidated calf serum. After gradual withdrawal of the serum, the cells were left in the medium alone for 24 hr more and then treated with estradiol (1 nM), estradiol (1 nM) and ICI 182,780 (100 nM), zearalenone (10 nM), zearalenone (10 nM) and ICI 182,780 (100 nM), genistein (0.1 μ M), or genistein (0.1 μ M) and ICI 182,780 (50 nM). The medium alone was used as the control. After 24 hr of treatment the cells were lysed and the luciferase assay performed using Promega's Luciferase Assay kit (Madison, WI) per the kit protocol. Light emission from treated MVLN cell extracts was measured using a scintillation counter in out-of-coincidence mode.

Cyclin-dependent Kinase 2 Assays

MCF-7 cells for Cdk2 analysis were exposed to estrogens for 20 hr. After incubation, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed by the addition of cold lysis buffer (Tris 20 mM, pH 7.5, NaCl 250 mM, 0.1% NP-40, NaF 10 mM, NaVO₃ 1 mM, PMSF 1 mM). After 15 min on ice, the lysates were centrifuged at 20,000 \times g for 15 min (4°C). Cdk2 was precipitated from equal amounts of cell extracts using purified rabbit anti-Cdk2 (Santa Cruz Biotechnology, Santa Cruz, CA) and protein albumin-globulin (A/G) agarose. Immunoprecipitates were washed three times with the lysis buffer and twice with kinase buffer (Tris 40 mM, pH 7.5, MgCl₂ 10 mM). The immunoprecipitates were suspended in 30 μ l of kinase buffer supplemented with 400 μ g/ml histones (Sigma Chemicals type II-SS), 5 μ M adenosine

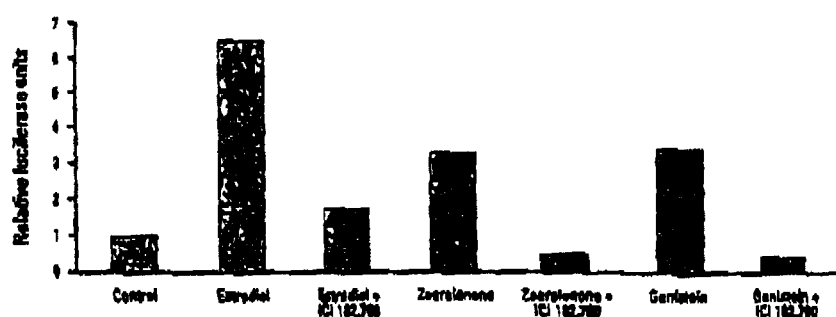


Figure 1. Genistein (0.1 μ M) and zearalenone (10 nM) at low concentrations mimic effects of estradiol by stimulating luciferase production in MVLN cells where the reporter gene is under the control of an estrogen-response element. The steroidal antiestrogen ICI 182,780 (100 nM) blocks genistein- and zearalenone-stimulated luciferase production.

triphosphate (ATP), 0.5 mM dithiothreitol, 0.5 mM ethylene glycol tetraacetic acid, and 5 μ Cl γ -[³²P]-ATP for 20 min at room temperature. The reaction was stopped using gel electrophoresis sample buffer, and the reaction products were separated on a 14% polyacrylamide gel (Novex, San Diego, CA).

Cyclin D₁ Synthesis

MCF-7 cells were plated in six well culture plates, grown to 60 to 80% confluency, and growth arrested as described for kinase assays. Deficient medium was replaced and the dietary estrogens added as indicated. At appropriate time points, medium in wells to be radiolabeled was changed to methionine-free DMEM/F12 (no serum or phenol red). After 15 min at 37°C, this medium was removed and replaced with methionine-free DMEM/F12 containing 50 μ Cl of Trans ³⁵S-label (1000 Ci/mmol). Following incubation for 2 hr at 37°C, labeling medium was removed and the monolayers were washed twice with ice-cold PBS. Lysates were prepared as given for kinase assays and stored at -80°C for later use. For immunoprecipitation analysis, lysates were equalized based on protein concentration and pre-cleared with 30 μ l of protein A/G agarose beads followed by incubation with antibodies to cyclin D₁ (mab HD-11, Santa Cruz Biologicals, Santa Cruz, CA), 1 μ g per sample, and protein A/G agarose beads. After 18 hr at 4°C, immune precipitates were washed 5 times with wash solution, suspended in 1X sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, boiled, and separated on 12% polyacrylamide gels. Following electrophoresis, the gels were fixed, washed in water, and soaked in 1 M sodium salicylate (pH 6.0) before drying. Fluorography was performed using Kodak XAR film at -80°C.

Results

Stimulation of Estrogen Receptor-controlled Luciferase Production

We hypothesized that if dietary estrogen exposures were mitogenic, they acted through the ER. Therefore, the expression of a reporter gene (luciferase) under the control of an estrogen response element must be increased in MVLN cells exposed to dietary estrogens. (Figure 1) shows that exposure of MVLN cells to genistein (0.1 μ M) or zearalenone (10 nM) stimulates the production of a luciferase reporter gene that is under the control of a *Xenopus* estrogen response element. The steroidal antiestrogen ICI 182,780 (100 nM) inhibited luciferase production in MVLN cells stimulated by all of the estrogens, demonstrating the requirement for ER in xenoestrogen action (Figure 1).

Stimulation of Cyclin D₁ Synthesis

Synthesis of cyclin D₁ occurs early in G₁ phase prior to activation of Cdk2 (9-11). Estradiol and xenoestrogens (e.g., DDT, Red Dye No. 3) (2) induce increased synthesis of cyclin D₁ in G₁-arrested MCF-7 cells (8). Increased synthesis of cyclin D₁ protein induced by genistein and zearalenone confirm that both dietary estrogens regulate expression of this protein critical for progression through the cell cycle at low concentration (0.5 μ M) (Figure 2).

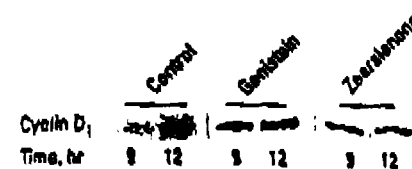


Figure 2. Increased cyclin D₁ synthesis is found in MCF-7 cells treated with genistein or zearalenone at low concentrations (0.5 μ M).

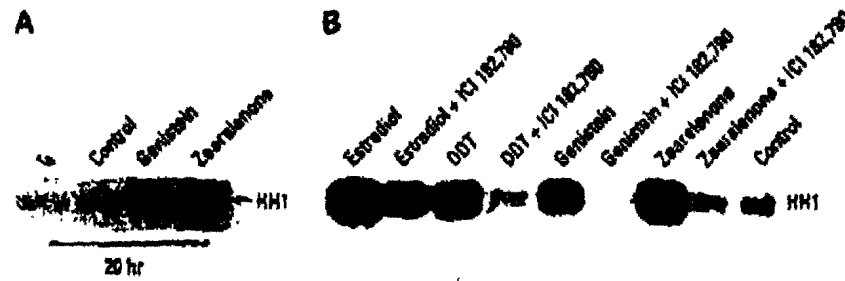


Figure 3. (A) Genistein and zearalenone increase Cdk2 activity in MCF-7 breast carcinoma cells; (B) increased activity in Cdk2 activity in genistein-treated (1 μ M) and zearalenone-treated (1 nM) MCF-7 cells was completely inhibited by the steroidal antiestrogen ICI 182,780 (50 nM). Cdk2 activity stimulated by DDT (0.3 μ M) was also completely inhibited by ICI 182,780 (50 nM). However, the same concentration of ICI 182,780 only partially suppresses Cdk2 activation stimulated by estradiol (10 nM).

Stimulation of Cdk2 Activation

Subsequent to cyclin D₁ synthesis and activation of Cdk4, transit of cells through G₁ and entry into S phase requires Cdk2 activation (9-11). Both genistein and zearalenone stimulated Cdk2 activity when added to growth-arrested MCF-7 cells (Figure 3A). Increased Cdk2 activity induced by genistein or zearalenone could be detected as early as 12 to 16 hr after they were added to MCF-7 cells (data not shown). However, maximum levels of dietary estrogen-stimulated Cdk2 activity occurred 18 to 22 hr after they were added to human breast cancer cells (Figure 3A).

The steroidal antiestrogen ICI 182,780 (50 nM) partially inhibits Cdk2 activation stimulated by estradiol (10 nM) and completely inhibits Cdk2 activation by DDT (0.3 μ M), genistein (1 μ M), and zearalenone (1 nM) (Figure 3B). Both genistein and zearalenone slightly increased Cdk2 activity induced in MCF-7 cells by DDT (Figure 4).

Phosphorylation of pRb105

Cdk activation results in the hyperphosphorylation and inactivation of pRb105, allowing for release of transcription factors

of the E2F family, which are required for S phase entrance (10). Therefore, if dietary estrogens mimic the effects of estradiol on the cell cycle, then increased phosphorylation of pRb105 should occur in breast cancer cells treated with dietary estrogens. Figure 5 shows that low levels of dietary estrogens (0.5 μ M) induce hyperphosphorylation of pRb105 that can be detected as a mobility shifted form of pRb105 by Western blot analyses.

Discussion

The role, if any, that xenoestrogens have in the etiology of human breast cancer is controversial (1,2,15,17,22). Some investigators have proposed that exposure to xenoestrogens may enhance the risk of developing breast cancer (1,2,22). Some epidemiologic studies have supported this hypothesis (3), but others do not find any correlation with xenoestrogen exposure and breast cancer (14). Additionally, it has been proposed that exposure to estrogenic chemicals in the diet may neutralize or somehow prevent xenoestrogens from having any significant biologic effect (17).

Human diets contain plant-derived nonsteroidal estrogenic compounds (e.g., genistein). Other estrogenic molecules in the diet may include compounds produced by fungi (e.g., zearalenone) (20). Additional estrogenic compounds in the diet may

include zeranol, a synthetic derivative of zearalenone, which has been used as a hormonal growth-promoter in cattle (20). Dietary derived estrogenic compounds like genistein have been proposed to act in preventing proliferation of ER-positive breast cells (17). However, one previous report failed to find any chemopreventive action of genistein or zearalenone because these compounds stimulated the growth of MCF-7 human breast cancer cells (20). More recent reports suggest that genistein is a potent inhibitor of MCF-7 cells (18,19). The apparent disparity in the reported effects of genistein on MCF-7 cells may depend on the concentration of genistein used. Genistein is a potent inhibitor of protein tyrosine kinases and can inhibit cell cycle progression in tumor cells independent of action at the ER (23,24). Other inhibitors of MCF-7 cells have been shown to have concentration-dependent estrogenic effects and can also display antiestrogenic activity (25). We surmised that dietary estrogens may have estrogenic activity at low concentrations and antiestrogenic activity or toxicity at higher concentrations.

It has been previously demonstrated that estrogen induces Cdk2 activity, cyclin D₁ synthesis, and hyperphosphorylation of pRb105 in growth-arrested MCF-7 cells (8). Two xenoestrogens also have been shown to mimic estrogen's ability to stimulate MCF-7 cells to enter the cell cycle (2). Therefore, using Cdk2 activation, cyclin D₁ synthesis, and hyperphosphorylation of pRb105, we examined the effects of low concentrations of genistein and zearalenone on growth-arrested MCF-7 cells.

In all assays the dietary estrogens stimulated molecular changes, indicating that they stimulated cell cycle entry. Additionally, rather than antagonizing the effects of DDT, both dietary estrogens stimulated Cdk2 activation. Hyperphosphorylation of pRb105 was induced by both genistein and zearalenone. Flow cytometric data has confirmed that both genistein and zearalenone induce S phase entry in MCF-7 cells as has been demonstrated with estradiol (8) (data not shown). Therefore, at low concentrations, dietary estrogens appear to stimulate MCF-7 cells to enter the cell cycle. In addition, the steroidal antiestrogen ICI 182,780 was found to inhibit stimulation of Cdk2 activity by genistein and zearalenone (Figure 3B). Genistein and zearalenone also stimulated the production of a reporter molecule under the control of an estrogen response element that was also inhibited by ICI

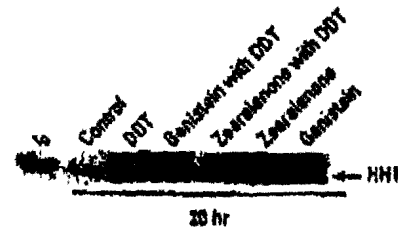


Figure 4. Cdk2 activity in MCF-7 cells is increased by DDT, genistein, and zearalenone. When added with low concentrations of DDT, genistein and zearalenone increase the Cdk2 activity above that produced by DDT, genistein, or zearalenone alone.

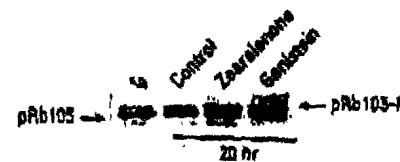


Figure 5. Genistein and zearalenone induce hyperphosphorylation of pRb105. Hyperphosphorylated pRb105 is evident as a slower migrating band.

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182,780 (Figure 1). Therefore, genistein and zearelenone stimulation of human breast cancer cells to enter the cell cycle is mediated through the dietary estrogens' effects on the ER and transcriptional control via estrogen-responsive elements. Further studies are required to determine if genistein and zearelenone inhibit Cdk2 activity, cyclin D₁ synthesis, and hyperphosphorylation of pRb105 at higher concentrations.

Our studies using molecular assays to evaluate the effects of dietary estrogens agree with previous reports (19,20) that at low concentrations, genistein and zearelenone produce proliferative effects on human breast cancer cells. The effects appear to be concentration dependent, which would agree with a recent study that shows stimulation of MCF-7 cell growth at genistein concentrations of less than 10 μ M. Our studies do not support the suggestion

that dietary estrogens neutralize the effects of DDT. In contrast, the effects of dietary estrogens at low concentrations on DDT-induced Cdk2 activation appear to be additive or perhaps synergistic (Figure 4).

Under the proper conditions and concentrations, genistein has been reported to be a potent inhibitor of MCF-7 cell growth (18,19). However, our studies suggest that women should not consume particular foods (e.g., soy-derived products) to prevent breast cancer. Further, our results suggest that low concentrations of genistein may stimulate MCF-7 cells to enter the cell cycle. If the amount of estrogens that can be derived from the dietary sources does not contribute a level high enough to suppress ER-positive cell growth, dietary estrogens may increase the risk of breast cancer, especially in combination with dietary derived xenoestrogens. The risk of exposure

to xenoestrogens and low levels of dietary estrogens may be further increased if developing fetal tissues are exposed (23).

The molecular effects of dietary derived and xenoestrogens on ER-positive breast cancer cells appear to be complex. The effects of dietary estrogens may be concentration dependent and may interact with synthetic and natural estrogens. It may be premature at this time to suggest dietary changes that significantly alter the amount of dietary derived estrogens until additional research can fully elucidate the effects they have on reproductive tissues in terms of dose, tissue-specific effects (like tamoxifen), and potential interactions with other estrogenic compounds. It remains to be determined if dietary estrogens are beneficial or, as suggested by our *in vitro* studies, an additional carcinogenic risk factor for tissues where proliferation is controlled by estrogens.

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Phytoestrogens in Soy-Based Infant Foods:
Concentrations, Daily Intake and Possible Biological Effects

C.H.G. Irvine, M.G. Fitzpatrick[†] and S.L. Alexander

Animal and Veterinary Sciences Group, Lincoln University, New Zealand, and

[†]Chemistry Department, University of Auckland, Auckland, New Zealand

Corresponding author: Prof C.H.G. Irvine, Animal & Veterinary Sciences Group,
PO Box 84, Lincoln University, Canterbury, NEW ZEALAND
Phone 64 3 3252 811 Fax 64 3 3253 851, Email: irvinech@whio.lincoln.ac.nz

ABSTRACT

Exposure to estrogenic compounds may pose a developmental hazard to infants. Soy products, which contain the phytoestrogens, genistein and daidzein, are becoming increasingly popular as infant foods. To begin to evaluate the potential of the phytoestrogens in these products to affect infants, we measured total genistein and daidzein contents of commercially-available soy-based infant formulae, infant cereals, dinners and rusks. We also assayed phytoestrogens in dairy-based formulae and in breast milk from omnivorous or vegetarian mothers. In most cases, the glucoside forms of the phytoestrogens were hydrolyzed before separation by HPLC. Mean (\pm SEM) total genistein and daidzein contents in four soy infant formulae were 87 ± 3 and 49 ± 2 $\mu\text{g/g}$, respectively. The phytoestrogen content of cereals varied with brand, with genistein ranging from 3 to 287 $\mu\text{g/g}$ and daidzein from 2 to 276 $\mu\text{g/g}$. By contrast, no phytoestrogens were detected in dairy-based infant formulae or in human breast milk, irrespective of the mother's diet (detection limit = 0.05 $\mu\text{g/ml}$). When fed according to the manufacturer's instruction, soy formulae provide the infant with a daily dose rate of total isoflavones (i.e. genistein + daidzein) of approximately 3 mg per kg body weight, which is maintained at a fairly constant level between 0 and 4 months of age. Supplementing the diet of 4-month old infants with a single daily serving of cereal can increase their isoflavone intake by over 25%, depending on the brand chosen. This rate of isoflavone intake is much greater than that shown in adult humans to alter reproductive hormones. Since the available evidence suggests that infants can digest and absorb dietary phytoestrogens in active forms and since neonates are generally more susceptible than adults to perturbations of the sex steroid milieu, we suggest that it would be highly desirable to study the effects of soy isoflavones on steroid-dependent developmental processes in human babies.

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INTRODUCTION

The use of soy-containing infant foods is increasing as the public has been made aware of the health promoting properties of soy. Even in 1986, approximately 25% of the liquid infant formulae sold in North America were soy based (1). Although soy also contains antinutritive factors (2), these are largely eliminated by processing or counteracted by supplementation for infant feeding (3). By contrast, the isoflavone phytoestrogens, genistein and daidzein, present in raw beans primarily as the glucosides, genistin and daidzin (4), are heat stable and show substantial carry-over through regular processing methods (5). Recently, concern has been expressed that exposure to soy isoflavones may pose a developmental hazard to infants, particularly to the reproductive system (6-9). This is because the isoflavones may cause perturbations of the sex steroid milieu, which are poorly tolerated by neonates (7). In vitro experiments have shown that the soy isoflavones can bind to estrogen receptors and act as competitive agonists or antagonists to endogenous estrogens depending on relative concentrations and affinities (10-12). Moreover, they can influence endogenous steroid metabolism by inhibiting 17β -hydroxysteroid oxidoreductase Type 1, which is the enzyme responsible for converting relatively impotent estrone to the more potent estradiol and, to a lesser extent, androstenedione to testosterone (13). Genistein can also inhibit protein tyrosine kinases, which phosphorylate intracellular proteins and are necessary for the action of insulin-like and epidermal growth factors (14,15). For example, genistein blocks transforming growth factor- α induction of aromatase by inhibiting protein tyrosine kinase (16) and thus decreases the aromatization of androgens to estrogens. There are therefore several pathways by which soy isoflavones might affect sex steroid synthesis and activity in vivo.

The first step in evaluating the potential of the phytoestrogens in soy-based infant foods to affect the reproductive system of infants is to determine the isoflavone intake of infants. Because there are very few data available on the genistein, genistin, daidzein and daidzin contents of typical infant foods (17), we measured their concentrations in: 1) several brands of soy- or dairy-based infant formulae commonly used in New Zealand and U.S.A., 2) breast milk from omnivorous or vegetarian women, and 3) several brands of infant cereal, dinners and rusks. We were then able to calculate the daily phytoestrogen intake of infants fed breast milk or the commercial foods given according to the manufacturer's instructions.

MATERIALS AND METHODS

Samples: Infant formulae and other food items were commercially available and were purchased from a local supermarket. Four commonly-used brands of powdered soy-based infant formulae, and one liquid 'ready to feed' variety were used. All dairy-based formulae were powdered; three were cow's milk, while the fourth was goat's milk. The dinners were two brands of chicken and vegetable puree.

Milk samples from each of 11 breast-feeding mothers were frozen immediately following their collection. Two of these women were vegetarians. Although the diets of the mothers were not controlled, we monitored their intake of soy products for 48 hr before the breast milk samples were collected. The soy consumption by the women over this 48-hr period was classed as follows: 1) No known soy consumed (n=6), 2) soy consumption < 10 g (n=3), 3) soy consumption between 10 and 50 g

(n=1), and 4) soy consumption > 50 g (n=1).

Chemicals: All solvents (Mallinkrodt, ChromAR grade), hydrochloric acid and acetic acid (BDH, AnalaR grade) were purchased from Lab Supply Pierce (Auckland, New Zealand). Genistein (Sigma Chemical Co., St Louis, MO) and daidzein (ICN Biomedicals, Aurora, OH) were used without further purification.

Extraction and hydrolysis of isoflavones: Genistein and daidzein were extracted from food items according to the method of Franke et al (18). Samples (approximately 2.5 g for solid samples and 5 ml for liquid samples) were refluxed in 50 ml ethanol:hydrochloric acid (4:1) for 2 hr. The extracts were cooled and immediately passed through a 0.45 μ filter before 20 μ l was injected onto the HPLC system.

Instrumentation and chromatographic conditions: HPLC determinations were carried out on a Waters WISP 710B equipped with a 20 μ l injection loop and an ICI LC1200 UV/Vis detector. A 3.9 x 300 mm uBondapak C18 reversed-phase column (Waters, Milford, MA) connected to a uBondapak C18 GuardPak (Waters, Milford, MA) pre-column insert was used. Elution was carried out as described by Franke & Custer (19): the mobile phase 20:80 acetonitrile:10% acetic acid for 15 min and then 70:30 acetonitrile:10% acetic acid for 15 min at a flow rate of 0.8 ml/min. Analytes were monitored at 260 nm during each run.

The mean spike recovery of isoflavones was 93% from solid samples and 85% from liquid samples. The detection limits were 0.1 μ g/g for solid samples and 0.05 μ g/ml for liquid samples.

Most commercial infant formulae and cereals were analyzed in triplicate, however occasionally the batch analyzed differed between runs. The mean percentage coefficient of variation (i.e. $\text{sd}/\text{mean} \times 100$) of concentration estimates was 12.5%, which takes into account variation due to batch and to measurement error.

Isoflavone analysis without prior hydrolysis: To determine the distribution of isoflavones into glucosylated and aglucone forms, selected formula and cereal samples were extracted in 80:20 methanol:water for 4 hr (20) and submitted to HPLC analysis as described by Murphy & Wang (21).

RESULTS

Glucosylated isoflavones were the predominant form in both soy-based infant formulae and cereals (Fig. 1). Isoflavone concentrations in commercially available infant formulae and food are shown in Table 1. In this case, glucosylated isoflavones were hydrolyzed before the HPLC separation and therefore results are expressed as total genistein and total daidzein concentrations. The soy-based formulae were $13.8\% \pm 0.2$ protein according to the information on each product's label, with soy being the major protein source. The isoflavone concentrations in the soy formulae were $17.7\% \pm 0.5$ mean values in soy protein isolates (20), which is consistent with the formulae being simple dilutions of soy isolates.

The total genistein and daidzein concentrations in dairy-based infant formulae were less than the detection limit of the analytical method (i.e. < 0.1 μ g/g). Similarly, genistein and daidzein concentrations in all breast milk samples were also less than the method's detection limit (i.e. < 0.05 μ g/ml). By contrast, the ready-to-feed soy

formula contained total genistein and daidzein concentrations of 18 and 15 µg/ml, respectively.

We used the isoflavone concentrations measured in infant formulae and cereals to estimate the mean daily intake (i.e. total genistein plus total daidzein consumed) and the daily isoflavone dose per kg body weight basis received by infants when the products were fed as recommended by the manufacturer (Table II). For soy formulae, the dose rate received by infants remained fairly constant between <1 and 4 months, with a mean (+ SEM) value of 3.2 ± 0.2 mg/kg per day. There was little variability in intake due to the brand of formula chosen (Table I, also see the small SEM in Table II). By contrast, the isoflavone intake provided by cereals differed markedly with brand. For example, feeding 4-month old infants one serving of Cereal A each day would increase the daily isoflavone dose by 28% (Table II).

DISCUSSION

Our results show that considerable amounts of isoflavones remain after processing and formulation of most soy infant foods. By contrast, the isoflavone contents of cow- or goat- based formulae were less than the method's detection limit of 0.05 µg/ml. Likewise, isoflavones were not detectable in human breast milk regardless of the mother's soy consumption.

Although endogenous steroids are present in human breast milk, total concentrations (i.e. conjugated plus free steroids) in most women are only 1-5% those in plasma (22). Similarly, steroids in oral contraceptive pills are transferred into breast milk in small quantities, but the amounts are usually very low or insufficient to allow detection in the infants (23,24). Moreover, in cattle, ovarian steroids used to induce lactation are not excreted through the milk in detectable concentrations (25). It seems likely therefore that the transfer of plasma phytoestrogens into milk may also be inefficient. In young vegetarian women, plasma total genistein and daidzein concentrations have been reported to be 118.5 nmol/L (i.e. approximately 0.04 µg/ml) (26). Even if plasma phytoestrogens passed into milk with 100% efficiency, the resulting milk concentrations would be less than we could detect. On the other hand, the total isoflavone concentrations in soy infant formula diluted as used were at least 660 times higher than the maximum level possible in breast milk (i.e. our detection limit). This indicates that for infants exposure to phytoestrogens via milk is much less significant than exposure via soy formulae.

As in other soy products (4,20,21), the isoflavones in soy- based infant foods exist predominantly as glucosides which are biologically inert (5). However, in adult humans, these glucosides are readily hydrolyzed in the acidic environment of the stomach and by intestinal bacteria (27,28). Consequently, isoflavones are rapidly and efficiently absorbed and digested (29), with plasma concentrations of genistein and daidzein rising significantly after ingestion of soy products (27).

Elimination of isoflavones occurs largely via the urine, mainly as glucuronide conjugates (28,30). Fecal excretion is only 1-2% of intake (30).

Although gut microflora appear during the first week of life (31), the ability of infants to digest ingested isoflavones has not been investigated before four months of age (32). An earlier abstract in which efficient digestion was reported at two months of age has not yet been confirmed (33). In a recent study, male babies, fed from birth

on soy formulae, were found to excrete significantly more daidzein and genistein in the urine at 4 months of age than did infants drinking cow's or mother's milk (32). This shows that 4-month old infants can hydrolyze the soy isoflavones into active forms and absorb these (32). Furthermore, the small amount of phytoestrogen excretion by babies fed on cow's or mother's milk is consistent with our finding of undetectably low phytoestrogen concentrations in breast milks. Interestingly, urinary concentrations of equol, a more potent estrogenic metabolite of daidzein (10,32), are similar and low in infants fed soy, cow's or mother's milk (32). The absence of equol in the urine of soy fed infants may be because the gut microflora needed to produce it from daidzein have not yet developed (32). Alternatively, it may simply reflect the variability in its production observed in adult humans (34 cf 26, 35, 30 cf 31, 36).

Our data show that as the infant's weight increases from 3 to 7 kg (32) the daily rate of intake of soy isoflavones provided by infant formulae is held fairly constant at 3.2 ± 0.2 mg/kg when formulae are fed as recommended by the manufacturers. Supplementing the diet of 4-month old infants with a single serving of cereal each day can increase their daily intake by over 25%, depending on the brand chosen.

Assuming efficient absorption as suggested by the work discussed above, would this isoflavone intake be sufficient to exert biological effects? Since species may differ in responsiveness to phytoestrogens, this question is best answered by considering data from humans <nothing>. Unfortunately, few controlled studies have been reported on the effect of dietary soy on the human reproductive system. Two groups have used postmenopausal women to search for estrogenic effects of soy-supplemented diets (estimated intake = 1.2 - 2.5 mg total isoflavones/kg (27) assuming an average body weight of 65 kg) on the reproductive system. These have yielded contradictory results, with one group observing increased vaginal cell proliferation (37) and the other finding no significant changes in the vaginal epithelium, plasma gonadotropin or sex hormone binding globulin concentrations (27). Baird et al (27) speculated that the soy isoflavones may act primarily as antiestrogens in the reproductive system. Their effects would therefore be easier to detect in premenopausal women than in postmenopausal women whose endogenous estrogens are already very low (27). Supporting this deduction, Cassidy et al found that in premenopausal women a high soy intake significantly alters reproductive hormones (38). That is, when young women consume 45 mg total genistein and daidzein (i.e. 0.73 mg/kg body weight) daily, the pre-ovulatory follicle-stimulating hormone (FSH) and luteinizing hormone (LH) surges fall to one-third and one-half, respectively, of control values, while follicular phase estradiol levels are raised significantly (38). These hormonal changes are accompanied by a lengthened follicular phase and/or delayed menstruation (38). Although treatment lasted one month, the effects persisted for up to three months (38).

The isoflavone dose rate given infants fed on soy-based formulae is more than four times that shown to alter reproductive hormone secretion in cyclic women. Since neonates are generally more susceptible than adults to perturbations of the sex steroid milieu (7), what consequences might result from such a high isoflavone intake by infants?

It has long been known that modification of the sex steroid milieu in neonatal rodents alters reproductive axis function and sexual behavior, and leads to structural

changes in specific areas of the brain. The effects of neonatal steroid treatment, although irreversible, are often not manifested until the reproductive system is activated at puberty (39-40).

Moreover, there is only a limited window during development, the 'critical period', when sex steroids can markedly influence neuronal structure and function. In humans, unlike rodents, this critical period was thought to occur before birth (41,42). However, recent theories on human sexual differentiation propose that there are several critical periods for development which occur not only prenatally but also during the early postnatal period (43). The timing of these critical periods seems to vary from tissue to tissue so that a temporary perturbation of the sex steroid environment may affect the development of only the one tissue that was passing through its critical period at that time (43). In extreme cases, this can lead to the development of sexual mosaics in which masculinized and feminized tissues coexist within the same body (43).

Because the cerebral cortex develops late relative to other neural regions, the postnatal critical periods may be particularly important for cognitive development and other aspects of behavior that are mediated cortically (43).

In both humans and monkeys, the reproductive axis is active soon after birth. In males (44-47), plasma testosterone concentrations increase post-partum and are maintained at levels similar to those in adults for 20-90 days. In females, plasma estradiol concentrations approximately double during the first month of life (47). After this, gonadal steroid levels decline and remain at low basal levels until puberty (44-47). Observations in males strongly suggest that the postnatal period of raised testosterone secretion is a critical period for normal sexual development. For example, blocking the testosterone surge in male monkey infants significantly delays puberty (46). Once pubescent, treated monkeys have lower plasma LH and testosterone concentrations and reduced testicular volumes and sperm counts compared with normal controls (46). Moreover, there appears to be permanent impairment of the central nervous system pathway regulating gonadotropin-releasing hormone (GnRH) secretion (48), sexual behavior is compromised (49), and bone density is reduced (48). Interestingly, no adverse effects of blocking the postnatal testosterone surge were noted before puberty.

Although ethical considerations prevent intentional blocking of the postnatal testosterone surge in human infants, studies of boys with congenital hypogonadotropic hypogonadism suggest that early gonadal steroid deficiency may subsequently contribute to impaired testicular descent and maturation leading to oligospermia in the adult (46-50). The postnatal androgen surge may also prime the urogenital tract by promoting early growth and by potentiating the maturational effects of testosterone at puberty (51). For example, boys born with micropallus related to androgen lack have inadequate androgen-mediated growth of the external genitalia if therapy is not commenced until puberty. Responses are normal if androgens are replaced during infancy (51). With regard to cognitive development, prepubertal androgen deficiency in boys results in impaired spatial perception which normally is more acute in men than women (52). Likewise, in monkeys gender differences in maturation rate of learning ability (53) and performance of delayed visual discrimination tasks (54) can be manipulated by altering the postnatal sex steroid milieu.

The effect of modifying the sex steroid milieu in female primates has been little studied. This could be because the female's postnatal rise in plasma estradiol concentrations is much more subtle than the male's postnatal testosterone surge (47). However, it is still possible that these low levels of estrogen are needed for normal sexual development as has been clearly shown in female rats (55,56).

While obliteration of the postnatal testosterone surge in primate males unquestionably impairs many aspects of sexual development, there is no experimental evidence that a high level of phytoestrogen intake by primate infants does or does not alter either the sex steroid milieu or sexual differentiation. Nevertheless, in neonatal rodents isoflavone administration during the critical period can alter brain structure and the adult regulation of LH secretion in a dose-dependent manner (57). Feeding infant female pigs on soyameal causes reproductive tract abnormalities (58). Likewise, other phytoestrogens given to female neonatal mice (59) or infant pigs (60) also affect reproductive tract anatomy. When adult, phytoestrogen-treated mice develop the premature anovulatory syndrome (61), while pigs show abnormal regulation of LH secretion for weeks after phytoestrogen exposure ceases (60). Furthermore, marked effects on post-pubertal reproductive parameters have been shown in male and female rats nursed by phytoestrogen-fed mothers during early infancy (55). Early phytoestrogen exposure may also have beneficial effects. Mice injected with large doses of genistein soon after birth have reduced susceptibility to chemically-induced tumor development when adult (62). However, this treatment also impairs ovarian follicular development and cyclicity in the adult (63).

One reason for the lack of evidence linking isoflavone consumption with altered gonadal steroid-dependent developmental processes in human infants may be the probable delay in expression of the effects of early isoflavone exposure until after puberty. For example, another exogenous estrogen, diethylstilbestrol, was administered under controlled conditions to large numbers of women for over 20 years before its connection with postpubertal disorders in their offspring was observed, and several more years before the evidence of adverse effects was considered sufficiently convincing to cause withdrawal of the substance (7,8,64).

Because of these observations and the increasing use of soy products as infant foods, it would be highly desirable to study the effects of soy isoflavones on steroid-dependent developmental processes in human babies.

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Table I: Concentrations (mg/kg) of total genistein and total daidzein in commercial infant formulae and foods commonly fed in New Zealand. Shown for comparison is the isoflavone content of soy protein isolate (20)¹.

Product	Total Genistein	Total Daidzein
Soy-based Formulae		
Formula A	92	55
Formula B	81	50
Formula C	91	48
Formula D	83	44
Soy Isolate	514	248
Infant Foods		
Cereal A	287	276
Cereal B	104	95
Cereal C	3	2
Dinner A	58	45
Dinner B	32	31
Rusks	<0.1	<0.1

¹ Published weights corrected for the molar conversion of glucosides into aglucones for comparison with the present data.

² Most values are the mean of three analyses. The mean coefficient of variation was 12.5%, which includes variation due to measurement and to product batch.

Table II: Mean (+ SEM) daily total isoflavone (total genistein + daidzein) intake and daily isoflavone dose calculated on a body weight basis received by infants fed soy-based infant formulae as recommended by the manufacturer.

Formula means are based on the isoflavone contents of four infant formulae commonly used in New Zealand (See Table I). Also shown are the isoflavone intakes provided by three infant cereals. Because of the variability of their isoflavone contents, cereal values have not been averaged.

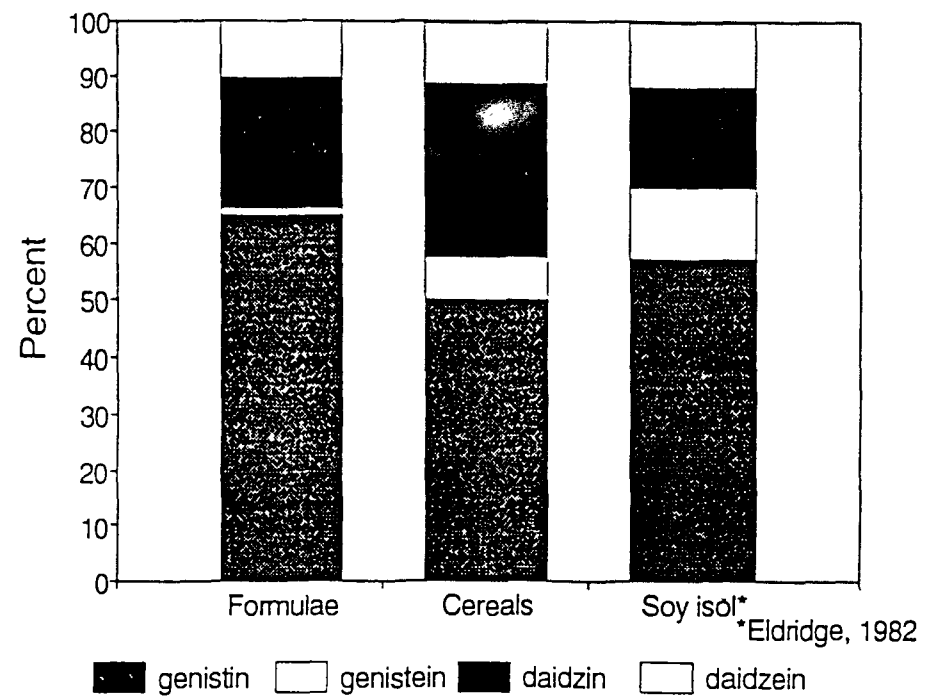
Age	Weight ¹ (kg)	Total Isoflavones (mg/day)	Isoflavone Dose (mg/kg/day)
Soy-based Formulae			
<1 month	3	9.1 ± 0.7	3.0 ± 0.2
1 month	4	14.1 ± 0.6	3.8 ± 0.2
2 months	5	16.6 ± 1.1	3.3 ± 0.2
4 months	7	20.0 ± 2.0	2.9 ± 0.3
Cereals			
Cereal A ²	7	5.6	0.8
Cereal B	7	2.0	0.3
Cereal C	7	0.05	0.01

¹ Weights based on data from Cruz et al., 1994 (32).

² Manufacturers recommend that cereal feeding should start when the infant is 4 months old. The isoflavone intake has been calculated on the basis of one 10-g cereal serving per day.

Legend to Figure

Figure 1. The percentage distribution of isoflavones into glucosides (genistin, daidzin) or aglucones (genistein, daidzein) in four soy-based infant formulae and three infant cereals. Shown for comparison is the mean isoflavone distribution in five soy protein isolates (soy isol; data from Eldridge, 1982 (20)).



Herbal Medicines, Phytoestrogens and Toxicity: Risk:Benefit Considerations¹ (44248)

DANIEL M. SHEEHAN²

Division of Reproductive and Developmental Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, DHHS, Jefferson, Arkansas 72079

Abstract. There are several suggested health benefits of phytoestrogens, particularly those found in soy products. Herbal medicines are also widely thought to confer health benefits. Additionally, drugs are prescribed to improve human health, but unlike phytoestrogens and herbal medicines, toxicities are defined in experimental animals and monitored in humans before and after marketing. Knowledge of toxicity is crucial to decrease the risk:benefit ratio; this knowledge defines appropriate conditions for use and strategies for development of safer products. However, our awareness of the toxicity of herbal medicines and phytoestrogen-containing foods is dramatically limited compared to drugs. Some aspects of the toxicity of herbal medicines are briefly reviewed; it is concluded that virtually all of our knowledge is derived from human exposures leading to acute toxicities. Importantly, detection of toxicity is sporadic, and little information is available from prior animal experimentation. Additionally, well-organized monitoring of human populations (as occurs for drugs) is virtually nonexistent. Important toxicities with long latencies are particularly difficult to associate with a causative agent during or even after large scale exposures, as exemplified by tobacco smoking and lung cancer; estrogen replacement therapy and endometrial cancer; diethylstilbestrol and reproductive tract cancers; and fetal alcohol exposure and birth defects. These considerations suggest that much closer study in experimental animals and human populations exposed to phytoestrogen-containing products, and particularly soy-based foods, is necessary. Among human exposures, infant soy formula exposure appears to provide the highest of all phytoestrogen doses, and this occurs during development, often the most sensitive life-stage for induction of toxicity. Large, carefully controlled studies in this exposed infant population are a high priority.

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Several lines of evidence suggest significant health benefits of phytoestrogens, plant chemicals possessing estrogenic activity. This evidence is reviewed in a number of papers presented in this volume (1-5), and while not the subject of this paper, clearly needs to be considered as part of an overall evaluation of potential health benefits. However, here I wish to discuss certain characteristics of herbal medicines, long used for health purposes, and to explore some broad cultural and scientific relationships that exist between herbal medicines and phytoestrogens. Specifically, both herbal medicines and phytoestrogens are

widely believed to be beneficial but can display toxic effects, and these, like the health claims, also need consideration in order to evaluate overall health effects properly.

Toxicologists can be perceived as having a negative impact on the development of a wide range of marketable products that may benefit society. This naive perspective is challenged by one of the important goals of the toxicologist: to provide information on risks to be included as part of a risk:benefit evaluation. The appropriate decision is directly dependent on the proposed use of a product. For example, drugs useful in diseases with high mortality can display serious toxicity but still be appropriate for use, whereas lower toxicity may not be accepted in a product for common minor ailments, such as colds. An important additional consideration is voluntary versus involuntary exposure: risks associated with involuntary exposures are less acceptable than risks from voluntary exposures (6). Thus toxicologists contribute to decision-making regarding whether a product should be on the market, and if so, under what specific

¹ This review, as presented in this paper, does not necessarily reflect the views of the FDA.
² To whom requests for reprints should be addressed at Division of Reproductive and Developmental Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, DHHS, Jefferson, AR 72079.

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HERBAL MEDICINES, PHYTOESTROGENS, AND TOXICITY

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conditions of use. These decisions are important to protect human health, which can be improved by having knowledge of toxicity. An example is provided by tamoxifen, a drug that is widely used for its beneficial effects as an antiestrogen in preventing recurrence of breast cancer. However, recent findings demonstrate that tamoxifen acts as an estrogen and increases endometrial cancer incidence in breast cancer patients (7). This knowledge has not resulted in the removal of tamoxifen for intended beneficial effects; rather, clinicians now know to monitor patients closely for clinical signs of endometrial abnormalities and to take appropriate medical action when these are found. This knowledge decreases the risk/benefit ratio by lowering the population risk.

This, then, is the context within which the information presented herein should be considered: How can we improve human health by understanding the toxicity of both herbal medicines and phytoestrogens?

Plants are chemical factories that directly provide about 25% of currently used drugs; another 25% of drugs are chemically altered natural products (8). Likewise, phytochemicals with known or potential health benefits are found in plants or plant products marketed either as herbal medicines (9, 10) or foods (11). The latter group includes soybeans, which have a high phytoestrogen content and are a growing component of the human diet. There is, however, a fundamental difference in the safety evaluation of drugs compared to herbal medicines or foods (excluding, in the latter case, chemicals added to foods). Marketing approval for drugs requires careful preclinical, clinical and post-market evaluation of both safety and efficacy; this is not a general requirement for herbal medicines or foods.

Preclinical safety testing in animals follows well-defined protocols involving short- and long-term dosing for evaluation of organ toxicity and death, and tests for mutagenic and carcinogenic activity, reproductive toxicity, and adverse effects on development, among others. Additional specific investigations may be necessary depending on the drug class or the nature of toxicity found in standard preclinical tests. A significant proportion of potential drugs never get to the market because toxicity data suggest a poor risk/benefit ratio.

Clinical testing, which follows animal testing, involves drug administration to volunteers with close monitoring of both efficacy and safety. Again, a number of drugs fail these evaluations. Once marketed, drugs continue to be scrutinized through post-market surveillance; for example, physicians report adverse effects possibly associated with drug treatment, and reports must be maintained by the drug sponsor. Drugs are occasionally found to have toxicities in post-market surveillance that were not detected in the rigorous preclinical and clinical testing. These findings can influence marketability, conditions of use, and patient monitoring as appropriate. Most drugs are available only by prescription. Patients are informed of possible adverse effects both by their physician and the drug label, and are monitored by

their physician. By these measures, most people are aware that drugs may have toxic effects. This knowledge can lead a patient to associate their drug ingestion with adverse outcomes.

Another difference between many drugs compared to herbal medicines and foods is the purity of the chemical of interest. Many drugs are pure chemicals (with fillers, excipients, etc., added for pharmacological purposes); others, however, are complex mixtures that may be partially purified, such as alcoholic extracts (tinctures). Foods and herbal medicines generally are complex mixtures.

Unlike drugs, herbal or folk medicines and food products directly derived from plants are not generally required to be tested for safety or efficacy. Food safety laws are complex and not the subject of this discussion, but it should be appreciated that chemicals added to foods during processing (e.g., antioxidants, emulsifiers, etc.) do require safety testing.

Herbal products have a long history of use based on religious and cultural traditions in which plants are viewed as sources of health remedies (12). This is clearly shown by the prevalence of plant products among prescription drugs. However, it is also true that plants have evolved defense mechanisms against animal and pest predation. These include thorns and other types of physical protection, as well as chemicals that either make plants unpalatable or that sicken or kill their predators and are widely distributed among plants. Such toxicity occurs in humans. The coexistence of beneficial and adverse effects is as true for plant products as for drugs. An important distinction is that our knowledge of drug safety is much superior to our knowledge of herbal product or food safety; we depend on the mostly random accumulation of reports of adverse effects in humans for the latter products (12), and this reporting system is poorly organized and greatly dependent both on luck and consumer as well as physician alertness.

What do we know about the use of herbal medicines and the attitudes of consumers? In a survey of HIV-positive patients, 22% reported regular use of an average of 4.5 herbal tablets per day. Over one-quarter of these reported adverse effects that could be caused by the herbal products (13). Approximately one in two Hong Kong residents use herbal medicines (14). Brown and Marney (15) report that over 90% of 100 surveyed adults used at least one botanical remedy or another, with a median number of seven (range, 0-33). Of those with chronic conditions, more (58%) used home remedies than physician-prescribed treatments (31%). These findings demonstrate a strong belief in, and highly prevalent use of, herbal products, a phenomenon that cuts across cultures and economic classes. Additionally, in part due to the lack of adequate safety data, toxicities may not be expected; in fact, the possibility may be vigorously denied. Adverse outcomes, therefore, may not be recognized as being associated with herbal products.

However, numerous herbal products demonstrate toxicity; this relationship between known toxicity and the per-

ception of consumers stands in direct contrast to the more widespread knowledge of drug toxicity detailed earlier. The toxicity of herbal products may be classified as due to misidentification of plants or to toxicity of properly identified plants (16).

Misidentification has been documented for both self-collected plants and in commercial products. Foxglove is the original source of digitalis compounds and some leaf preparations remain available. Death due to arrhythmias and hypertension is well described (17). An elderly man consumed tea prepared from leaves of a foxglove plant found in his back yard; acute cardiotoxicity resulted (17). A couple died following ingestion of tea prepared from foxglove mistaken for comfrey; other cases of foxglove poisoning are known (18). A red variety of common vetch was misidentified and sold as red lionel; vetch contains neurotoxins (19). Two infants were poisoned with a tea prepared with the herb *Senecio longilobus* mistakenly substituted for *Gordalibo yerba* (12, 18). One infant died and the other suffered chronic liver toxicity. Both of the latter examples were due to commercial products. Likewise, a large American marketer of herbs misidentified deadly nightshade as comfrey, resulting in atropine poisoning (20, 21). In any case, comfrey itself is hepatotoxic and, although widely marketed, should be completely avoided (12, 22, 23).

Misidentification also results from nomenclature problems. Ginseng (English common name) is also called ren-shen (transliteration), radix ginseng (Latinized pharmaceutical name) and *Panax ginseng* (scientific name) (14). Additionally the same common name may be applied to different plants (14). "Cobosh" is used in New England for barberry, which is toxic, while in areas of Appalachia it refers to black or blue cohosh; all three are different genera that show different patterns of toxicity (24). Huxtable (16) provides more detailed examples of nomenclature problems as described above; he as well describes the common confusion that one plant may have many different names (e.g., *Helleborium angustifolium* has 31 common synonyms). Additionally, it is common to be unable to identify components of herbal medicines or teas when consumers present with clear signs of toxicity associated with their consumption (14). For example, an unidentified herbal tea was consumed by four women; one developed a skin rash, whereas the other three had veno-occlusive disease of the liver, from which one died. The tea contained pyrrolizidine alkaloids at a high concentration (25, 26).

However, proper identification alone cannot provide assurance of safety. Ridker (23, 27) lists 26 herbs with known toxicities; all are used to prepare teas, and most are available commercially. Almost 400 different herbs and spices are used for teas, and while more than 10% contain psychoactive ingredients, it is unclear if they induce response (28). One chemical class of toxicants found in a number of herbal preparations is the pyrrolizidine alkaloids; over 8,000 cases of veno-occlusive disease of the liver have

been reported to be caused by this class of chemicals (16, 29) including probable human embryotoxicity (12, 30).

In addition to teas, herbs are also consumed by smoking. Some 20 years ago, 192 different herbs were available for such use (28). Of the mixtures used in 18 different products, almost half contained psychoactive ingredients.

Sassafras, long consumed as a tea in the southeastern United States, causes diarrhea and is hepatotoxic and hepatocarcinogenic (23). It contains the experimental animal carcinogen, safrole. Licorice (*Glycyrrhiza glabra*) can induce a syndrome of toxicities that appears clinically similar to primary aldosteronism (23). Alexander the Great's army used licorice during desert crossings, probably to conserve water by reducing urine output. Licorice contains glycyrrhizic acid, a metabolic precursor of an 11 β -steroid dehydrogenase inhibitor which is almost certainly the cause of the primary aldosteronism of licorice (9). Ginseng is consumed by 5-6 million people in the United States. Frequent consumption can produce a syndrome of toxicities (hypertension, confusion, depression, insomnia) and severe hypotension upon withdrawal which together mimic corticosteroid poisoning (8).

Several plants in the southwestern United States contain adrenergic chemicals. One of these, ephedrine, has been consumed as a "natural amphetamine." About 500 reports of adverse effects, including eight deaths, were received in less than two years by the state of Texas (31, 32). Natives of Curacao consume teas prepared from *Croton flavens* and suffer a high incidence of esophageal cancer (33). This plant contains a family of diterpene esters that increase the risk of malignancies when given with a chemical carcinogen (co-carcinogen) or after a carcinogen (tumor promoter). Their potency is comparable to the phorbol-12,13-diester, such as TPA, which are widely used in experimental carcinogenesis studies. This is the first example that an herbal tea containing co-carcinogens and tumor promoters likely represents the primary carcinogenic risk in epidemiologically identified human malignancy (33).

The examples provided here, as well as numerous others (see Refs. 16, 23, 24, 26) demonstrate that a long history of accepted use of herbal medicines cannot provide great confidence in their safety. In fact, it has been asserted that plants injure or kill more people than animals (12).

Estrogen toxicity is well-known to be associated with plant exposures; phytoestrogens induce infertility (34, 35) and developmental toxicity (36-38) in animals. However, we have little evidence of the adverse effects of herbal preparations that contain phytoestrogens, although attention to phytoestrogens in herbal medicines is increasing (39). Chaparral (*Larrea tridentata*), a desert plant found in the Southwestern United States and Mexico, has long been used as an infusion (tea) for a number of diseases. The high content of nordihydroguaiaretic acid in chaparral appears responsible for hepatitis in users of the tea (40, 41). It has also been used as a contraceptive preparation (42), consistent with experimental data showing estrogenic activity and

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actions as a reproductive toxicant. It has been marketed as an herbal medication. Obermeyer *et al.*, (40) have shown that the phenolic content, which is 80%-90% of the dry weight, is more effectively extracted in methanol than water. The major phenolic chemicals extracted are flavonoid aglycones and glycosides (quercetin, kaempferol, and luteolin) and lignans (43). Because chaparral is marketed as capsules or tablets, the bioavailability of these estrogenic chemicals is expected to be higher than in teas (40). One of the chemicals that may be responsible for the reproductive toxicity (anti-implantation activity) is 3'-demethoxy-6-O-demethyl-isogualacin, which is estrogenic in rats (44). Given that one traditional use is as a contraceptive agent (consumed as a tea), increased phytoestrogen bioavailability from capsules or tablets may induce involuntary infertility in unsuspecting consumers. Other herbal products contain phytoestrogens that have been detected in bioassays using either extracts of the herbal medicines or saliva from individuals consuming them (39). An herbal medicine derived from *Vitex agnus castus* may increase follicular phase estradiol concentrations and induce an ovarian hyperstimulation condition (45). The phytoestrogen content is unknown.

Despite the fact that numerous herbal medicines are traditionally recommended for various disorders and conditions of female reproduction and pregnancy, and that numerous plants contain estrogenic chemicals, no information unambiguously links the phytoestrogen content of herbal medicines to estrogenic effects in humans. Given the poor monitoring of exposure and effects in humans, it cannot be considered that such a relationship does not exist.

In addition to the high phytoestrogen content in soy products, which are estrogenic and developmentally toxic in animals (38), there are other well-described examples of phytoestrogen-containing plants inhibiting fertility via estrogenic activity. These include "sheep clover disease" due primarily to the phytoestrogen coumestrol (34, 46) and "moldy corn syndrome" in pig and cattle fed corn contaminated by *Fusarium* sp., which produce the estrogenic β -resorcylic acid lactone, acaralene (47). Both of these chemicals display typical estrogen effects during reproduction and development. Another example is inhibition of reproduction of California quail by phytoestrogens produced by plants growing in dry conditions (34).

These examples in animals suggest that the phytoestrogen content of herbal medicines and soy products may induce unintended adverse effects on reproduction and development in humans. Herbal product use is prevalent and perceived as safe. Some herbal medicines induce toxicity, and these outcomes are not usually detected by an organized and systematic monitoring of the exposed population. How can we apply these findings to a consideration of the health effects of soy products? First, soy product use is prevalent and perceived as safe; it demonstrates toxicity in livestock and experimental animals; and exposed populations are not systematically monitored for adverse effects. Based on this

comparison with herbal medicines, confidence that soy products are safe is clearly based more on belief than on hard data.

A general argument can be made that the long history of apparent safe use of soy argues that it is not toxic, similar to assertions made for herbal medicines. It is important to point out that almost all known human toxicities of herbal medicines are acute; toxicities with long latencies to appearance are infrequently described and are usually associated with long-standing use of a product. Adverse outcomes with long latencies following discontinuation of herbal medicine use have rarely been demonstrated. Does this mean that such toxicities do not exist or that our abilities to detect them are sharply limited? Without numerous well-designed studies, we simply cannot answer the first question, but there are clear examples that demonstrate the difficulty in associating long latency toxicities with a specific chemical exposure. Four such examples are provided.

Since its introduction to Europe 5 centuries ago, tobacco use has increased. However, heavy smoking was relatively infrequent soon after use in Europe began and was not suggested to be associated with lung cancer until 1761 (48). Not until the middle of this century were convincing studies presented linking tobacco use to malignancies, primarily lung cancer (48). To this day, most tobacco companies and some consumers deny the clear and compelling evidence that smoking causes lung cancer, which shows a latency from initiation of smoking to disease detection of several decades. Thus does belief trump data.

Likewise, the use of unopposed estrogen replacement therapy (i.e., lacking a cyclical progestin component) for menopausal symptoms is now well known to increase the risk of endometrial adenocarcinoma (49). The relative risk increases about one unit for every year of use (e.g., 5 years of exposure results in a 5-fold higher risk of disease occurrence). Prescription drugs were causative. Physicians, drug companies, consumers aware of possible drug toxicities, and the Food and Drug Administration were all involved in defining and advising against unopposed estrogen replacement therapy. Yet even under much more favorable conditions than for detection of adverse effects caused by tobacco, almost 3 decades elapsed before a high level of human exposure to unopposed estrogen therapy was unambiguously associated with this serious toxicity.

In both of these examples, most of the individual exposures were continuing at the time of detection of the malignancies. Thus while there was a long latency to disease appearance, exposure was generally concurrent with disease detection, allowing the association of cause and effect to be made more easily.

Two other examples suggest that when exposure is brief, a long latency to disease appearance may be an even more difficult obstacle to finding the causation. Diethylstilbestrol (DES) exposure of 3-5 million women occurred during pregnancy; a majority of female offspring and a

...most striking showed various developmental abnormalities and malignancies of the reproductive tract that were diagnosed some 12-25 years following the exposure (50). Treatment with this estrogenic drug continued for over 20 years before this association was made by alert physicians who saw, in a short space of time, a handful of young women with clear-cell carcinoma of the vagina or cervix (51). This malignancy is extremely rare, particularly in young females, and it was this unusual feature that led to the demonstration of DES causation. If this had been a more prevalent disease in young women, or if it had not occurred at all or more rarely following DES exposure, it is questionable that the much higher prevalence (50) of benign abnormalities of the female reproductive tract would have been associated with DES exposure. This is due to the fact that similar benign abnormalities occur at a lower prevalence in young women not exposed to DES, and thus an increased incidence might have gone undetected. Thus, simple luck and alertness appears to have played a large role in understanding the role of DES in inducing human malignancies and malformations.

Finally, a very informative example is provided by Fetal Alcohol Syndrome (FAS). FAS occurs in some infants of mothers who consumed alcohol during a critical stage of fetal development. It is characterized by a distinctly recognizable pattern of facial abnormalities and other significant problems, primarily but not exclusively, in the central nervous system (52). This syndrome and its association with maternal alcohol consumption was first described in the 1970s (53, 54). Yet alcohol consumption during pregnancy has certainly occurred over several thousands of years. There is no reason to assume FAS is a recent occurrence; it has been described in every species examined, including nonhuman primates, and in all ethnic groups examined (52). Humankind has looked closely at the faces of its newborn infants over the entire period that maternal alcohol consumption has occurred, yet FAS was only described from a casual and after perspective three-quarters of the way through this century. The latency between exposure and detection is less than nine months, and the features of FAS are clearly visible at birth. This is an extraordinary demonstration of our inability to associate a clear externally displayed manifestation of toxicity with a well-defined exposure over thousands of years in an untold number of cases.

To my knowledge, there are no long-term studies in humans in which a possible association between soy exposure and toxicity has been systematically and rigorously explored. Given the prevalence of soy exposure and the possible health benefits, it is appropriate to include adverse effects in any future large-scale, long-term epidemiological studies. Because reproductive and developmental toxicity have been demonstrated in animals and humans with a wide variety of estrogens, and phytoestrogen exposure has been shown to induce reproductive and developmental toxicity in experimental animals and livestock, these endpoints should

receive particular attention. Given the parallels with herbal medicines with respect to attitudes, monitoring deficiencies, and the general difficulty of detecting toxicities with long latencies, I am unconvinced that the long history of apparent safe use of soy products can provide confidence that they are indeed without risk.

One use of soy, in infant formulas, results in a high phytoestrogen exposure during development (55). Consumption of phytoestrogen-containing soy products by women produces demonstrable estrogenic responses at phytoestrogen doses about 5-fold lower than in soy infant formula exposed infants (55-57). Unfortunately we know very little regarding the toxicity of estrogens generally in human infants. Premature breast development, (gynecomastia in males and premature thelarche in females), can be caused by infant exposure to oral contraceptives via mothers milk (58). Rhesus monkeys are used as an animal model for human reproduction and development. A low dose of DES (500 ng/Kg) administered daily to infant rhesus monkeys altered the normal postnatal gonadotrophin pattern (59). Such findings raise concerns for the potential adverse effects from an infant diet exclusively composed, in many cases, of only soy infant formula.

Additionally, goiter has been described in soy formula fed infants (60), although iodine supplementation of the formula was thought to reverse this problem (61). However, a recent study shows an increased risk of autoimmune thyroid disease in infants consuming soy formula (62). Some isoflavones found in soy formula inhibit thyroid peroxidase, the key enzyme in thyroid hormone synthesis. Inhibition can be reversible or irreversible depending on whether iodine is present (63). Inhibition of thyroid peroxidase would lower thyroid hormone (T3 and T4) serum levels and thus increase Thyroid Stimulating Hormone (TSH) levels in a homeostatic attempt to increase thyroid hormone production. The increased TSH also increases thyroid growth, potentially leading to goiter and malignancy (63). These findings, taken together, suggest that careful studies of the soy infant formula-exposed population should be undertaken, as it is a well-identified group, and phytoestrogen doses can be estimated with some accuracy. Such studies should include not only the infants currently consuming soy infant formulas, but older children, adolescents, and adults previously exposed. They should incorporate estrogenic and thyroid hormone related endpoints, as well as a wide variety of other endpoints of toxicity, as history has shown us that the specific type of toxicity encountered is not always obvious *a priori*. Additionally, given the potential health benefits of soy (1-4) and particularly the finding of inhibition of chemically-induced breast cancer by developmental treatment of rats with genistein (5), measures of possible beneficial effects should be included. Only by such studies can risk/benefit data be collected in order for health professionals to provide appropriate advice to the public. At present, we are conducting a "... large, uncontrolled, and basically

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unmonitored human infant experiment . . . with uncertain risks and benefits (64-65).

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Biochemical and Molecular Roles of Nutrients



Dietary Genistein Exerts Estrogenic Effects upon the Uterus, Mammary Gland and the Hypothalamic/Pituitary Axis in Rats¹

Ross C. Santell, Yu Chen Chang,* Muralee G. Nair* and William G. Helferich²

Department of Food Science and Human Nutrition, *Department of Horticulture, Michigan State University, E. Lansing, MI 48824

ABSTRACT These studies were undertaken to assess the estrogenic and antiestrogenic effects of dietary genistein. To determine estrogenic effects, genistein was mixed into a modified AIN-76 or AIN-93G semipurified diet at 0 (negative control), 150, 375 or 750 $\mu\text{g/g}$ and 17, β -estradiol at 1.0 $\mu\text{g/g}$ and fed to ovariectomized 70-d-old Sprague-Dawley rats. Estrogenic potency was determined by analyzing uterine weight, mammary gland development, plasma prolactin and expression of uterine c-fos. Dietary genistein (375 and 750 $\mu\text{g/g}$) increased uterine wet and dry weights ($P < 0.05$). Mammary gland regression following ovariectomy was significantly inhibited by dietary genistein at 750 $\mu\text{g/g}$ ($P < 0.05$). Plasma prolactin was significantly greater in ovariectomized rats fed genistein (750 $\mu\text{g/g}$) compared with comparable rats not receiving genistein. The relative binding affinity of genistein to the estrogen receptor (ER) was ~ 0.01 that of estradiol. Genistein (750 $\mu\text{g/g}$) induced the uterine expression of c-fos. To evaluate potential antiestrogenic effects, genistein and estradiol were mixed into the modified AIN diets at the doses noted above and fed to ovariectomized rats. Dietary genistein (375 or 750 $\mu\text{g/g}$) did not inhibit the effects of estradiol on uterine weight, mammary gland development or plasma prolactin. Serum concentration of total genistein (conjugated plus free) in rats fed 750 $\mu\text{g/g}$ was 2.2 $\mu\text{mol/L}$ and free genistein was 0.4 $\mu\text{mol/L}$. Administration of dietary genistein at 750 $\mu\text{g/g}$ can exert estrogenic effects in the uterus, mammary gland and hypothalamic/pituitary axis. Dietary genistein (750 $\mu\text{g/g}$) did not antagonize the action of estradiol in estradiol-supplemented ovariectomized rats or in intact rats. J. Nutr. 127: 263-269, 1997.

KEY WORDS: • rats • genistein • uterus • mammary gland • prolactin

Phytoestrogens include the isoflavones, lignans and other nonsteroidal chemicals found in plants and plant products. These compounds can bind to the estrogen receptor and are thought to exert their estrogenic effects through mechanisms similar to those of estradiol.

The consumption of certain plants and plant products can result in impaired reproductive function in livestock. Over five decades ago, clover disease, a syndrome with effects ranging from temporary to permanent infertility, was described in sheep foraging upon subterranean clover in western Australia (Bennets et al. 1946). Additional studies have documented impaired reproductive function in a number of species (reviewed in Price and Fenwick 1985) including desert quail feeding upon desert brush (Leopold et al. 1976). Furthermore, a decrease in reproductive performance was observed in female rats fed either a soy-based diet or a diet supplemented with genistin at 2 g/kg diet (Carter et al. 1955), and in male mice fed genistin at 36 mg/(mouse \cdot d) (Matrone et al. 1955). All of these effects were attributed to the phytoestrogen content of the diets.

It was later discovered that genistein was responsible for the impaired reproductive performance seen in sheep ingesting

subterranean clover (Bradbury and White 1951). Genistein an isoflavone (4',5,7-trihydroxyisoflavone), which has estrogenic activity (Folman and Pope 1966), present in various plants including soybeans (Nam et al. 1974). Since the initial discovery of its estrogenic activity, there have been a number of studies in which the effects of soy and genistein on the uterus of mice and rats were evaluated (Carter et al. 1953). All of these studies demonstrated estrogenic effects except the work of Farmakalidis and Murphy (1984). In their study, the potent estrogen agonist diethylstilbestrol also did not promote uterotrophic effects in this strain of mouse (CD-1).

The estrogenic effects of genistein in the uterus are well documented. However, few experiments have been conducted to assess estrogenic effects in other tissues, and to our knowledge, none have examined the effects of dietary genistein on the mammary gland or the hypothalamic/pituitary axis. The studies were undertaken to provide additional insight into the actions of dietary genistein by analyzing its effects on the uterus, pituitary gland and mammary gland. In addition, the plasma concentration of genistein responsible for these estrogenic effects was also determined.

MATERIALS AND METHODS

Chemicals. Genistein was synthesized by the demethylation of biochanin A or from organic precursors as described by Chang et al. (1994). In both processes, chemical identity was assessed by nucle

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² To whom correspondence should be addressed.

magnetic resonance (NMR)¹ and purity assessed at >98%. ³H-17, β -Estradiol (3.59 TBq/mmol) and ³²P-deoxycytidine 5'-triphosphate, tetra(triethylammonium) salt (111 TBq/mmol) were purchased from Dupont New England Nuclear (Boston, MA). All other chemicals, unless otherwise specified, were purchased from Sigma, St. Louis, MO.

Animals. All rats were maintained according to guidelines in the *Guide for the Care and Use of Laboratory Animals* (NRC 1985). Intact and ovariectomized 60-d-old Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing from 200 to 225 g were used in Experiments 1 and 2. In Experiment 3, intact 30-d-old rats weighing from 80 to 90 g were used. Upon receipt, rats were weighed and sorted to equalize animal weights within each treatment group. Unless otherwise noted, rats were acclimated to the animal care facility and diet for 7 d prior to initiating the studies. Rats were maintained in the animal care facility with temperature $22 \pm 2^\circ\text{C}$, relative humidity (40–70%), and a 12-h light:dark cycle. Rats were housed in polycarbonate cages (3 rats/cage) with aspen woodchip bedding and were allowed unrestricted access to food and water.

Diets. Animals were fed the American Institute of Nutrition-93G (AIN-93G) or modified AIN-76 diets prepared in our facilities. For the AIN-76 diet cornstarch was substituted for sucrose to lower the sucrose concentration from 50 g/100 g to 10 g/100 g of the diet. Semipurified diets are required because the potential presence of genistein and other phytoestrogens in the soy portion of commercial nonpurified diets could confound the experimental results. Genistein and 17, β -estradiol were mixed into the AIN-76 or AIN-93G diets at the concentrations specified in Experiments 1, 2 and 3.

Experiment 1: estrogenic effects of dietary genistein and estradiol. Forty-two rats were ovariectomized at 56 d of age and dietary treatments were begun at 70 d of age. The ovariectomized rats (6/treatment) were given free access to food and water for a period of 5 d. Dietary treatments consisted of genistein at 150, 375 and 750 $\mu\text{g/g}$ or estradiol at 0.5, 1.0 and 1.5 $\mu\text{g/g}$. Eight intact and six ovariectomized 70-d-old rats were fed a modified AIN-76 diet and served as positive and negative controls, respectively. Estrogenic activity was assessed by uterine wet and dry weights.

Experiment 2: anti-estrogenic activity of dietary genistein. Forty-six rats were ovariectomized at 56 d of age and fed the modified AIN-76 diet until 70 d of age at which time dietary treatments were begun. Rats, six per group, were given free access to food and water for a period of 21 d. Dietary treatments included estradiol at 1.0 $\mu\text{g/g}$, genistein at 750 $\mu\text{g/g}$, genistein at 150, 375 and 750 $\mu\text{g/g}$ plus estradiol at 1.0 $\mu\text{g/g}$ and an untreated control group. Ten ovariectomized rats were killed at the start of the experiment to obtain baseline values for uterine weight and mammary gland development. Estrogenic activity was assessed by comparing uterine weight, mammary gland development, plasma prolactin and uterine expression of an estrogen responsive gene, *c-fos*, to those of ovariectomized, untreated controls. Antiestrogenic activity of genistein was assessed by comparing the uterine weight, mammary development and plasma prolactin of rats consuming diets with varying combinations of genistein and estradiol to the rats fed the diet containing 1.0 $\mu\text{g/g}$ estradiol.

Experiment 3: effects of dietary genistein on the development of the mammary gland and uterus of intact rats. Thirty-four 30-d-old rats were fed the AIN-93G diet for 1 d and treatments began on the second day. Rats, eight per group, were given free access to food and water for a period of 14 d. Dietary treatments consisted of genistein at 375 and 750 $\mu\text{g/g}$. Eight rats fed the AIN-93G diet without genistein served as positive controls. Ten rats were killed at the start of the experiment to establish baseline values for uterine weight and mammary gland development.

Analysis of uteri. Rats were weighed, anesthetized by CO_2 exposure and then killed by cervical dislocation. Uteri were excised, trimmed of fat and connective tissue, weighed and immediately placed in liquid nitrogen for later RNA analysis. Wet uterine weight was determined in all experiments. In Experiment 1, dry weight was also

determined for each uterus by incubating approximately one-half the uterus at 100°C for 16 h.

Analysis of mammary glands. Rats were weighed, anesthetized by CO_2 exposure and then killed by cervical dislocation. Mammary glands were prepared according to the procedure of Banerjee et al. (1976). The inguinal mammary gland was excised, fixed in a (v/v) solution of ethanol:acetic acid for 2 h, transferred to 70% ethanol for 3 d with ethanol changed each day, rinsed in water, stained in alum-carmine for 16 h. The glands were then rinsed water, placed in 70, 95 and 100% ethanol for 30 min each, transfer to toluene for 24 h for clarification and then stored in absolute methanol. Lobulo-alveolar and ductal structure (extent of branching) were assessed using a dissecting microscope and assigned values from 0 to 4, with the higher number representing maximal development. Analyses were performed twice on each gland with identity of the glands unknown to the analyst.

Northern blot analysis of *c-fos*. Total RNA was isolated by procedure of Chirgwin et al. (1979) as modified by Helfferich et al. (1990). Uteri from four rats in each group (randomly selected from the following groups: ovariectomized control, 1 $\mu\text{g/g}$ estradiol, 750 $\mu\text{g/g}$ genistein) were removed from liquid nitrogen, placed in mL of 4.0 mol/L guanidinium thiocyanate (GITC) and homogenized on ice with a polytron (Brinkman Instruments, Westbury, NY). Lauryl sarcosyl was added (100 μL 10% solution per milliliter GITC) the mixture vortexed and cellular debris removed by centrifugation at $12,000 \times g$ (g max) for 10 min. The supernatant was removed, layered over 1.0 mL of 5.7 mol/L CsCl followed by centrifugation at $110,000 \times g$ (g max) for 16 h. The pellet was resuspended in 7.1 L guanidine-HCl, 20 mmol/L sodium acetate, 1 mmol/L dithiothreitol, 10 mmol/L iodoacetic acid and 1 mmol/L Na_2EDTA and transferred to a 1.5-mL microfuge tube. RNA was precipitated with 1 volume absolute ethanol and 0.1 volume 300 mmol/L sodium acetate at -20°C . RNA precipitates were washed once with 3 mol/L sodium acetate and 10 mmol/L iodoacetate, pH 5.0 at 4°C , then with ethanol and 33 mmol/L sodium acetate, pH 5.0, and then with absolute ethanol at -20°C . Ethanol was removed and the RNA precipitate dissolved in Tris-EDTA (pH 8.0) and spectrophotometrically quantified at 260/280 nm. Ten micrograms of RNA was loaded onto a 1% agarose formaldehyde denaturing gel and electrophoresed for 8 h at 35 V. The RNA was transferred to membrane (Hybond-N, Amersham Life Sciences, Cleveland, OH), and the membrane exposed to UV light at 120 kJ/cm² for 2 min. Rat *c-fos* cDNA, 2100 bp, kindly provided by Tom Curran (Roche Institute of Molecular Biology, Nutley, NJ) and probes made by random priming (Boehringer Mannheim, Indianapolis, IN) with incorporation of ³²P-deoxycytidine 5'-triphosphate. The membrane was blocked by prehybridization with herring sperm DNA at 42°C for 4 h followed by hybridization for 12 h and subsequent washing at 65°C with 2X saline sodium citrate buffer (SSC) + 0.1% sodium dodecyl sulfate (SDS) for 1 min each, then 0.3X SSC + 0.1% SDS for 25 min. The hybridized membrane was exposed to Kodak X-OMAT film at -70°C for 48 h and then developed.

Plasma prolactin analysis. Plasma prolactin concentration was determined in the laboratory of Keith Lookingland at Michigan State University utilizing a double antibody RIA employing reagents and procedures of the National Institute for Diabetes and Digestive and Kidney Disease (NIDDK) assay kit (generously supplied by A. F. Low and S. Ratti, National Hormone and Pituitary Program, F. F. R.ville, MD). NIDDK rat PRL RP-3 was used as the standard. Usual 100- μL aliquot of plasma, the lower limit of sensitivity was 10. L. Samples from each rat were assayed in duplicate at two different dilutions in a single radioimmunoassay.

Competitive binding analysis. Competitive binding analysis of ³H-estradiol was performed using uterine cytosol prepared from treated rats consuming a nonpurified diet (22/5 Rodent Diet 8640, Harlan-Teklad, Madison, WI). Rat uteri were placed in cold TEDG (10.0 mmol/L Tris-HCl pH 7.4, 1.5 mmol/L EDTA, 7.4, 1.0 mmol/L dithiothreitol added fresh, and 10% glycerol) homogenized with a polytron (Brinkman Instruments). The homogenate was centrifuged at $800 \times g$ for 10 min, and the resulting supernatant removed and centrifuged at $110,000 \times g$ (g max) for 1.5 h at 4°C . Protein concentration was 5.0 g/L as determined by the Bradford assay (Bradford 1976). Aliquots were quickly frozen in a isopropanol

¹ Abbreviations used: DCC, dextran-coated charcoal; ER, estrogen receptor; GITC, guanidinium thiocyanate; NIDDK, National Institute for Diabetes and Digestive and Kidney Disease; NMR, nuclear magnetic resonance; TEDG, Tris-HCl, EDTA, dithiothreitol, glycerol.

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dry-ice bath and stored at -70°C . Binding assays were composed of 200 μL uterine cytosol (1.0 mg total protein), TEDG, and genistein or estradiol in ethanol vehicle bringing the total volume to 500 μL . Assays containing 5.0 nmol/L ^3H -estradiol and 0–20 nmol/L 17, β -estradiol or 0–50 $\mu\text{mol/L}$ genistein were incubated at 4°C for 3 h. After incubation, the contents were removed and placed in a microfuge tube containing the pellet from 250 μL of dextran-coated charcoal (DCC) solution (5% Norit A, 0.5% dextran T-70 in TEDG) to remove the unbound ^3H -estradiol. The microfuge tube was vortexed to disperse the DCC pellet and incubated for 3 min at 23°C followed by centrifugation at $13,000 \times g$ to pellet the DCC. Two 200- μL aliquots of the supernatant were collected and counted on a scintillation counter (Beckman Instruments Model LS100, Fullerton, CA). The counts were averaged, divided by the total counts and expressed as a percentage of the total radioactivity.

Serum genistein analysis. Rats were anesthetized with diethyl ether, and blood (~ 6 mL/rat) was collected from the tail artery. Blood was placed at 4°C for 16 h to allow clotting. The blood was then centrifuged at $350 \times g$ for 10 min and the serum removed and stored at -70°C . For genistein analysis, 50 μL of serum, from each of four rats fed 750 $\mu\text{g/g}$ genistein in Experiment 2, was sampled in duplicate with one set receiving 5 μL (515 units) of β -glucuronidase Type H-1 (Sigma). All aliquots were incubated in 0.5-mL microfuge tubes at 37°C for 48 h. Following the incubation, 50 μL of absolute methanol was added to each tube, the tubes vortexed and then centrifuged at $15,000 \times g$ for 10 min. Approximately 75 μL was removed and placed at -20°C until analysis. For analysis, the samples were centrifuged at $15,000 \times g$ for 15 min and 20 μL injected onto a C_{18} column (Microsorb-MV, 5 μm 100A, Rainin Instrument, Woburn, MA) with a flow rate of 1.0 mL/min of 50:50 methanol:water, with 17 mmol/L acetic acid. Recovery was determined by spiking serum from control rats with genistein and then assessing recovery of genistein. Mean recoveries were determined to be 74% (SEM 1.68%). The data presented are corrected for recovery. No genistein was detected in control rats fed the AIN-76A diet.

Statistical analyses. All statistical tests were performed using a PC-based version of the Statistical Program for the Social Sciences (SPSS) Version SPSS/PC 2.0, Chicago, IL 60611. Uterine weight and plasma prolactin data were analyzed by one-way ANOVA. Variances in uterine weights [Tables 1 and 2] were nonhomogeneous with respect to treatment; thus these data were log transformed prior to ANOVA. Data are means and standard errors before transformation. When a significant ($P < 0.05$) treatment effect was detected, treatment means were compared using the least significant difference method (Steel and Torrie 1980). Mammary gland data were analyzed by the Kruskal-Wallis nonparametric test (Shavelson 1989). When a significant ($P < 0.05$) treatment effect was detected, treatment rank means were compared using the least significant difference method (Steel and Torrie 1980). Values in the text are means \pm SEM.

RESULTS

Competitive binding analysis. Competitive binding studies utilizing rat uterine cytosol, ^3H -estradiol, unlabeled estradiol and genistein were performed to determine the relative binding affinity of genistein to the estrogen receptor. The concentration of unlabeled 17, β -estradiol required to displace 50% of the bound ^3H -17, β -estradiol in rat uterine cytosol in this study was ~ 5 nmol/L (Fig. 1). Competitive binding analysis indicated that the relative binding affinity of genistein was ~ 0.01 that of 17, β -estradiol.

Uterotrophic effect of dietary genistein. Genistein administered in the diet to ovariectomized adult rats in Experiment 1 at 150, 375 and 750 $\mu\text{g/g}$ produced a dose-dependent increase in both uterine wet and dry weights (Table 1). In rats fed genistein at 375 and 750 $\mu\text{g/g}$, significantly greater uterine wet weights and dry weights than those in the control group were measured. Rats fed 750 $\mu\text{g/g}$ genistein had uterine weights similar to those of rats fed 1.0 $\mu\text{g/g}$ estradiol.

In Experiment 2, the potential of genistein to antagonize

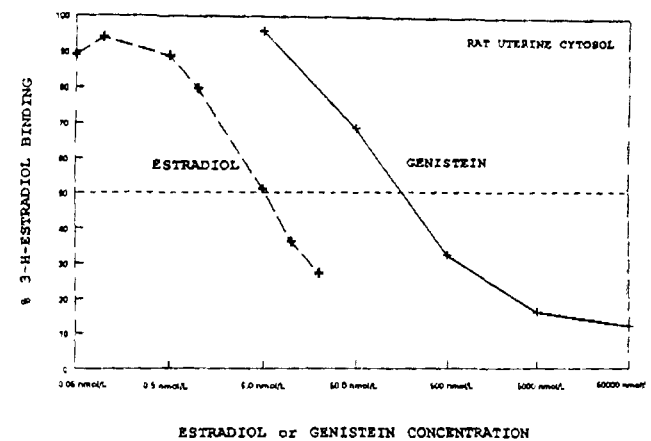


FIGURE 1 Competitive binding analysis of genistein and estradiol using rat uterine cytosol. Genistein or 17, β -estradiol and ^3H -estradiol were incubated with rat uterine cytosol for 2 h. Following incubation free compounds were removed with dextran-coated charcoal and aliquots counted in a Beckman LS-100 scintillation counter. Each point represents the mean of two replicates. Values are expressed as a percentage of the total radioactivity.

the uterotrophic effect of 1.0 $\mu\text{g/g}$ 17, β -estradiol was evaluated by comparing uterine weights of the 17, β -estradiol treated group with those of the groups receiving 17, β -estradiol plus genistein. Uterine weights in the base-line group (rats killed at the start of dietary treatment, 14 d after ovariectomy) indicated that substantial regression had occurred (Table 2) when compared with intact rats at the same age used in Experiment 1 (Table 1). Rats receiving genistein and estradiol at all doses had significantly greater uterine weights than those in the control and base-line groups (Table 2). Genistein at 150, 375 or 750 $\mu\text{g/g}$ did not inhibit the uterotrophic effect of concurrently administered 17, β -estradiol (Table 2).

In Experiment 3, the effect of genistein on the development of the uterus of intact immature rats was evaluated. Ten ra

TABLE 1 000540

Effect of dietary genistein and estradiol on uterine weight in ovariectomized mature rats¹

Treatment	n	Uterine wet weight	Uterine dry weight
		mg	
Control ²	8	76.5 \pm 3.2 ^a	20.7 \pm 1.1 ^a
Intact ³	6	386.6 \pm 41.1 ^b	73.7 \pm 6.5 ^d
Estradiol, $\mu\text{g/g}$			
0.5	6	122.1 \pm 6.6 ^b	17.6 \pm 1.7 ^a
1.0	6	194.8 \pm 8.8 ^c	40.4 \pm 6.0 ^c
1.5	6	255.0 \pm 8.8 ^d	54.0 \pm 2.2 ^d
Genistein, $\mu\text{g/g}$			
150	6	92.4 \pm 2.6 ^a	28.0 \pm 2.8 ^b
375	6	135.6 \pm 9.8 ^b	30.0 \pm 3.6 ^{bc}
750	6	189.3 \pm 26.6 ^c	39.0 \pm 5.6 ^{bc}

¹ Values are means \pm SEM. The experimental groups included negative control (control), positive control (intact), genistein 150, 3 and 750 $\mu\text{g/g}$, estradiol 0.5, 1.0 and 1.5 $\mu\text{g/g}$. Values in a column with different superscripts are significantly different ($P < 0.05$). ANOVA was performed on log-transformed data followed by multiple means comparison using the least significant difference method.

² Controls were ovariectomized, nontreated rats.

³ Intact rats were not ovariectomized.

TABLE 2

Effect of dietary genistein and estradiol on uterine weight and plasma prolactin in adult ovariectomized rats¹

Treatment	n	Uterine weight mg	Plasma prolactin µg/L
Baseline ²	10	130.7 ± 5.5 ^b	—
Control ⁴	6	96.5 ± 3.9 ^a	5.68 ± 1.05 ^a
Gen 750 µg/g	6	343.6 ± 24.3 ^d	12.0 ± 2.73 ^b
E ₂ 1.0 µg/g	6	220.1 ± 17.3 ^c	16.0 ± 2.53 ^b
E ₂ 1.0 + Gen 150	6	241.3 ± 24.9 ^c	—
E ₂ 1.0 + Gen 375	6	312.4 ± 13.4 ^d	16.7 ± 1.52 ^b
E ₂ 1.0 + Gen 750	6	305.6 ± 24.3 ^d	18.8 ± 2.33 ^b

¹ Values are means ± SEM. The experimental groups included an ovariectomized control, baseline, genistein (Gen) 750 µg/g, estradiol (E₂) 1 µg/g, E₂ 1 µg/g + 150 µg/g Gen, E₂ 1 µg/g + 375 µg/g Gen, and E₂ 1 µg/g + 750 µg/g Gen. Values in a column with different superscripts are significantly different ($P < 0.05$). ANOVA was performed on log-transformed uterine weight data followed by multiple means comparison using the least significant difference method.

² Baseline rats were killed at the beginning of dietary treatment.

³ Plasma prolactin was not determined in the baseline and E₂ + Gen 150 treated rats. Uterine weights in these groups did not differ.

⁴ Control rats were ovariectomized, nontreated.

were killed at the beginning of the study to obtain base-line data for uterine weights. Baseline uterine weights were similar to the uterine weights of the ovariectomized rats observed in Experiments 1 and 2, thereby confirming the immaturity of the rats in this study. Dietary genistein administered at either 375 or 750 µg/g for 14 d had no effect on the development of the uterus as indicated by uterine weight, relative to the control, intact rats (Table 3).

Induction of uterine c-fos. Uterine tissue from ovariectomized rats administered 750 µg/g dietary genistein or 1.0 µg/g 17,β-estradiol and untreated controls were analyzed for the presence of c-fos mRNA (Fig. 2). Gels were stained with ethidium bromide to confirm equal loading of RNA and to assess the integrity of the RNA (data not shown). Both dietary genistein and estradiol induced the expression of c-fos mRNA relative to that of the untreated control rats.

Mammatrophic effect of dietary genistein. In Experiment 2, the mammatrophic effects of dietary genistein were evaluated in ovariectomized rats by analyzing the following two

TABLE 3

Effect of dietary genistein on uterine weight of intact immature rats¹

Treatment	n	Uterine weight mg
Baseline ²	8	99.1 ± 14.2 ^a
Control ³	8	230.7 ± 17.9 ^b
Genistein 375 µg/g	8	243.2 ± 10.8 ^b
Genistein 750 µg/g	8	234.4 ± 13.8 ^b

¹ Values are means ± SEM. The experimental groups included baseline, genistein 375 µg/g, genistein 750 µg/g and control. Values in a column with different superscripts are significantly different ($P < 0.05$). ANOVA was performed on log-transformed data followed by multiple means comparison using the least significant difference method.

² Baseline rats were killed at the start of dietary treatment.

³ Control rats were not treated.

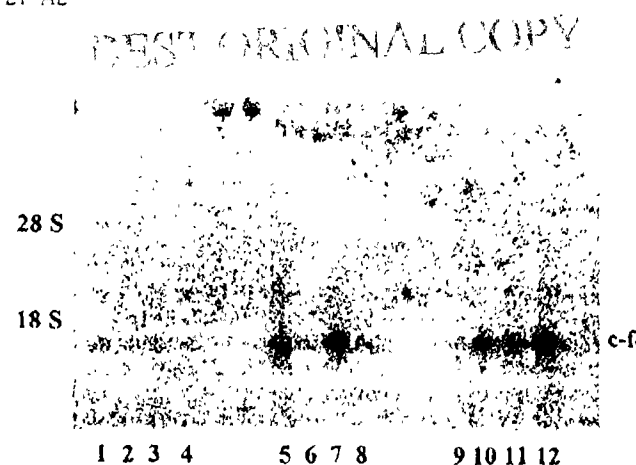


FIGURE 2 Effect of dietary genistein and estradiol on the uterine expression of c-fos in ovariectomized mature rats. Total RNA was prepared from four rats randomly selected from each of the following treatment groups: lanes 1–4 control, 5–8 750 µg/g genistein and 9–12 1.0 µg/g 17,β-estradiol. Ten micrograms of total RNA was electrophoresed on a 1.2% denaturing agarose gel, transferred to nylon and hybridized with random primed rat c-fos DNA probe labeled with ³²P. The film was exposed for 3 d.

criteria: 1) lobulo-alveolar structure and 2) ductal structure including side branching and infiltration of ducts into the fat pad of the mammary gland. Dietary treatment of ovariectomized rats for 21 d with 750 µg/g genistein prevented mammary gland regression, seen primarily in lobulo-alveolar structure, relative to that of the ovariectomized, untreated control rats (Table 4 and Fig. 3). Average lobulo-alveolar development in the 17,β-estradiol-treated rats did not differ from controls. Average ductal development did not differ for the genistein- or estradiol-treated groups compared with the controls. Lobulo-alveolar development was significantly greater for the groups receiving 750 µg/g genistein or genistein 750 µg/g + 17,β-estradiol 1.0 µg/g, compared with the control group. The potential of genistein to antagonize the mammatrophic effect of estradiol was also evaluated. Co-administration of dietary genistein at 150, 375 or 750 µg/g, with 1.0 µg/g 17,β-estradiol, did not result in lower mammary scores compared with rats receiving 17,β-estradiol alone (Table 4).

In Experiment 3, the effect of genistein on the mammary gland in immature rats was evaluated. Ten rats were killed at the beginning of the study to obtain base-line data for mammary gland development. Dietary genistein had no effect on the development of the mammary gland, as assessed by lobulo-alveolar and ductal development, relative to the control untreated intact rats (data not shown).

Plasma prolactin analysis. The effect of dietary genistein on plasma prolactin in ovariectomized rats was determined in Experiment 2. Plasma prolactin was significantly higher in the dietary genistein- and estradiol-treated rats relative to the control group (Table 2). Genistein co-administered with estradiol did not stimulate or inhibit the effects of estradiol.

Serum genistein analysis. The serum concentration of genistein was assessed in four rats from Experiment 2 to determine the concentration present in rats fed 750 µg/g genistein. Total genistein (conjugated plus free) concentration was 2.2 ± 0.01 µmol/L and the free concentration of genistein was 0.4 ± 0.03 µmol/L. Efficiency of recovery was determined by quantifying recovery of a genistein spike from control serum. Average recoveries were $74 \pm 1.68\%$.

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TABLE 4

Effect of dietary genistein and estradiol on the mammary gland of ovariectomized adult rats¹

Treatment	n	Mean raw score		Mean rank score	
		Lob/av ²	Duct	Lob/av	Duct
Baseline ³	10	2.27	2.89	35.65 ^c	34.35 ^c
Control ⁴	6	1.38	2.29	11.75 ^{ab}	22.83 ^{abc}
Gen 750 μ g/g	6	3.42	2.50	34.42 ^c	27.08 ^{bc}
E ₂ 1.0 μ g/g	6	0.96	1.63	7.83 ^a	11.83 ^a
E ₂ 1.0 + Gen 150	6	1.86	1.92	16.58 ^{ab}	13.42 ^a
E ₂ 1.0 + Gen 375	6	1.83	1.72	18.75 ^b	19.17 ^{ab}
E ₂ 1.0 + Gen 750	6	3.21	2.63	31.25 ^c	28.58 ^{bc}

¹ The experimental groups included an ovariectomized control, base-line, genistein (Gen) 750 μ g/g, estradiol (E₂) 1 μ g/g, E₂ 1 μ g/g + 150 μ g/g Gen, E₂ 1 μ g/g + 375 μ g/g Gen, and E₂ 1 μ g/g + 750 μ g/g Gen. Values in a column with different superscripts are significantly different ($P < 0.05$). Data were ranked and analyzed with Kruskal-Wallis nonparametric ANOVA. Raw mean and rank mean scores are included in the table. When a significant treatment effect was found, the rank means were compared with the least significant difference method.

² Lob/Av = lobular/aveolar.

³ Baseline rats were killed at the start of dietary treatment.

⁴ Control rats were ovariectomized, nontreated.

DISCUSSION

Competitive binding analysis and induction of c-fos. Competitive binding analysis demonstrated that the relative binding affinity of genistein for the rat uterine estrogen receptor (ER) was ~ 0.01 that of estradiol. The binding of a compound to a receptor does not necessarily result in the production of a complex capable of initiating the biological response; therefore, additional studies were undertaken to ascertain whether dietary administration of genistein would induce the expression of an estrogen-responsive gene, c-fos (Weisz and Rosales 1990), in an estrogen-responsive tissue. Uterine expression of c-fos was induced in ovariectomized rats following the dietary administration of genistein or 17 β -estradiol. The variable expression of c-fos may be due to several factors, including the following: 1) the timing of food consumption, 2) variability in food consumption, 3) metabolism of genistein, and 4) the short half-life of c-fos mRNA (Greenburg and Ziff 1984). The induction of c-fos by genistein suggests that genistein is acting through the ER, similar to estradiol, and is capable of forming an active complex with the ER in uterine tissue.

Uterotrophic effects of dietary genistein. Phytoestrogens have long been recognized for their uterotrophic activity in a variety of animal species. These effects range from temporary to permanent infertility (reviewed by Adams 1989). In the present study, there was a dose-dependent increase in uterine weight with effects seen at a dietary dose of genistein as low as 375 μ g/g diet, suggesting that genistein acts in the uterus in a manner similar to that of estradiol. That is, genistein binds to the ER, and the ligand:receptor complex induces the expression of estrogen-responsive genes which ultimately result in increased uterine mass.

Genistein competes with estradiol binding to the ER and has shown estrogenic effects in estrogen-responsive tissues. Many antiestrogens, including tamoxifen, possess agonistic properties when administered in low amounts; however, in higher concentrations, they are antagonists (Martinez-Campos et al. 1986). As a weak agonist, genistein also has the potential to antagonize the effects of estradiol. Effects of antagonistic properties have been reported in mice coadministered subcutaneous injections of genistein and estrone. Folman and Pope (1966) inhibited the uterotrophic effect induced by subcutaneous injections in mice of 0.4 μ g estrone (total dose) by administering concurrent subcutaneous injections of either 800 or 1600 μ g genistein (total dose) twice daily over a 3-d period.

In our study, the coadministration of 150, 375 or 750 μ g/g genistein with 1.0 μ g/g 17 β -estradiol did not inhibit the effects of estradiol on uterine weight. In addition, dietary genistein did not affect the development of the uterus, when administered to immature rats, as assessed by uterine weight during maturation of the organ. In the studies reported here, genistein was administered in the diet to rats, whereas in the study by Folman and Pope (1966), genistein was administered subcutaneously to mice. The dose of genistein administered in our studies, relative to the dose of estradiol, was much lower than that of Folman and Pope. Furthermore, variability in species as well as strain in response to compounds with estrogenic activity is well documented (reviewed by Adams 1989, Farmakalidis and Murphy 1984). The amount of genistein absorbed from the gut is currently unknown. All of these variables could account for the different results obtained in this study compared with that of Folman and Pope.

Effects of dietary genistein on the mammary gland and plasma prolactin. Dietary genistein consistently elicits an estrogenic response in the uterus of ovariectomized and immature rodents; however, the effect of dietary genistein on the mammary gland, another estrogen-responsive tissue, has not been assessed. Increased differentiation of the mammary gland has been observed in prepubertal rats following the subcutaneous injection of 5 mg genistein per rat on d 2, 4 and 6 postpartum (Lamartiniere et al. 1995). The model employed in these studies utilized 60-d-old rats and assessed the ability of dietary genistein and/or estradiol to inhibit mammary gland regression postovariectomy.

Development and maintenance of the mammary gland in rats are controlled by many factors including estrogen, progesterone, growth hormone and prolactin (reviewed by Topper and Freeman 1980). Estrogen acts directly at the mammary gland by inducing gene transcription and the subsequent translation of many proteins, including the progesterone receptor required for progesterone action. Estrogen also acts indirectly through the induced synthesis and release of prolactin from the anterior pituitary gland which then exerts its mitogenic effects on the mammary gland. Removal of endogenous estrogen results in regression of the mammary gland, particularly the lobulo-alveolar structures. Mammary gland regression at 35 d postovariectomy in untreated control rats was greater than that in the untreated base-line rats measured at 14 d postovariectomy (the start of dietary treatment). Dietary gen-

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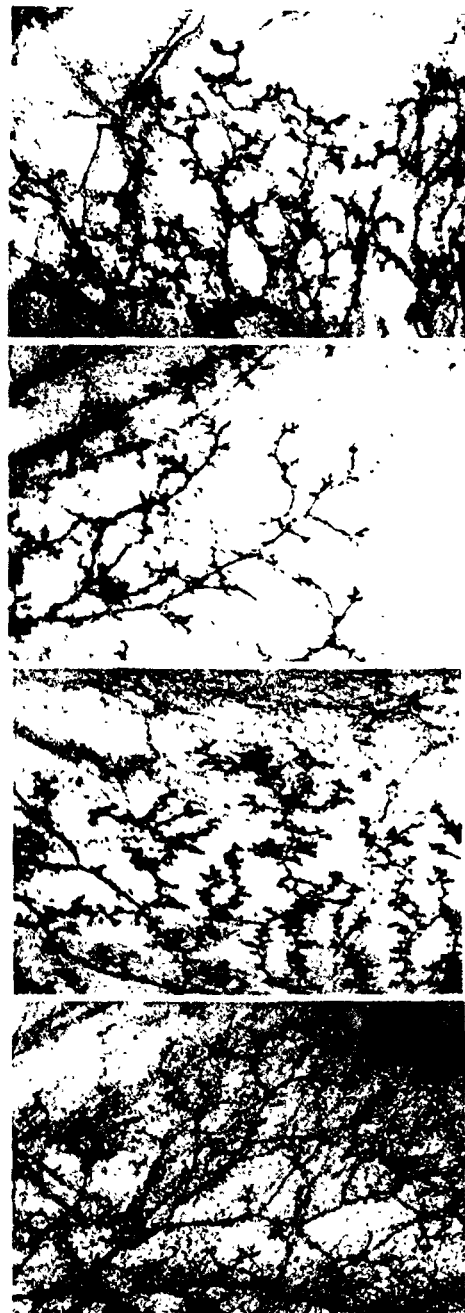


FIGURE 3 Photographs of the mammary glands from rats treated with dietary genistein and estradiol. Mammary glands were dissected from the rats and stained with alum-carmin. Photographs were taken through a dissecting microscope under 43X magnification. Photograph a is from the base-line group (killed at the start of dietary treatments), b from the control group (ovariectomized nontreated), c from the genistein 750 µg/g treatment group, and d from the 1.0 µg/g estradiol treatment group.

Genistein prevented regression of the mammary gland compared with the untreated controls. Our goal in this set of experiments was to evaluate whether the concurrent administration of genistein at different doses would inhibit the effects of estradiol in preventing regression of the mammary gland. Orally administered estradiol (1 µg/g) was effective in increasing uterine weight and plasma prolactin; however, it was ineffective in the

mammary gland. The reason dietary estradiol exerts estrogenic effects in the uterus and not in the mammary gland is unclear. The ability of genistein to inhibit the effects of estradiol in the mammary gland could not be determined with the dose of estradiol used in this study.

It is unlikely that genistein alone is directly responsible for the effects observed in the mammary gland. Maintenance of the mammary gland in rats is also dependent on prolactin. Prolactin synthesis in the anterior pituitary gland is under tonic inhibition by dopamine produced in the hypothalamus (Jones and Naftolin 1990). Estrogen is thought to decrease the activity of tyrosine hydroxylase, thereby decreasing the concentration of dopamine in the hypothalamus and pituitary (Jones and Naftolin 1990), resulting in increased plasma prolactin. Genistein or estradiol administered in the diet resulted in increased plasma prolactin compared with the ovariectomized control animals. The increase in plasma prolactin in this study suggests that dietary genistein functions in the hypothalamus and pituitary gland in a manner analogous to that of estradiol, leading to the synthesis and release of prolactin from the anterior pituitary gland.

Estradiol increased uterine weight and plasma prolactin yet did not affect regression of the mammary gland. This suggests that dietary estradiol (1.0 µg/g) is estrogenic in the uterus and on the hypothalamic/pituitary axis. However, this dose was not sufficient to affect the mammary gland.

Serum genistein analysis. Our results indicate that dietary genistein can elicit uterotrophic effects at a dose as low as 375 µg/g, mammatrophic effects at 750 µg/g and effects upon the hypothalamic/pituitary axis at 750 µg/g. The plasma concentration of genistein in rats fed 750 µg/g genistein was 2.2 µmol/L (conjugated plus free). This concentration was sufficient to elicit estrogenic effects in ovariectomized rodents. The intestinal metabolism, absorption, and half-life of dietary genistein, all of which could affect its bioactivity, are currently unknown. Genistein is present in soy products at concentrations as high as 1500 µg/g (Eldridge 1982). Women who consumed soy milk powder daily, which contained 227 mg genistein, had plasma genistein concentrations (conjugated plus free) of up to 6.0 µmol/L (Xu et al. 1995).

At present, there are no human studies in which the biological effects of pure genistein have been assessed and only a few which have evaluated the effects of a diet supplemented with soy. Published studies in which postmenopausal women consumed diets supplemented with soy have yielded conflicting results. Wilcox et al. (1990) showed that soy supplementation produced changes in the uterus similar to those produced by estrogen. However, other studies have failed to show effects different from controls by supplementing soy in the diet of postmenopausal women (Baird et al. 1995).

In the studies described here, genistein did not inhibit the effects of estrogen in either intact or estrogen-fed ovariectomized rats. However, in ovariectomized rats, genistein stimulated the growth of estrogen-responsive tissues, particularly the mammary gland. Perhaps in premenopausal women, genistein would have little, if any, estrogenic activity, whereas in postmenopausal women, the effects would be more pronounced. This raises some concern with regard to mammary tumorigenesis, which initially requires estrogen, because these studies have demonstrated that dietary genistein has estrogenic effects in the mammary gland of ovariectomized rodents. Further research is required in this area to clarify the effects of dietary soy, particularly in postmenopausal women in whom circulating estradiol is low.

Genistein is receiving much attention as a potential chemopreventive/therapeutic agent in the treatment and or preven-

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tion of various cancers. The results of these studies demonstrate the need for additional experiments on the biological effects of dietary genistein, particularly in tumor models, before any dietary recommendations can be made or supported.

ACKNOWLEDGMENTS

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Linda Kahl, 15:20 29/04/98 +1, GRAS Notice GRN 000001

To: Linda Kahl
 From: Dave Woodhams <woodhams@iprolink.co.nz>
 Subject: GRAS Notice GRN 000001
 Cc:
 Bcc: Me
 Attached:

HARD COPY.

Dear Dr Kahl

On 31 March 98 I sent you an Email relating to GRAS Notice GRN 000001.

Would please confirm receipt of this and advise what action is being taken by FDA in the issue.

I advise that I am today mailing a letter to you with copies of my correspondence with the US Nutrition Foundation attached. As ADM quote conclusions from the Foundation's position paper on soy infant formula this correspondence is relevant to ADM's GRAS determination.

Also enclosed with the letter is a hard copy of this and my previous Email, together with a copy of the Japanese paper, and its translation, referred to in the latter communication: Y. Ishizuki, S. Hirooka, M. Murata and M. Togashi, "The effects on the thyroid gland of soybeans administered experimentally in healthy subjects" Nippon Naibunpi gakkai shassi, 67, 622-623 (1991).

I have also enclosed a brief CV to place my credentials on record.

Under separate cover I am mailing copies of the first three newsletters of the Soy Information Network which cover the basis of our concerns, critiques of the soy industry response to our concerns and an invited article from Dr Messina, a soy proponent, in response to previous issues of the newsletter.

Under the same cover I am also sending a copy of my March 1995 paper on infant formula which is derived from the original Aspell Report by Dr Mike Fitzpatrick. This latter report, my introduction and my derivative paper on soy infant formula are available from the NCTF Library in Jefferson, Arkansas. My own paper was never intended to be submitted for independent publication and thus has not been peer reviewed but Dr Fitzpatrick's reports were independently reviewed before they were placed before the NZ Ministry of Health in November 1994. The three page independent review by a University of Auckland toxicologist is also included in the volume held by the NCTF Library.

Yours sincerely

BEST ORIGINAL COPY

Printed for Dave Woodhams <woodhams@iprolink.co.nz>

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With compliments from:

Soy Information Network

PO Box 32 236

Devonport 1309

NEW ZEALAND

Tel: +64 9 445 8721

Fax: +64 9 445 9834

ATTN: Dr LINDA S KAHL.

DR DAVID J WOODHAMS

**CEng, MChemE, M.IPENZ, MNZIC
DAIRY PROCESS CONSULTANT**

2/47 Church Street
Devonport 1309
New Zealand

Telephone: 09 445 8721
Facsimile: 09 445 9834
Email: woodhams@iprolink.co.nz

~~*[Signature]*~~

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NUTRITIONAL DEFICIENCIES IN SOY PROTEIN BASED INFANT FORMULAS

by

Dr David J Woodhams CEng
MChemE, MIPENZ, MNZIC

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Introduction:

In this paper, attention is concentrated on just one product: soy protein based infant formulas. In the main scientific report on the project, Dr MG Fitzpatrick identifies and discusses several groups of toxins and their effects in more general terms. The purpose here, however, is to illustrate with this one product the dangers that soy products pose to the consumer. By focussing on this single product, it is possible to capture in miniature a glimpse of the whole picture that the main report of the project portrays.

It must be said that the product selected for discussion seems to pose special dangers. Human infants are particularly at risk because, if they are being fed a soy based formula, it provides virtually the whole of their diet for a period of several months and this at a time when major body development and growth is occurring. In fact the investigating team believes that a serious threat to infants has been identified in the course of this study.

Soy protein based infant formulas:

Soy protein based infant formulas command about 20 to 25% of the infant formula market in the US and 13% in NZ although only about 1% of infants are allergic to milk protein. The reasons why the product is used to this extent, in the absence of clear medical requirements, are not known. It would seem that a mythology has been generated which has persuaded mothers or doctors that soybeans are somehow more natural than milk and that cow's milk is the cause of all infant maladies. It is often used as the first line of defence in cases of suspected lactose intolerance in infants and in cases of suspected milk protein allergy. It is doubtful whether these afflictions are positively diagnosed before the soy formula treatment is employed.

In the dairy industry, great strides have been made in the past 30 years in the understanding of the fine composition of both human and bovine milk. With this understanding has come a growing ability to emulate human milk in the production of dairy-based infant formulas. At the same time, an appreciation has been gained of the immense and apparently inexhaustible subtlety and complexity of nature in the evolution, over long ages, of these foods for the young. A recent symposium, "New Perspectives in Infant Nutrition" [Renner and Sawatzki, 1993] provides ample evidence of the importance of the minor constituents of human milk. At least with cow's milk the basic components [proteins, fats, carbohydrates, vitamins and minerals] are very similar to those in human milk and have evolved to perform similar functions [the building of bone, muscle and other tissue and the provision of metabolic energy]. If there are any minor constituents in human milk which, in the formulation of a dairy-based formula, are overlooked in ignorance, they may well be partly compensated for by similar or identical factors from the cow's milk.

The problem of emulating human milk with plant proteins, and other components that are taken out of the role for which they developed naturally, is of an entirely different order, particularly when the subtlety and complexity of the biochemistry of the plant world, evolved for plant functions, is taken into account. In this situation, factors which are essential for the complete nutrition of the infant must be known before they can be provided for. If they are unknown, obviously they cannot be included.

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Any toxins or anti-nutritive components in the raw materials which adversely affect the infant must first be identified and then removed. If they are unknown or unidentified, of course, their removal will be a matter of chance.

These problems are inherent in the manufacture of soy formulas. The soybean has evolved for the survival of the soybean species, not for the benefit of human infants. The process of evolution has bequeathed us human milk for our newly born and young children; the further we stray from that natural standard, the greater is the risk that we will seriously compromise the nutritional needs of the infant child.

In this paper several classes of natural constituents of soybeans are discussed which are not naturally present in human milk. Some of these components are at least partially inactivated during processing but others are neither removed nor destroyed by so-called "adequate treatment".

Trypsin Inhibitors:

The first components of soybean products which may affect infant nutrition and health are the trypsin inhibitors. These are proteins which bind the enzymes trypsin and chymotrypsin so that they are unavailable to break down the protein in the diet. Trypsin and chymotrypsin are two of the main digestive enzymes secreted by the pancreas into the small intestine to digest proteins in the food. They work by breaking the bonds between amino acids in the middle of the protein chains. The amino acids, which are the building blocks of proteins, are then absorbed and used to build the new proteins required for a multitude of tasks in the body. If the actions of the trypsin and chymotrypsin are inhibited, the body is deprived of these essential building blocks. The body then either fails to grow properly or wastes away.

The heat treatment of soy products is designed to denature the trypsin inhibitors [see Introduction]. However, Wormsley [1988] expresses concern that some soy milk preparations contain quantities of residual heat-stable trypsin inhibitory activity that are sufficient to inhibit most of the trypsin secreted by normal infants in response to a meal.

Newborn and very young infants are particularly vulnerable to the effects of these anti-nutritive proteins. Not only is the acidity in the infant stomach much lower than in the adult¹ but food in the infant stomach also has a shorter residence time² so that biologically active proteins are very much less likely to be inactivated by digestion in a baby than in an adult. Thus the reasons for particular concern regarding the residual trypsin inhibitor content of soy-based infant formulas and the effect on infant nutrition is apparent.

There are further concerns with this component, however. In a major review of soy products and the human diet by Erdman and Fordyce [1989] the authors state that:

"For monogastric animals a reduction of approximately 80% of the trypsin inhibitor activity by heat processing is sufficient to produce optimal protein nutrition and growth."

¹ Infant stomach pH = 3 to 5, adult = 1.5 to 2.5

² Infants 5 minutes to 30 minutes adults 3.5 hours to 4 hours

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The remainder is, however, still worrying. They go on to say:

"Of ultimate concern is whether prolonged exposure of the human pancreas³ to trypsin inhibitors from soy and other sources may eventually lead to pancreatic cancer. This question . . . is unresolved currently. The questions must be asked because many infants and vegetarian groups consume significant quantities of soy protein [and] could be chronically exposed to low levels of trypsin inhibitors. The long term effect of low intake of trypsin inhibitors has not been delineated and is now being investigated in humans. Research studies with humans are needed before this concern can be resolved."

The reason for the concern is that soybean trypsin inhibitors promote pancreatic cancer in rats once it has been initiated by a carcinogenic agent [Gumbmann, 1986; Roebuck, 1987].

It has been long known that trypsin inhibitors cause pancreatic hypertrophy⁴ and hyperplasia⁵ in rats, mice and chickens. On the basis of data obtained from experiments with larger animals, [the pig, the dog and especially the primate Rhesus and Cebus monkeys], it is assumed that trypsin inhibitors in the human diet do not cause abnormal growth of the pancreas. However, the information that deaths in the USA from pancreatic cancer have increased during the same period that the use of soybeans products in the diet has increased [Roebuck 1987] means that the "ultimate concern" of Erdman and Fordyce is still an open question.

According to Hathcock [1991] of the Center for Food Safety and Applied Nutrition, US Food and Drug Administration, more data are needed for the risk of the influence of long term exposure to low levels of residual trypsin inhibitors on human pancreatic cancer to be assessed properly. The diet for the two year [animal] trial suggested would have to be carefully controlled with respect to the type and amount of fat, because fat also promotes pancreatic cancer, and account would have to be taken of the effect of the level of trypsin inhibitor in the diet on protein nutrition.

Estrogens⁶:

A second group of components of soybeans, known as estrogens or phytoestrogens⁶ are naturally present in the beans and survive "adequate processing" to appear in soy based infant formulas. The principal phytoestrogens in soybeans are genistein and daidzein, together with their derivatives, collectively known as the isoflavones.

³ The pancreas is a body organ consisting of glandular tissue. The larger part manufactures enzymes involved in the digestion of proteins, carbohydrates and fats and secretes them into the small intestine. The smaller part secretes the hormones insulin and glucagon which between them control the glucose level in the blood.

⁴ Hypertrophy means an abnormal enlargement of a cell or organ, usually because of disease.

⁵ Hyperplasia means excessive multiplication of cells in the body or an overgrowth of tissue.

⁶ Estrogen [US spelling] or oestrogen [British] is the generic term used for a group of hormones which influence sexual physiology and behaviour. The active principals in oral contraceptives are estrogens. Estrogens produced by plants rather than animals are called phytoestrogens. Strictly speaking, phytoestrogens are not hormones which, by definition, are substances produced by cells of the body for export to other parts of the body. They are better called estrogen-mimickers or xenoestrogens [alien estrogens]. Many oral contraceptives are estrogens but not hormones.

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Hormonal Effects:

Genistein first came to the attention of agricultural research workers because of its effects on the fertility of sheep. Reproductive disorders in sheep fed soybean plant forage were first recorded by Hunt in 1935. An investigation of infertility in sheep in Australia, by Bennetts *et al* in 1946, revealed that excessive consumption of subterranean clover was to blame. These two observations were linked by Bradbury and White in 1951 when it was found that genistein was the principal phytoestrogen in red clover as well as in soybean fodder. Alfalfa, or lucerne, a leguminous crop used for animal feed, is also known to be a rich source of genistein and daidzein, also coumestrol. Coumestrol is a more potent estrogen than the other two and is also found in soybeans but at lower concentrations. Research on the influences of the isoflavones genistein, daidzein and coumestrol on the fertility of sheep continues today.

As part of the present study, analyses of the genistein and daidzein contents of four locally available soy-based infant formulas were conducted and the results are recorded in Table 1. These data, in conjunction with the instructions on the containers regarding the quantities and frequency of feeding, were used to calculate the daily quantities of genistein and daidzein consumed by a baby at different ages [Table 2]. The calculated daily quantities consumed from birth to 6 months of age vary from 7.4 to 25.7 mg per day, depending on the age of the baby and on which soy formula is used.

TABLE 1
Concentrations of total genistein and daidzein
in soy-based human infant formulas^{*}

Sample	Genistein, Total [mg/kg]	Daidzein, Total [mg/kg]
Infasoy	98	53
Isomil	72	53
Prosobee	118	59
Karicare	91	48

^{*} Analysis by MG Fitzpatrick, Grayson Laboratories Ltd

In a study by Cassidy *et al* [1994], adult women were fed a daily dose of 45 mg of soy estrogens⁷ for one month, equivalent to 0.73 mg isoflavone per kg body weight. The researchers found that the normal mid-cycle surges in the hormones which trigger ovulation were significantly suppressed and that there was a significant delay in the onset of menstruation, or lengthening of the menstrual cycle. These effects were measured in the course of only one month on the soy diet but the effects in some of the subjects persisted for up to 3 months after soy feeding stopped.

⁷ The diet was 60 g/day soy protein containing 0.75 mg/g soy isoflavones, or estrogens

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TABLE 2
Daily isoflavone consumption based on the recommended daily intake of soy-based human infant formulas

Age [months]	Infasoy [mg/day]	Isomil [mg/day]	Prosobee [mg/day]	Karicare [mg/day]
< 1	9.4	6.9	12.9	10.8
1 - 2	16.0	12.0	19.1	14.3
2 - 4	19.9	15.4	22.8	17.9
4 - 6	26.6	17.6	24.4	17.4

Using the isoflavone quantities consumed by a baby calculated above, and taking into account relative body weights^a, during the first six months of life the baby ingests daily 2.4 to 5.8 times the dose [mg isoflavone per kg body weight] of soy estrogens that induced significant physiological effects in adult women in the Cassidy study. Note that, for the infant, this feeding regime and estrogen exposure may continue for a year or more, whereas the trial for the women lasted only one month.

The impact of the soy estrogens on human infants is not known. There is no research published. The issues have not been explored. However, the effects of the synthetic estrogen diethylstilbestrol [DES] on the children of mothers who were treated with this drug during pregnancy are well documented. The use of DES during pregnancy was approved by the USFDA to reduce the chances of miscarriage [It was also approved for suppression of lactation in women who did not wish to breast feed their infants, as therapy in advanced breast cancer, as an agent in hormone replacement therapy and as a growth stimulant in cattle and sheep]

It was 20 years before the effects of such DES treatment on the infants exposed *in utero* was discovered and another 5 or more years before it was withdrawn from use. DES daughters have a much higher probability of cervico-vaginal abnormalities, affecting both their fertility and the probability of their suffering a miscarriage [Kincl, 1990; Apfel and Fischer, 1984]. The latter researchers also report a strong association between fetal exposure to DES and clear cell adenocarcinoma, a rare form of malignant vaginal cancer, as well as a higher than normal incidence of genito-urinary abnormalities and infertility in fetally exposed DES males.

DES is about 100,000 times more potent than the phytoestrogens in soy [Bickoff *et al.* 1962]. However, there are large quantities of phytoestrogens in processed soy products, more than enough to counteract the lower intrinsic potency.

Estrogens are metabolised in the liver. When DES was used as a growth stimulant in cattle, if the beef was "mishandled" [ie slaughtered with too short a withholding period], the liver could contain as much as 0.5 ng/g of DES. Because of the relative abundance of the weaker soy estrogens, soy protein isolate contains the equivalent

^a The average weight of the women was 64 kg and weights for a baby of 3.6 kg at birth rising to 7.2 kg at 6 months of age have been assumed

of 3.9 ng/g of DES [Murphy, 1982]. Soy protein isolate comprises about 15 to 20% of soy infant formula. Thus the estrogenic effect of soy infant formula is closely comparable with that of liver tissue from "mishandled" DES beef.

The effects of phytoestrogens in fetal and neonatal animals other than humans have been studied and the effects observed parallel those that have been observed in human infants exposed to DES. While care must always be exercised in extrapolating results from one species to another, in the absence of any other evidence the warning signs should be heeded.

Hormonal imprinting of the neonate, leading to the full development of the reproductive system, occurs during the period immediately after birth. Normally, an endogenous^a hormone attaches to a receptor within a cell. By doing so it initiates a series of biochemical reactions which result in normal development of the neonate's reproductive system. At the same time it initiates the reactions that are needed to metabolize the hormone, thus maintaining the necessary hormonal balance in the body. Professor Cliff Irvine, a reproductive endocrinologist at Lincoln University, uses the analogy of a key and a lock. Normally, the key fits both the lock and the tumblers and, turning, unlocks the needed processes. However, if the receptor site becomes occupied by a foreign estrogen or estrogen-mimicker, it is as if the key fits the lock but not the tumblers. It cannot be turned. Nor can a more appropriate key, an endogenous hormone, gain access to the lock. The required processes either to develop the reproductive system or to metabolize the hormone are thus not initiated. If a sufficient number of receptors are so blocked, hormonal imprinting does not take place and the potential exists for:

- disturbance in reproductive functions,
- abnormal development of the sex organs,
- atypical sexual behaviour patterns;
- predisposition of certain tissues to the development of cancers

As was the case with the effects of DES, it is expected that the impact of these potential problems would become apparent only in later years, after puberty

It has been argued that an infant being fed naturally on breast milk receives high levels of the maternal hormones estradiol, estrone and estrone, which are more potent than the soy phytoestrogens. [Genistein and daidzein are respectively 1200 and 7500 times weaker than estradiol, the most potent of the maternal estrogens (Markiewicz *et al.* 1993).] However, three points need to be considered in rebuttal of this claim:

- the quantities of maternal hormones in breast milk are at a maximum during the first week after the birth and thereafter decline rapidly;
- Even during this first week *post partum*, the estrogenic effect of the maternal hormones is only about 1% of that of the phytoestrogens in soy formula, because of the abundance of these latter components in soy protein isolate;
- Over uncounted generations the human infant has adapted to the presence of the maternal hormones in breast milk immediately after birth and thus has

^a An endogenous hormone is one that originates within the infant's own body

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the means to metabolize them, or otherwise deal with them. It is only over the last one or two generations that infants have been challenged with the alien estrogen-mimickers from soybeans

It has also been argued that Japanese and Chinese babies would get high levels of the soy phytoestrogens from their mothers' breast milk because of their mothers' high consumption of soy products. Thus, the argument runs, if soy estrogens caused problems in infants, the effects would have been observed already in Japanese and Chinese babies. Because no adverse effects have been observed, the isoflavones must be harmless.

We have found no direct measurements of the phytoestrogen content of Japanese breast milk. There are, however, data available on the concentration of the soy isoflavones in the blood serum of women who are high soy consumers [Xu et al., 1994]. Working with the assumption that the phytoestrogen concentration in breast milk will be no higher than in the serum, soy infant formula contains at least 35 times the concentration of soy isoflavones likely to be found in Japanese breast milk. Thus the apparent absence of observed effects in Japanese babies is irrelevant.

Immunosuppressive Effects:

Quite separate from the hormonal effects of the soy isoflavones are the now well established effects of genistein in suppressing the immune system. Genistein was identified as a powerful immunosuppressive agent by Atluru & Atluru [1991], who were seeking improved immunosuppressive agents for use after human organ transplants. Similar evidence is presented by McCabe [1993].

While immune suppression has not been investigated specifically with respect to infants, most mothers would not choose to feed a powerful immune suppressant to infants as part of their sole diet in the first six months of life if they were able to make an informed decision.

Lectins¹⁰:

Another component of soy products which poses an especially significant threat to infants is a class of proteins called lectins, also known as hemagglutinins because of their ability to cause blood to clot. Lectins vary in their toxicity from the very poisonous ricin, a lectin found in the castor bean, to the lectin in the ordinary green pea, which is essentially harmless to humans.

Apparently, one of the main functions of the lectins in soybeans is to bind the nitrogen-fixing bacteria in the soil to the roots of the plant as they form the root nodules which are typical of legumes. Within these nodules the bacteria convert atmospheric nitrogen to a form that the plant can use, at the same time enriching the surrounding soil with nitrogen.

¹⁰ Lectins are defined as carbohydrate-binding proteins of non-immune origin which agglutinate cells. It is their ability to bind to the carbohydrate structures that form the walls or outer membranes of cells that permits the characteristic agglutination

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Lectins, as they are now defined, have two sites, each of which binds to a specific carbohydrate structure. This ability to bind to carbohydrate structures in cell walls is the basis of their blood agglutinating properties and carries over into the effects of lectins on the cells and membranes of the intestine.

While soybean lectins are assumed to be readily destroyed by the heat of "adequate processing" [Smith and Circle, 1978], the presence of lectin in soy protein isolate is reported by Hisayasu et al. [1992]. No attempt was made in the present investigation to measure lectins in the soy-based infant formulas.

In a recent text book on plant lectins, Pusztai [1991], a leading research worker in this area, summarises the following "common features of toxic [anti-nutritive] effects in lectin-gut interactions"¹¹

- 1 High degree of resistance to gut proteolysis¹²,
- 2 Binding to brush border cells; damage to microvillus¹³ membrane, shedding of cells, reduction in the absorptive capacity of the small intestine,
- 3 Increased endocytosis¹⁴, induction of hyperplastic growth¹⁵ of the small intestine, increased turnover of epithelial cells¹⁶,
- 4 Interference with the immune system, hypersensitivity reactions,
- 5 Interference with the microbial ecology of the gut, selective overgrowth,
- 6 Direct and indirect effects [hormones etc.] on systemic metabolism "

Newborn and very young infants are particularly vulnerable to the effects of soybean lectins [Grant, 1994] for the same reasons as were quoted for the trypsin inhibitors, the higher pH and the reduced residence time in the infant stomach. Again there is cause for particular concern regarding the lectin content of soy-based infant formulas.

Pusztai concludes his discussion of the effects of soybean lectin on the cells of the small intestine by saying:

"The anti-nutritive effects of soya lectin are now well established. Its binding to the small intestinal epithelium induces a number of changes in intestinal function and morphology¹⁷ some of which reduce the food conversion efficiency in soya-fed rats."

¹¹ Quoted directly from Table 6.9 [p179] of "Chemistry and Pharmacology of Natural Products: Plant Lectins" by A. Pusztai, Cambridge University Press, [1991]

¹² Proteolysis is the digestive breakdown of a protein into its component parts. The resistance of lectins to digestion means that they are more likely to survive passage through the stomach to reach the small intestine intact and viable.

¹³ Brush border cells and the microvillus membrane form the lining of the intestine and are the primary sites for absorption of nutrients into the body's circulation system.

¹⁴ Endocytosis is the uptake by cells of particles that are too large to diffuse through the wall.

¹⁵ Hyperplastic growth is a tumour.

¹⁶ Epithelial cells are those that form the lining of a cavity that opens to the body surface.

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He goes on to say :

".... most soya preparations used in feeding studies contain appreciable amounts of functional lectin. Moreover, brush border membranes have many binding sites for the soya agglutinin. Thus, potentially damaging interactions between the small intestinal epithelium and the dietary soya lectin may be of a regular daily occurrence in both humans and animals."

As noted earlier in the summary table by Pusztai, lectins are also known to interfere with the immune system. Pusztai says [p 95]:

"It has been recognised for some time that mitogenic stimulation of lymphocytes¹⁷ with lectins can produce suppressor cells which are capable of inhibiting all biological activities of both the T and B cells¹⁹."

It is understood that lectins were at one time used as immune system suppressants after transplant surgery. Now, however, they have apparently been supplanted by alternative drugs, partly because of the unwanted side effects of lectins.

The investigating team has found no evidence of any standards for the permissible levels of soy lectin in infant formulas. It is apparently assumed that "adequate treatment" of the soy meal is all that is required and, at least in New Zealand, even this is not checked by the authorities.

Phytic Acid²⁰:

A fourth anti-nutritive component in soy products is phytic acid, a substance which is only slightly affected by the "adequate processing" heat treatment. Phytic acid is composed of molecules which have the capability of chelating²¹, or binding, certain mineral atoms, thus making them unavailable, or available only with difficulty, to the body. Examples of the minerals needed by humans, in larger or smaller amount, that are easily chelated are calcium, magnesium, zinc, iron, copper, selenium, cobalt.

For infants who depend solely on soy formula for nutrition there is concern that the daily intake of important minerals may be lower than required. For instance, the absorption efficiency of zinc from soy-based infant formulas is approximately 14%, compared with 31% from cow's milk. Erdman and Fordyce [1989] have noted that the issue of bioavailability of zinc from soy-based infant formulas is clearly an area of particular concern. While further fortification with zinc is possible, the variability of the phytate content of soy protein could lead to excessive zinc absorption by the

¹⁷ Morphology refers to structure as opposed to function

¹⁸ Lymphocytes are a type of leucocyte found in lymph. Leucocytes are the white blood corpuscles which are the scavengers of the immune system. Lymph is a colourless alkaline fluid from tissues and organs of the body, like blood but without red corpuscles

¹⁹ T and B cells are the workhorses of the immune system which identify and attack foreign bodies

²⁰ Phytic acid is an cyclic organic acid containing six phosphate groups. It is commonly found in cereals and is in most soy products in considerable quantities, typically 1 to 2%

²¹ Chelation is a process whereby an organic molecule combines with a metallic ion so that it is less available to react with other molecules in the environment

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infant. The margin between enough zinc and too much is not wide [McGillivray, 1994].

The phytic acid content of soy-based infant formulas is not reported on the containers and has not been measured in this study. However, on the assumption that the formulas contain approximately 15 to 20% soy protein isolate, the phytate contents are estimated to be in the range 0.23% to 0.50%. At this level, it is a distinct possibility that the zinc status of infants fed soy formulas may be diminished by the presence of phytate [Fitzpatrick, 1994]. As calcium and phytate work synergistically to reduce zinc bioavailability even further [Likuski and Forbes, 1965], the high calcium contents of the soy formulas will aggravate the problem.

The role of phytates associated with dietary fibre in the diet of adults is still a matter for discussion and dispute in the scientific literature, with some investigators attributing the lower rates of colon cancer from high fibre diets to the phytate rather than the fibre [Graf and Eton, 1985] and others maintaining that the level of dietary fibre recommended by many human nutritionists will result in a compromised mineral utilization because of the associated phytate content [Sanstead, 1992].

In the case of the infant, however, the effects of phytate on mineral availability in a fibre-free diet during the period of maximum growth must be the dominant concern

Conclusion:

Erdman and Fordyce [1989] report an estimate [by others] that 0.3 to 0.7% of infants in the United States are sensitive to cow-milk protein and that of these approximately 25% are also allergic to soy preparations²². Thus it would seem that only one infant in about 200 is allergic to cow's milk protein and at the same time is not allergic to soy protein and so might both require and benefit from the alternative soy formula. Even for these, however, a soy-based infant formula may not be the best alternative. According to Erdman, "A variety of alternative formulas such as those based on extensively hydrolysed casein, whey, or other protein sources²³ may be superior." [Erdman 1990] He suggests that the National Committee on Nutrition of the American Academy of Pediatrics should convene to reassess the issue of soy protein based formulas which they had last addressed in 1983. At that time their recommendations, quoted by Erdman, read:

"The use of soy-protein formula should be approached with thoughtful consideration of indications for use. Based on the information given here, specific recommendations for the use of soy protein formula include

[1] in vegetarian families in which animal protein formulas are not desired.

²² In an exchange of letters following the publication of this paper between Witherly [1990] and Erdman [1990] two other references are quoted which put this latter figure at greater than 50% and 35% respectively

²³ Hydrolysed casein etc. refers to milk proteins, and other proteins, which have been broken down or pre-digested by some form of processing so that the structures that elicit the allergic response are no longer present in the food

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[2] in the management of galactosemia, primary lactose deficiency, or the recovery of secondary lactose intolerance, because these formulas are the least expensive and most available formulas not containing lactose, and

[3] in potentially allergic infants with a family history of atopy who have not shown clinical manifestations of allergy. However, these infants should be monitored closely for allergy to soy protein."

Instances when soy-protein formula should *not* be used included:

"[1] for the routine feeding of pre-mature and low-birth-weight infants, use should be for limited periods, if at all,

[2] in the dietary management of documented clinical allergic reaction to cow's milk protein and/or soy protein formula, and

[3] in the routine management of colic."

These 1983 recommendations are by no means an open endorsement of soy formulas and the indication is that Erdman would expect later recommendations to be more restrictive. It is not known whether the National Committee on Nutrition of the American Academy of Pediatrics has met again. No reference to their findings has been located in our database searches.

In this paper I have outlined potential dangers to infants from consuming soy based infant formulas, as identified in the scientific literature, drawing on specific analyses done on four soy infant formulas. Previous warnings by other workers about possible risks from the high levels of weak phytoestrogens in processed soy products have gone unheeded but have not been as fully articulated as herein. I have drawn particular attention to the fact that there are no studies available that provide definitive answers to the questions raised. Such studies are urgently needed.

The argument that has been most frequently raised against our proposition that phytoestrogens and other anti-nutritive factors in soy infant formulas are dangerous is that no evidence of harm has been observed in 40 years of use in the US or in 25+ years of use in New Zealand.

It is entirely possible that evidence of harm has in fact been observed but that the symptoms have not been linked to the cause. Given that some of the harmful effects, for instance infertility, may appear only in later life, this lack of causal connection is understandable. Because a number of commonly used pesticides, together with PCB's and dioxin, are also estrogen mimickers, the role of soy estrogens may be overlooked or even masked. Even more immediate effects for which we have only anecdotal evidence, such as severe temperature fluctuations, hyperactivity and aggression, may not have been linked to soy formula consumption because the medical profession has not previously been alerted to the possibility. The questions have not been asked. The data do not exist.

Thus the statement that "millions of babies have been successfully raised on soy formula world wide over the last 20 to 40 years" is fundamentally flawed. The word "successfully" presupposes that someone has in fact done a long term retrospective study of the health and behavioural histories of people who, as infants, were fed soy

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formula. It also presupposes that, as a result, it has been determined that in all respects their histories are the same as those of people who were raised on breast milk or on alternative, non-soy formulas.

The retrospective epidemiological studies have not been done. It is unethical and misleading for the soy industry and promoters of soy infant formula to act as if the results of such a study will undoubtedly clear soy infant formulas.

The investigators consider that the effects of the soy isoflavones, found to be present in all four of the soy infant formulas tested in the present study, constitute potentially serious threats to the development and life processes of infants including development of the reproductive system and suppression of the immune system. The presence of phytic acid threatens the bioavailability of essential minerals if not adequately controlled. Residual trypsin inhibitors have not been cleared of the suspicion that they might contribute to pancreatic disorders. The soy lectins present their own potential for harm to the gastrointestinal system.

Until such time as a major retrospective study has been completed so that decisions can be taken on the basis of established fact rather than on extrapolating results from other species, we believe that mothers and their medical advisers should avoid the use of soy infant formulas wherever possible. In the presence of such significant doubt the issue must be resolved in favour of the safety of the infants.

We have recommended to the Minister of Health that the Ministry:

- require that soy infant formula be made available only with medical advice,
- advise all medical practitioners, pharmacists and other health professionals of the potential dangers,
- promote a retrospective study to establish the comparative health and behavioural histories over a period of 25 years of groups with different infant feeding histories, including breast feeding, dairy-based formula and soy-based formula.

Before taking our case to the Ministry the report of our work was reviewed independently by Dr Iain Robertson, HRC Senior Research Fellow and Senior Lecturer in Toxicology at the University of Auckland Medical School. His first recommendation [out of a total of eight] was "To discontinue the use of soybean products in infant formula..."

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The literature research by Dr MG Fitzpatrick and his analysis work, both at Allan Aspell and Associates and at Grayson Laboratories, were privately funded by Mr RF and Mrs VA James, aviculturists of McLeod's Bay, Whangarei Heads. I have made liberal use of this work throughout the paper.

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Soy Information Network

PO Box 32 236
Devonport 1309
New Zealand

SIN Newsletter # 1

7 November 1995

WELCOME

Welcome to the Soy Information Network and to its first newsletter. The network has been set up by concerned scientists, parents and citizens to provide information on soy products that the soy industry and the Ministry of Health are reluctant to provide.

In a later article we set out the basis of our concerns about soy infant formula. In later newsletters we will provide more information and an opportunity for you to express your own opinions.

At this early stage we are gathering information to assist in any enquiry that may result from our suggestion that research into the long term effects of soy infant formula is required. Initially we need to get a feel for the range of experiences that people report so that we can design a questionnaire which covers the ground properly. We are grateful for your contribution to this pool of information.

We are interested in receiving details of your particular experience, especially with soy infant formula. If we receive such information from you we undertake to treat both the information and your own details with complete confidentiality. Personal information will not be released. If a media representative wants to contact members of the network, we will write to you and invite you to contact the particular reporter. In this way you, and only you, will be in control of whether contact is made. If it becomes apparent that particular symptoms are appearing regularly, we will give a generalised account in a newsletter without divulging personal details.

There are at least two broad groups of users of soy infant formula.

- 1 Those whose infants are unable to tolerate cow's milk formula for some reason. If your child was/is in this category, please tell us how your child's problem was diagnosed.
- 2 Those who choose to use soy formula for some reason other than a diagnosed allergy or intolerance. If your child was/is in this category, please tell us why you chose to use soy formula.

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If you still have Plunket records of your infant's feeding regime, please let us know. The existence of such records may be of great value if an epidemiological study is undertaken in the future. Please note that we are interested in hearing from you whether your experience of soy formula was good or bad.

Feel free to share the information in this newsletter with your friends and invite them to write to us if they wish to become a member of the Soy Information Network.

If you would like to assist with a donation towards our costs please send a cheque made out to "Soy Information Network". Thank you.

NZ SCIENTISTS TO ATTEND INTERNATIONAL CONFERENCE ON PHYTOESTROGENS IN DECEMBER

Two New Zealand scientists will be attending the Third International Conference on Phytoestrogens in Little Rock, Arkansas, from December 3 to 6. Dr Mike Fitzpatrick, the independent consultant who prepared the reports which revealed the potential for problems with soy products, and Professor Cliff Irvine, a reproductive endocrinologist at Lincoln University, will both be at the conference to present an invited paper on the soy infant formula issue to this scientific forum. The Conference is organised and sponsored by the Division of Reproductive and Developmental Toxicology, part of the US Food and Drug Administration's National Center for Toxicological Research.

The Conference lasts for two and a half days and one and a half days are to be devoted to papers exploring the benefits and the risks of dietary phytoestrogens. The wide publicity which has resulted from the New Zealand work has caused a great deal of interest in this conference and a number of scientists from around the world will be attending. We understand that the soy industry will also be present in force. We hope to present some notes from the conference in the next SIN Newsletter.

SOY INFANT FORMULA: THE QUESTION

The following article sets out the basis of the concerns of those who have been involved with the attempt to have soy infant formula removed from general sales until an epidemiological study has been carried out. We believe that the mothers of young infants have a right to this knowledge so that they can exercise an informed choice.

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When we first approached the Ministry of Health a year ago and gave them the results of our analyses of soy infant formulas, together with the results of our search of the scientific literature, we expected that this would start a scientific debate and would trigger research that would reach some sort of conclusion within a year or two.

We also expected that mothers would be warned by the Ministry that soy infant formula contained certain substances derived from soy beans which are not normally present in a baby's diet when fed on breast milk. There are several of these substances but the ones we emphasised to the Ministry are called *isoflavones*. Because of their estrogenic effects in animals they are also called *phytoestrogens* which means "estrogens derived from plants". These substances are able to occupy the sites in cells which are normally available to hormones generated within the infant's body, hormones that influence how these cells develop. Although the isoflavones are very much weaker than the natural hormones, they are present in soy formula [and many other soy products], at very much higher concentrations. The higher concentration may cancel out the effect of them being weaker.

Unfortunately, the Ministry did not give this warning. This means that people are buying and using soy infant formula without full knowledge of what they are buying. It is also unfortunate that the research that is essential to determining whether soy infant formulas are safe in the long term has not been started. The Plunket Society was planning an investigation earlier this year but their plans were disrupted by the denial of research funds by the Ministry of Health. Research funding is being sought currently by about three people or groups of researchers that we are aware of but to date none has been awarded.

The Argument:

Our argument is quite straight forward.

- Recent research at Cambridge University shows that when women who have not yet reached menopause eat soy protein at normal dietary levels [60g soy protein per day], the isoflavones in the diet have a marked effect on their menstrual cycle and on the hormones that trigger ovulation. The menstrual cycle was lengthened by 1½ days and the two main ovulation hormones were reduced to a half and a third of their levels before the soy diet was started. This means that the soy isoflavones are biologically active in humans at dietary levels, a fact which was not known directly before this work was done. The amount of isoflavones in the diet of these women was 45 milligrams per day, their average weight was 64 kilograms, so the dose rate was 0.73 mg isoflavones per kg body weight per day. They were on the soy

diet for 30 days [one menstrual cycle] and it took up to three months for the effects to wear off. The validity of this research is widely accepted by scientists on both sides of this debate.

- There are four soy infant formulas on sale in New Zealand: Prosobee, Infasoy, Karicare and Isomil. Our analysis shows that they contain around 150 mg [range 177 to 125] of isoflavones per kg of dry formula. When the formula is made up as directed and fed as recommended on the labels on the cans, a baby will consume a dose of around 10 mg isoflavones per day during the first month of its life, rising to about 20 mg per day at four months and older. When these consumptions are converted to a dose rate, by dividing the dose by the approximate weight of the infant at these ages, we find that they are consuming about 2.5 mg per kg body weight per day at one month of age and 3.5 mg/kg/day at four months. These dose rates are 3.5 to 4.8 times higher than the rates which caused the changes in the women's menstrual cycles when fed to them for 30 days. However, babies may be fed this formula for up to six months as the sole source of nourishment and for much longer than that as part of a more diverse diet. The validity of these analyses can easily be checked in any competent laboratory having the right equipment and the appropriate pure isoflavone standards. The analyses are in line with those reported by other scientists. The dose rate calculation is, we believe, novel, in that we have not seen it reported elsewhere. However, the calculation is simple and straightforward.
- The argument at this point is more speculative because the facts are not available. Babies are growing and developing and are thus more likely to be at risk from interference from unusual estrogenic substances. They are also less likely to have developed the biochemical mechanisms necessary to detoxify foreign substances that evolution has not prepared them for. The difficulty is that developmental problems may not become apparent for many years, perhaps at puberty or beyond, and it is unlikely that problems observed at that stage of life will be related back to the infant feeding regime.

Those are some of the facts and considerations that we thought mothers had a right to know before they decided to feed their baby on soy formula, especially those mothers whose infants are not diagnosed as being allergic or intolerant of cow's milk formula. The Ministry and the soy formula manufacturers think otherwise.

Put very simply, we have asked the following question:

"If the soy phytoestrogens, as part of a diverse diet, have this marked effect on women's menstrual cycles at a dose rate of 0.73 mg per kg body weight per day for 30 days, what will the effects be on infants who consume 2.5 to 3.5 mg per kg per day for six months when soy formula is their total diet?"

We are still waiting for an answer.

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WHY FEED SOY FORMULA TO INFANTS?

There are several reasons why a mother might choose to feed soy formula to her baby. The usual reasons are:

- Because she believes her child is allergic to dairy protein,
- Because she believes her child is intolerant of lactose,
- Because she believes soy formula is more healthy than dairy formula.

Protein Allergy:

As far as we can determine, something less than 5% of infants (and less than 1/2% of adults) have a genuine allergic response to dairy protein. Of these, about a quarter to a half are also apparently allergic to soy protein. Children tend to outgrow milk allergy by the time they are 3 years old. Given the possibility of problems with soy formula, we would advise a prudent mother who suspects that her child is allergic to one of the cow's milk proteins to get a positive diagnosis of this through her doctor or through Plunket and to have the baby checked for sensitivity to soy protein at the same time. It is all too easy to assume that a problem is due to an allergy and not to check it out. There are several non-soy options for getting around allergenicity to a specific dairy protein. Your doctor should be able to advise you on these if you make it clear that you would prefer not to use soy. The options include formula based on goats' milk, formula based on cows' milk which has had some of the proteins removed, and formula made from hydrolysed dairy protein. [The allergic reaction depends on the shape of the protein molecule. When proteins are hydrolysed they are broken down by enzymes to the stage where the shape that causes the allergic reaction is no longer present.] There may be some flavour problems with the hydrolysed product and it is more expensive. In a case of need it may be possible to get a subsidy - ask your doctor.

For a more extended discussion of milk allergy we recommend that you write to the Dairy Advisory Bureau, PO Box 417, Wellington for their short publication "Milk Allergy - Is it an issue for you?" This includes information on lactose malabsorption and was prepared with the assistance of independent advisors.

Lactose Intolerance:

After watching the TV advertising for certain soy products, one could be forgiven for believing that lactose is some sort of insidious evil compound lurking in cows' milk. It isn't. Lactose, or milk sugar, is the main carbohydrate in the milk of almost every one of the mammal species on earth for which the composition is known. The only

exception we are aware of is the sea lion. Presumably there are evolutionary advantages in a mother providing her infant with lactose rather than some other sugar. It has been known for many years that lactose in the diet has a favourable effect on calcium absorption, especially in infants, and there are numerous other beneficial physiological effects known.

We understand that the incidence of real [primary] lactose intolerance in babies is very rare. It is not an allergy. It is caused by the inability of the cells lining the intestine to make the particular enzyme needed to break down the lactose into its two constituent sugars. If you suspect that your baby is intolerant of lactose, you should have your doctor check it out specifically.

Not so rare is a temporary [secondary] intolerance of lactose following a gastric infection that has damaged the lining of the baby's intestine. Use of a low lactose formula for a short period allows the gut to heal and to regain the ability to make the needed lactase enzyme. If you suspect that your baby is lactose intolerant, you should consult your doctor for a positive diagnosis.

Because it is Healthy:

Over eons of progressive development human breast milk has evolved as the most suitable food for the human baby and human babies have evolved to make the best use of it. Human breast milk is the "gold standard" of infant feeding and is preferred over all substitutes.

The primary alternative recommended by the Plunket Society is a formula based on cows' milk. A properly designed formula is very similar in overall composition to human milk. The protein content is reduced with respect to the level in cow's milk. The ratio of the whey proteins to the casein proteins is increased. The lactose content is substantially increased to match the level in human milk. The mineral and vitamin contents are adjusted. Some of the more sophisticated formulas, especially in Japan, have the fine makeup of the fat adjusted and may have specific enzymes and oligosaccharides added to simulate human milk more closely still. But we are still far from understanding in detail the purpose of some of the minor constituents of human milk. In fact it is probable that researchers have not yet even identified all the biologically active components of human milk. Even so, reasonably close simulation of human breast milk starting from cows' milk is possible as the basic building blocks, the proteins, fat, lactose and minerals are the same or very similar.

Over eons of progressive development the soy bean has evolved as the most suitable way for the soy plant to reproduce itself. It may well be that the evolutionary advantage that the phytoestrogens confer on soy beans is their ability to adversely

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affect the reproductive health of their predators. The phytoestrogens in subterranean clover are certainly capable of causing permanent infertility in some sheep.

In choosing to feed a baby on soy formula for reasons other than confirmed allergy or lactose intolerance, a mother is choosing not to provide the child with most of the particular natural substances that are in human milk as a result of evolution and natural selection. Are we so sure that we know enough about infant development to say with confidence that we know better than nature what an infant needs?

CONCLUDING REMARKS:

We have asked a question about the long term developmental effects of feeding soy phytoestrogens to infants. The answer is not known. If only a small proportion of soy formula children are affected and the effects are only observed in later life, it is not likely that a connection with soy consumption will be made. If your child has a problem, we suggest that you ensure that your doctor is aware that your child was raised on soy formula. In this way, if there are connections with particular conditions or symptoms, they will eventually come to the attention of the health system. To assist you in this we enclose a copy of the letter that four of the scientists who are familiar with the soy work wrote to the NZ Medical Journal earlier this year, together with a resulting summary of the letter in MedAlert.

OTHER ENCLOSURES:

We are also enclosing cuttings of some recent articles in newspapers. The first is copied from "The Times" of London and is an interview with Dr Richard Sharpe of the UK Government's Medical Research Centre for Reproductive Biology.

The remaining articles are from the NZ Herald written by Camille Guy. The first of these articles was branded by soy industry representatives as "irresponsible journalism" at a news conference held during the Nutrition Society's conference in late August. However, on 14 October they won first prize and \$4000 in the Food News category for Camille Guy at the NZ Guild of Food Writers Annual Conference.

The final page contains three of the letters to the NZ Herald which resulted from the Symposium on Phytoestrogens at the Nutrition Society's Annual Conference.

In an uncopied letter to the Herald replying to the letter from Trevor Johnston, Dr Pat Tuohy, the Director of Policy for Plunket wrote

"It would be incorrect to say that the Plunket Society endorses the safety of soy infant formula. Our official position is that there is no evidence that the product is harmful to babies, but we do not endorse its use."

Finally, in response to the letters in the Herald from both Trevor Johnston and Professor Tasman-Jones, we would like to emphasise the following point, which was made very clearly at the Symposium as well:

The issue of toxicity in parrots [found by at least 35 parrot breeders, not just one] is not relevant to our argument about soy infant formula. There is no "leap of logic" from bird food to soy formula and no calculation has been made based on the "unproven hypothesis that soy oestrogen-like substances caused the illness and death in parrots". The problems with the parrots led us to look closely at the scientific literature on the toxic or anti-nutritive compounds in soy beans. As was demonstrated earlier, our calculations have been based on the demonstrated fact that soy estrogen-like substances cause measurable disturbances to women's menstrual cycles. This fact proves that these substances are physiologically active in adult humans. The effects on parrots are irrelevant.

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This issue of the SIN Newsletter was edited by

Dr David J Woodhams CEng
Process Engineering Consultant and Food Technologist

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Soy Information Network

PO Box 32 236
Devonport 1309
New Zealand

SIN Newsletter # 2

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EDITORIAL

Dave Woodhams, editor

The main event of note since the issue of the first SIN Newsletter in early November last year was the holding of the Third International Conference on Phytoestrogens in Little Rock, Arkansas, in the first week of December. In this issue we bring you information on the conference and some of the issues raised plus comments from one of the scientists who was there [See "THE LITTLE ROCK CONFERENCE"]. Coverage of the last day and a half of the Conference, when the risks of dietary phytoestrogens were discussed, will be carried in the next issue. Prof Cliff Irvine, who presented the NZ information and concerns at the Conference, also provides some background on the recent upsurge of interest in phytoestrogens [See "PHYTOESTROGENS AND HUMANS"].

The extraordinary actions of soy industry people as they attempted to prevent the New Zealand information from reaching the Conference, and thereby the scientific community are described in the lead article [See "GAG THE MESSENGER", following this editorial]. I do not believe that an industry with nothing to hide would act in this way.

A number of the soy industry's persistent claims are now exposed as being incorrect. For example, the issue of the relative strengths of the soy phytoestrogen, genistein, and the natural human estrogen, estradiol, has been resolved in favour of the figure that we have quoted [See "HOW WEAK IS WEAK?"]. Still to be addressed by the soy industry are the combined issues of strength and concentration of the soy phytoestrogens [See "HOW STRONG IS WEAK?"].

Another issue laid to rest is the phytoestrogen content of human breast milk from mothers who are high soy consumers [See "SOY BOYS GET IT WRONG AGAIN"]. On the other hand, one of the soy (and dairy) formula manufacturers has brought out a no-lactose, no-soy formula for managing the rare primary and short-lived secondary conditions of lactose intolerance in infants [See SOY-FREE, LACTOSE-FREE INFANT FORMULA ALTERNATIVE].

It is my intention as editor of the SIN Newsletter to send copies of the first two issues to Dr Mark Messina, co-editor of "Soy Connection" and co-author of "The Simple Soybean and Your Health" and to invite him to respond to the issues raised in them. Look for his response in the next issue of your SIN Newsletter.

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GAG THE MESSENGER

Dave Woodhams

In October 1995, Dr Mike Fitzpatnck, then of Grayson Laboratories, was forbidden to speak or write publicly in the soy debate on pain of instant dismissal from his job. He was forbidden to present his invited scientific paper, "The Phytoestrogen Content of Soy-based Infant Foods", at the Little Rock Conference and strenuous (but unsuccessful) attempts were made to prevent him from even attending the Conference. He was able to attend the Conference on the condition that he did not ask questions in the open sessions or speak to the press on his return.

The news that Dr Mike Fitzpatnck had been invited to present a paper on phytoestrogens in soy infant formula at the Third International Conference on Phytoestrogens at Little Rock, Arkansas, broke in mid-August last year, with the publication of the proposed programme. This announcement, together with the publicity generated by the NZ Nutrition Society's Symposium a week or so later, initiated a series of actions designed to prevent Mike from attending the conference.

In the United States, a representative of the United Soybean Board in Washington DC phoned a senior staff member at the National Centre for Toxicological Research in Jefferson, Arkansas. She said that the industry was concerned that Dr Fitzpatnck had been invited to speak at the Conference. They were concerned he was going to speak and they wanted the invitation withdrawn or else they wanted an invited slot to rebut his arguments. NCTR informed her that the soy industry could not dictate who should speak and who should not speak at a USFDA sponsored international conference. However, he continued to get a similar phone call, from the same person, virtually every day, sometimes twice a day, throughout September, October and November.

Meanwhile, back in Auckland, members of the local soy industry were also getting into action. In one phone call it was made clear to Dr Fitzpatnck that it would not be in his interest to attend the Conference and there was no doubt in Mike's mind that this was intended to dissuade him from going. A local manufacturer of soy products made a complaint to Mr Bill Grayson, managing director of Grayson Laboratories, about Mike's appearance on Jenny Anderson's Radio Pacific talkback show on 31 August and the linking of Grayson's name with Mike's involvement in the soy debate. Preceded by a number of sporadic other complaints, this one was accompanied by a transcript of the show with various comments highlighted. (The points highlighted seemed mostly to be those where, being honest with the audience, we were pointing out our assumptions or where we were drawing a parallel that was not necessarily scientifically proven.)

The transcript of Mike's comments show that, as a result of the work he had done for the James as an independent consultant, he had reached some personal conclusions. Because there are no actual data on human infants, one way or the other, Mike made it clear that he assessed the risks as a father of small children, not specifically as a scientist. As a result of that assessment he said he would not feed soy infant formula to his own child.

During September Nestlé's somehow became involved in the matter because on 4 October Mike was formally instructed by Bill Grayson in person and in writing, to cease all communication with the media on soy matters.

"As this matter has now escalated to involve one of our most prestigious clients, Nestlé's, I must warn you that should you choose to disobey this instruction I would have little choice but to dismiss you over such misconduct."

The content of the letter makes it clear that the Company feared being sued. However, in a later letter to the James (13 Oct 95) Bill Grayson says:

"You assume that Nestlé's have applied pressure to us over this matter. Nothing could be further from the truth. We have had no communication of any kind from Nestlé's on this subject."

It was made clear to Mike that the prohibition included his presentation of the paper in Little Rock or his being named as an author of the paper. However, he still had leave of absence for the period of the Conference. (As a result, responsibility for writing and presenting the paper at Little Rock was taken over by Prof Cliff Irvine.)

It is clear that the two "anti-Mike Fitzpatnck" campaigns in the US and NZ were related or at least in close communication, because almost immediately after Mike was forbidden to present the paper, back in the USA the NCTR received a slightly different phone call from the United Soybean Board. They were so disappointed that Dr Fitzpatnck would not be coming to the conference. They were really heartbroken. What a shame he would not be there.

When told that Mike was still coming to the Conference, concern was again expressed at the prospect of his attendance. Apparently, the United Soybean Board didn't think he should be permitted to attend.

Meanwhile in NZ the pressure continued. On the Wednesday before his Saturday departure for Little Rock, Mike was called into Bill Grayson's office and told he was

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not permitted to attend the Conference, on pain of dismissal. He was also told to cease all communication with Dick and Val James. After getting legal advice Mike decided to go to the Conference anyway and decided that his employer had no right to dictate who his friends should or should not be.

It is interesting to note that even the announcement in the NZ Herald that Cliff and Mike were leaving to attend the Little Rock Conference triggered another spate of phone calls to Bill Grayson, complaining that this fact was reported!

Such behaviour by soy interests is somewhat familiar. After being advised by the Ministry of Health that the Northern Advocate in Whangarei was planning to print a critical article on soy in December 1994, a prominent soy importer, made 11 phone calls to the reporters and the editor of the paper in the course of two days. While it was never stated, the staff were in no doubt that the paper would be sued if they published the story. These implied threats delayed publication for a few days until the matter was made public in Parliament on 7 December. More recently, the editor of the NZ Herald has come under increasing pressure from soy interests to stop publication of further articles by Camille Guy.

Somewhere, someone doesn't want you to know something. I wonder why.

STOP PRESS - MIKE FITZPATRICK UNCHAINED

Bill Grayson did not fire Mike for going to the Conference. However, as of mid-February Mike Fitzpatrick's situation has changed. He has resigned from Grayson Laboratories and will take up a position in the Chemistry Department of Auckland University in time for the new Term. We wish him every success in his new job and look forward to printing his comments on the Little Rock Conference in due course.

THE LITTLE ROCK CONFERENCE by Prof Cliff Irvine

My initial impression of the delegates at the "ice-breaker" was that they regarded the NZ position as extreme and irrational. When we gave them the evidence for our position the hostility decreased and we wound up having a reasonable dialogue. Mike Fitzpatrick did a great job on our image and my talk was an unbiased report on our experiments and their logical interpretation.

It seems certain that NZ has provided the impetus for research into soy in infants which, in my view, is certain to keep going until some of the issues are resolved (Which is really all that we want.)

Nothing that was said at the Conference caused me to be any happier about soy for infants but now we need the proof. Considering the difficulties of doing experiments on infants, this will be hard to get.

At the Conference the 80 delegates were addressed by speakers from USA, Sweden, Finland and New Zealand. Attendance at the three conferences to date were 1990: 9, 1993: 40, 1995: 80 and there is little doubt that the soy infant formula issue was the reason for the increased interest in this Conference. The intensity of the debate and the quality of the research presented suggested that the delegates knew they were dealing with some serious and important issues.

On the first day of the conference there was a session on methods of measurement of phytoestrogens which brought out some differences between Dr Ken Setchell and Dr Adrian Franke. I don't know who won because I am not an expert in that area. *[Editor's note: Franke was advocating high performance liquid chromatography (HPLC) because, without "derivatisation", it allows the measurement of a variety of isoflavonoids, including aglycones and conjugated analytes in one run. Setchell uses gas chromatography followed by mass spectroscopy (GC/MS) and, as I understand it, challenged Franke over the detection limits of his method which he considered to be too high to be useful, at least for breast milk.]*

The second morning was a love affair with the bean, especially from Steve Barnes who believes it cures or prevents nearly all the serious diseases of mankind - heart disease by lowering cholesterol, kidney disease, autoimmune diseases by being an immunosuppressant, cancer of many types, inflammatory processes and so on. I do think that soy does have some very useful health properties in adults but that doesn't mean that it can't be harmful to infants - in fact quite the reverse. Anything that can have such effects on a mature adult is likely to have much stronger, and often deleterious, effects on the newborn who is much more susceptible. For instance, reining in a hyperimmune response in an adult can be very advantageous because so many diseases are caused by autoimmunity or hyperimmunity. However, suppressing an immune response in a neonate can suppress its defences against important invaders, with serious consequences.

[A discussion of the last two half days of the Conference, when the risks of dietary phytoestrogens were discussed, will be in the next issue of the SIN Newsletter. Ed.]

SOY BOYS GET IT WRONG AGAIN

Dave Woodhams

"Japanese women eat a lot of soy products. Obviously, they must have a lot of soy phytoestrogens in their breast milk. Thus, if there were any problems arising from soy phytoestrogens in infants, we would see it in Japanese babies. We don't see any problems in Japanese children so there are no problems with soy formula."

So runs one of the main arguments used by soy advocates to ridicule the concerns we took to the Ministry of Health in November 1994. Dr Mark Messina, US Co-editor of "Soy Connection" and co-author of the book "The Simple Soybean and Your Health", used this argument three times in the course of an interview with Kim Hill on National Radio, on 21 Dec 94. It has been used many times since. But as I pointed out in a letter to the Minister of Health (31 Dec 1994) nobody at that time had reported any actual measurements of the soy isoflavones in breast milk from women on high soy diets. The levels of genistein in the blood serum of such women had been measured and reported, however. Mike Fitzpatrick and I had calculated that the concentration of soy isoflavones in soy infant formula is about 35 times higher than the maximum reported concentration in the blood serum of women on a high soy diet. We argued theoretically that the level in breast milk would be no higher than that in the blood serum. This was one of a number of issues that did not get an airing during discussion at the NZ Nutrition Society's Phytoestrogen Symposium. However, when I presented it privately to Dr Setchell, keynote speaker at the Auckland Symposium, he said that he would be measuring it before the Little Rock Conference.

In the event, three different researchers measured soy isoflavones in the breast milk of humans on and off a soy diet and reported their results in Little Rock. Their findings? Dr Adrian Franke, the first to report his results, challenged women with up to 20 g of soy protein per day and measured a maximum of 0.02 mg phytoestrogens per litre of breast milk (mg/l). This is about 1/1000 of the concentration measured in soy infant formula. Prof Cliff Irvine presented the results of the analyses done by Dr Mike Fitzpatrick on samples from a number of New Zealand women, including some on a soy diet but not specifically challenged. Mike found that the level of soy phytoestrogens in all the milks analysed was less than 0.01 mg/l which is insignificant and bordering on the lower limit of the ability of his method (HPLC) to detect. Cliff Irvine said in presenting the results that HPLC was not the best method for analysing breast milk. However, Dr Ken Setchell, using GC/MS, said in reporting his results, "My results concur with those of Dr Irvine. The levels of phytoestrogens in soy formula are many times higher than in the breast milk of high soy consumers."

Setchell also stated that, from his measurements of intake and excretion, he was in no doubt that phytoestrogens accumulated in the body.

HOW STRONG IS WEAK?

by Dave Woodhams

Our soy infant formula argument relies on the measured effects of the soy phytoestrogens in humans, [see Newsletter #1]. We know that they are biologically active in humans at dietary levels. Thus the relative strengths of genistein and estradiol are of only minor concern to us. However, because soy interests have tried to use them to discredit us, we have had to take a continued interest.

We have found that soy advocates have been very keen to talk about the relative **strengths** of the soy phytoestrogens but fail completely to discuss the relative **quantities** in soy foods. As an example, they have made much of the fact that breast milk contains natural estrogens and have extrapolated from that to assume that soy phytoestrogens are safe, "because they are so much weaker". However, we have been able to show that the potency (strength x concentration) of the genistein in soy formula is 100 times greater than that of natural estradiol in human milk, one week after birth, in spite of the estradiol being 1200 times stronger than genistein.

How can this be? Because there is about 130,000 times more of the soy isoflavones in soy infant formula than there is estradiol in human breast milk.

It is quite another story, of course, that the level of natural estradiol in human milk declines with time and has almost disappeared after two or three weeks, while soy phytoestrogens remain at a high level forever. [See also "How Weak is Weak?", p 9].

PHYTOESTROGENS AND HUMANS

by Prof Cliff Irvine

What is the reason for the upsurge in interest in phytoestrogens?

Firstly, what are phytoestrogens? Phytoestrogens are defined as substances of plant origin (hence the 'phyto-') which cause effects like those of the body's natural oestrogens. Oestrogens are sex steroid hormones produced by the ovary which cause the female of many mammalian species (although not the higher primates, including women) to become sexually receptive during a fairly brief period known as 'oestrus'. Also, in all species, oestrogens cause marked changes in the mammary gland, cervix and uterus.

The primary reason for the upsurge of interest in phytoestrogens is the observation that people living in countries in which the consumption of soy products is high, Japan, for instance, have a reduced incidence of hormone-dependent cancers. These include cancers of the prostate, cervix and mammary gland or breast. Experiments with rats show that a high intake of soy phytoestrogens, especially the

isoflavone, genistein, increases their resistance to several cancer-producing substances. The evidence for a beneficial response to tumour development is increasing rapidly. As well, there is increased enthusiasm for the use of soy phytoestrogens as an alternative to hormone replacement therapy (HRT) in post-menopausal women.

It appears contradictory that increasing the consumption of soy phytoestrogens increases oestrogen availability for post-menopausal women, yet decreases oestrogen availability for pre-menopausal women, thus reducing the development of tumours that require oestrogen. The explanation may be that both phytoestrogens and the body's own oestrogens bind to a molecule found in many body tissues called an 'oestrogen receptor'. Binding to the receptor initiates all the actions induced by oestrogens. However, although phytoestrogens occupy the receptor adequately, they are much less potent in stimulating the receptor's activity. If there is no natural oestrogen available, as in post-menopausal women, phytoestrogen can partly make up for the deficiency and relieve the symptoms. On the other hand, if levels of natural estrogen are high, as in oestrogen-dependent cancers, large doses of phytoestrogens can displace the more potent natural oestrogens from the receptors, thus reducing their effectiveness. Thus phytoestrogens can act as either oestrogens or anti-oestrogens.

Now, although a reduction of sex steroid levels induced by phytoestrogens may have some beneficial aspects for mature women, in infant monkeys a decline in the level of sex steroids causes some very undesirable effects at and after puberty. Normally there is a burst of secretion of sex steroids in the first few months after birth. If this does not occur, physical and mental deficits occur at puberty. Investigations in which sex steroids are lowered experimentally cannot be done ethically in humans. However conditions in which there is a natural deficiency of male sex hormones in baby boys are associated with physical and mental deficits at puberty, just as in monkeys. Also, if the sex hormone deficiency is corrected for a brief period in the young infant, post-pubertal development is normal. There is a critical period during which sex steroids have to be high, at least in the male, otherwise the pubertal surge of growth and development is reduced. There are many experiments in animals which show that soy phytoestrogens in the newborn can inhibit sex hormone production and eventual post-pubertal development.

It is only in the last year or two that these very important actions of phytoestrogens have become established by experiments on several species. However there are still large gaps in our knowledge of the relevance of this to human health. This was reflected in the vigorous debate which occurred during question time and at meal breaks at the Little Rock Conference.

Apart from its actions on the reproductive hormones, genistein, which is in high concentration in soy, has a wide range of actions from immunosuppression to slowing down the breakdown of acetaldehyde (and thus increasing both the duration and the intensity of a hangover!). Speaker after speaker at Little Rock dealt with various aspects of the benefits and the drawbacks of soy. It emerged that, provided soy is properly processed, the benefits may outweigh the drawbacks for adults. Genistein does have the capability of inhibiting many important processes in young infants but whether it exerts that capability, at what age, and how it may be circumvented are important subjects for future research.

At present I have ceased, or at least suspended, my research programme in reproductive endocrinology which has been my major interest for many years. I would not have done that unless I thought that the soy infant formula subject was of major importance. After hearing both sides of the story from experts I have no regrets that all my spare time has been taken over by soy.

HOW WEAK IS WEAK?

by Dave Woodhams

Soy industry representatives have for many months been trying to convince the Ministry of Health, the public and others that soy phytoestrogens are safe because they are so weak and have stated on numerous occasions that genistein is 10 000 times weaker than the normal human estrogen estradiol. The Ministry of Health's latest official statement (Prescriber Update, October 1995) says that the relative oestrogenicity of the soy isoflavones relative to estradiol is disputed but is generally accepted as being somewhere between 1/1000 and 1/10,000 times as potent.

The figure we have used consistently is 1/1200, derived from the estrogenic effects ~~on laboratory mice~~ as reported in 1993 by Markiewicz and others. This is consistent with the findings in a number of research reports.

The first time the figure of 1/10,000 appeared was in an assessment of the Fitzpatrick report made by a Mr Anthony Hugget, a scientist at Nestlé's Research Centre in Lausanne, Switzerland. In this report he made reference to the same 1993 scientific report by Markiewicz as Dr Fitzpatrick did. Hugget had made a calculation error, misplacing the decimal point. Dr Fitzpatrick pointed this out to him during a visit to the Centre in September 1994 and even went through the calculation with him on a calculator to demonstrate the point.

However, the same 1/10,000 figure appeared in soy industry communications several times over the next few months, the most significant being in a report dated 14 December 1994 sent to the Ministry of Health by the World Health Organisation in Geneva. In a covering letter WHO explained that as they didn't have any information

of direct relevance to the Ministry's specific questions, they had referred the query to the Nestlé Research Centre which, they understood, had specialist knowledge in this regard. Mr Anthony Hugget was kind enough to provide four pages of information which the WHO was pleased to share with the Ministry

As the date of this report is well after Mike Fitzpatrick's September visit, the retention of the 1/10,000 figure would appear to be either a deliberate attempt to deceive or else gross negligence. It is interesting to note that neither Nestlé's nor WHO drew the Ministry's attention to the fact that Nestlé's, as a manufacturer of soy infant formula which they sell in a number of third world countries, had a vested interest

More was to come, however. In their statement of 7 December 1994, prepared for the Ministry of Health, Wyeth-Ayerst, manufacturers of Infasoy, state that the soy phytoestrogens "exhibit very weak biological activity, 1/1000 - 1/100,000 that of estradiol". Unfortunately they did not provide the Ministry with the page that contained the references to the two papers cited in support of these figures

In a press statement dated 22 December 1994, Sanitarium's nutritionist, K. Lindbeck, says that "isoflavones have a potency of about 1/100,000 that of estrogen hormones", without defining which "estrogen hormones" he was referring to. The figure is correct for a comparison with the synthetic estrogen diethylstilbestrol, DES, but incorrect for a comparison with the natural estrogen estradiol. As soy infant formula is supposed to simulate human milk, a comparison with estradiol would have been more honest

Even more interesting is the "Technical Brief" sent to the Ministry by Columbit (New Zealand) Ltd, the leading supplier of soy protein isolate (the product used in infant formula) in New Zealand on 26 January 1995. This technical brief was provided originally by Protein Technologies International of St Louis, Missouri, who supply the product sold by Columbit. In this document PTI state that the soy phytoestrogens "exhibit very weak biological activity, 1/1000 to 1/1,000,000 of the activity of estradiol", referring to two scientific papers in support of these figures

One of these two papers was written by a Dr Farmakalidis. When I checked the data I found that Dr Farmakalidis had compared genistein (the soy phytoestrogen) not with estradiol, as claimed by PTI, but with the synthetic estrogen diethylstilbestrol, DES, which is known to be 100 times stronger than estradiol

The second paper referred to was a review written by Dr Mark Messina, the soy expert brought to New Zealand by Wyeth-Ayerst last August to bury those of us who dared to question the safety of soy infant formula. In his review Messina says that genistein exerts "an estrogenic effect ranging from approximately 1×10^{-3} to 1×10^{-5} that of diethylstilbestrol (DES) or estradiol". He refers to a number of reports, including that of Farmakalidis, to support this statement

Now " 1×10^{-3} to 1×10^{-5} " is the same as saying "1/1000 to 1/100,000" but comparing the strength of one thing to that of two other substances that themselves differ in strength by a factor of 100 is nonsense. As I wrote to the Assistant Director General of Health last June, Dr Messina's statement is the same as saying that "a rat is 1/1000 to 1/100,000 times the size of an elephant or a man"

How PTI managed to turn Messina's 1/100,000 into an even more erroneous 1/1,000,000 is not clear but it is not supported by either of their referenced reports

At the Little Rock Conference the matter was resolved without argument. It was universally accepted that the strength ratio was about 1/1000 to 1/1200, as we have consistently maintained. I understand that even strong soy supporters, like Dr Steve Barnes (Univ of Alabama at Birmingham), accepted that figure

But the story doesn't quite end there. Work at the University of Missouri-Columbia, reported to the Conference by Dr Wade Welshons, indicated that the effective strength of natural estradiol in the body is influenced by the degree to which it is bound by "serum binding proteins". In the pregnant female rat and in the foetus more than 99% of the natural estradiol may be bound by these substances, thus protecting the foetus from the sea of estrogens it is swimming in. Welshons produced evidence that some phytoestrogens may not be bound in the same way and developed an assay that showed that the strength of genistein relative to estradiol was enhanced approximately 10-fold when it was in the presence of adult male serum. He went on to suggest that this enhanced strength could be 100- to 1000-fold in foetal serum. This suggests that in the body during pregnancy the relative strength of genistein could be between 1 and 1/10 that of estradiol, a very far cry indeed from the 1/10,000, 1/100,000 and 1/1,000,000 figures vainly advised by soy interests. Until this latest research is replicated elsewhere, however, we will be happy to use 1/1200

SOY-FREE, LACTOSE-FREE INFANT FORMULA ALTERNATIVE:

Bristol-Myers Company announced in November the release in NZ of a soy-free, lactose-free, dairy-based infant formula, O-Lac, for babies with lactose intolerance. In a press release they quite properly advised that if infants are being fed soy formula under medical advice then the child's doctor should be consulted before changing formula as O-Lac is not suitable for infants with an allergy to cows milk protein. However, where infants are being "fed soy because of personal preference of their parents [they] can be switched from soy formulas without increased risk"

We also suggest that parents read again the comments on lactose intolerance in SIN Newsletter #1, page 6. Primary (ie permanent) lactose intolerance is very rare in infants and secondary lactose intolerance, after a gastric infection, tends to revert to normal tolerance after a few days

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SUPPORT GROUPS:

Parents of children who may have been adversely affected by soy formula or other soy products may like to contact other parents in their area who have similar problems. If you are one of these parents, please write to the Soy Information Network and we will provide you with a contact name and phone number for a support group near you, if one exists. If you are willing to be the contact name in your area, please let us know.

DONATIONS:

If you would like to support the work of the Soy Information Network, please send a cheque to us at PO Box ~~400-212~~, North Shore Mailing Centre. Make out the cheque to the "Soy Information Network".

ENCLOSURES:

We are enclosing a copy of the most recent article from the NZ Herald, written by Camille Guy, which covers the Little Rock Conference and current research directions.

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Soy Information Network

PO Box 32 236
Devonport 1309
New Zealand

SIN Newsletter # 3

June 1996

EDITORIAL

Dave Woodhams

In the last issue I recorded my intention to invite Dr Mark Messina to respond to the matters I had raised in the first two issues of the SIN Newsletter. Shortly after I had sent the copies to him, I learned that Dr Messina was in New Zealand on his way to give an address to an Australian conference of dietitians. While here in Auckland he addressed a meeting of the Vegetarian Society and had unspecified "discussions with officials". Before the meeting I suggested to him that, in addition to writing an article for the SIN Newsletter, he should address the issues at that meeting. A short extract from an edited transcript of his remarks is included in this Newsletter. (See "THE CASE FOR RESEARCH IS ABSOLUTE") I have now received Dr Messina's invited article. (See "DR MESSINA REPLIES" and "THE EDITOR RESPONDS")

For the last year and a half we have been trying to get the Ministry of Health to make a further statement on the risks of soy infant formula for the information of parents. We have not been successful. Therefore I have decided to publish in this Newsletter the text of the Ministry's advice to the Minister in the days preceding Sandra Lee's question in Parliament of 7 December 1994, together with the Minister's response. (See "OFFICIAL ADVICE" and "PARLIAMENTARY QUESTIONS AND ANSWERS") The disparity between the Ministry's advice and the Minister's answers is quite apparent. However, even more to the point is the fact that there is absolutely no discussion in the Ministry's advice of the potential developmental and health dangers to children. Indeed we have yet to see any evidence of concern by the Ministry for infant reproductive and developmental health. Their main concerns appear to have been the "great potential to cause mischief, particularly in the media" and that "world-wide soybean is big business". It was after the question in Parliament that the Ministry moved to "allay fears" in a press statement carried by several newspapers. The Minister's initial reaction to the Ministry's briefing, however, is handwritten across the front page: "Are we to issue a 'warning' press statement?" The answer should have been, "Yes". The MoH advice to the Minister was obtained under the Official Information Act in June 1995.

As promised in the last SIN Newsletter, we publish Mike Fitzpatrick's description of the second half of the Little Rock Conference, when the risks of dietary phytoestrogens were discussed. (See "THE LITTLE ROCK CONFERENCE") And the Editor reflects some of the comment he has received from parents of consumers. (See "WHY WEREN'T WE TOLD?")

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WHY WEREN'T WE TOLD?

Dave Woodhams

If there is one thing that unites the parents whose children have been exposed to the unknown dangers of soy infant formula it is the question "Why weren't we told?"

As long ago as 1984 and 1985 the possible danger of soy infant formula to human fertility was raised in scientific publications by Dr Ken Setchell, one of the acknowledged world leaders in research into phytoestrogens. For instance, in 1985, in a book called *"Estrogens in the Environment"* he wrote:

"Soya formula milks for infant feeding have seen greatly increased use over the last five years yet the potential implications of the long term exposure of the infant to the phytoestrogens which are present in soya based products appears to have been overlooked. Since the newborn infant will be subject to chronic exposure to soya milk, in some cases for up to two years, their situation could be considered analogous to the sheep grazing on clover."

The comment "sheep grazing on clover" refers to an earlier passage which reads

"Although the short term estrogenic effects of these isoflavones in sheep was reversible, prolonged grazing resulted in the infertility syndrome which became known as clover disease and led to permanent histological changes to the uterus and ovaries."

In a later item in this Newsletter we reproduce the Ministry of Health's advice to the Minister in December 1994. Hand written on the front page, presumably by either the Minister or the Associate Minister of Health is the comment "Are we to issue a warning press statement?"

It is difficult to imagine how, in the light of the Ministry's written advice, the content of the Fitzpatrick Report, the report of the independent toxicologist who reviewed it and the scientific warnings published ten years previously, the Minister failed to issue such a warning. The prudent action in the interests of infant health and welfare would have been to withdraw soy infant formula from supermarket shelves and make it available only under medical supervision until research into the matter had been completed. At the very least information on the possible dangers should have been made public so that parents could make their formula decisions themselves. The sales of soy formula in New Zealand far exceed the amount needed to meet the requirements of infants with a cow's milk allergy. These children in particular have been put to quite unnecessary risk by the failure of the Minister and the Ministry to make information published in the scientific literature available to parents. The decision to try to "allay fears" in a press release that ignored the Ministry's own assessment of the potential dangers may yet return to haunt them.

* Setchell K D R, "Naturally occurring non-steroidal estrogens of dietary origin" in *"Estrogens in the Environment"*, John A. McLachlan, Editor, Elsevier Science Publishing Co. Inc. pp 79/80 and p 70

THE CASE FOR RESEARCH IS "ABSOLUTE"

The following is an extract from an edited transcript of comments by Dr Mark Messina in Auckland on 2 March 1996. The issues are addressed at greater length in the invited article, "Dr Messina Replies" which follows the transcript.

"I think the issue boils down to two key observations. I showed you earlier a study [the Cassidy study] that reported that soy consumption increases the length of the menstrual cycle. I said that we don't know what that means for breast cancer risk. The hypothesis is that it is beneficial. But certainly it shows or strongly suggests that soy is having a profound physiological effect. Well, if you accept that soy can do all these other things then clearly you are accepting that soy and these components are quite potent. So then the question is, "What are the effects of these pretty potent compounds in infants?" When you are on soy formula you are consuming large amounts of these isoflavones, these phytoestrogens. In fact on a bodyweight basis infants are consuming much more than these adult women were.

So what evidence is there? Are there studies that would definitively show it to be safe? Have they been conducted? Absolutely not. Should they be conducted? Absolutely." [Editor's emphasis]

DR MESSINA REPLIES

Dr Mark Messina

I would like to thank Dr. Woodhams for providing me an opportunity to express my opinion in this newsletter. Important issues are being raised and they need to be addressed. Before commenting on these issues however, I want to briefly clarify my professional role as it relates to soyfoods since I have been erroneously portrayed as a soy industry spokesperson.

I am a nutritionist by training, and for the past 6 years have been involved in facilitating research on soybeans and soy products. During these years I have also spent a good deal of my time promoting awareness among both consumers and professionals of the hypothesized health benefits of soy. My formal involvement with soyfoods began while I was a program director for the National Cancer Institute (NCI) in Washington, D.C. In 1990, I organized a workshop on the potential role of soyfoods in reducing cancer risk -- as a result of the recommendations of that workshop, the NCI allocated \$2.9 million to study the anticancer effects of soybean components. In 1992, I left the NCI to devote full time to the study of soyfoods.

Although industry and soybean farmer groups sponsor some of my consulting activities, I am an independent consultant and have no direct financial relationship with any soy manufacturer or farmer group, nor do I hold any patents. The book my wife and I wrote on soybeans (*The Simple Soybean and Your Health*, Avery Publishing, 1994) was not connected in any way with the soy industry.

Now to the issue at hand -- the relative risks and benefits of consuming soyfoods. Soy has been hypothesized to have beneficial effects against a number of chronic diseases including heart disease, cancer and osteoporosis. In addition, some research suggests soy may be useful in the relief of menopause symptoms (hot flashes, night sweats, etc.). The evidence in support of soy's protective effects ranges from the speculative (soy reduces cancer risk) to the fairly solid (soy protein or some component associated with soy protein reduces blood cholesterol levels). Although there are a number of factors that might contribute to the hypothesized beneficial effects of soy, most focus has been on a group of chemicals relatively unique to soybeans called isoflavones.

As has been indicated in previous issues of this newsletter, isoflavones are considered to be weak estrogens. Despite their relative weakness, the isoflavones are clearly thought to be potent enough to exert physiological effects; otherwise they could not be hypothesized to exert beneficial effects. It is important to note though, that the critical physiological effects of the isoflavones in relation to chronic disease prevention/treatment may not be at all related to their weak estrogenic activity. The estrogenic activity of the isoflavones does however, seem to be the primary reason for concerns being raised about the possible adverse effects of soy consumption. Since this newsletter is aimed at discussing the possible adverse effects of soy consumption, I will not discuss the potential benefits any further.

Of primary concern is the safety of soy infant formula. The key questions are whether isoflavone ingestion during infancy exerts physiological effects and if so, are these effects harmful? Unfortunately, as is so often the case with science, in my opinion, the types of data needed to definitively answer these questions are not available. There does exist a theoretical basis for raising concerns however, since isoflavones possess estrogenic activity and estrogens play a role in development. Also, on a body weight basis, the amount of isoflavones ingested by infants is several fold higher than the amount of isoflavones typically ingested by people who eat soyfoods and certainly, than the amount of isoflavones ingested by infants breast-fed by mothers consuming soyfoods.

Is there evidence that soy infant formula is safe? I believe the answer is yes, and have written so in the past. It seems to me given the long history of soy formula use (30-40 years in the U.S.) if soy formula had adverse effects, that effects in infants would have been noted sometime during these many years. To my knowledge there are no letters or case studies published in scientific journals citing problems in soy-fed infants that might be attributed to estrogenic effects. Furthermore, many short term studies that have evaluated infants and children fed soy infant formula have concluded that soy formula promotes normal growth and development.

Does this lack of observed adverse effects definitively prove that soy is safe -- absolutely not, but in my opinion this does speak to the safety of soy formula. If however, isoflavone exposure during infancy caused adverse effects later in life in a small minority of individuals, it would be unlikely that any connection with soy formula would be made. I think this is unlikely to be the case, but this is one reason why more research is needed.

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My understanding is that although infants are likely to be the most sensitive to the estrogenic effects of isoflavones -- young children consuming soymilk and soy products are also theoretically at risk. Definitive data are lacking, but vegetarians and Seventh-day Adventists are two groups who typically consume soy products beginning early in life and who have been studied extensively. Again, I am not aware of reports indicating that soy is associated with any adverse effects in these groups, quite the contrary since both groups enjoy above average health. Also, for centuries soy products have been a common staple in the diets of many Asian children seemingly without leading to any adverse effects. It is conceivable however, that there could have been some adaptation to any potential adverse effects over the generations in Asians.

Concern has also been raised about the effects of soy consumption on fertility -- this concern is based primarily on two observations. First, isoflavone exposure in animals has caused reproductive problems (although animals were typically exposed to very high levels). Second, a recent study [the Cassidy study, see SIN Newsletter #1] found that soy consumption lowered the levels of hormones involved in ovulation although all the women in this study did ovulate successfully. Nevertheless, some questions remain about soy and fertility. But again, to my knowledge, there are no data indicating fertility problems among vegetarians and Seventh-day Adventists. Some data indicate vegetarians may be at an increased risk of menstrual irregularities, although there are recent data disputing this contention but in any case menstrual irregularities have not been associated with soy consumption.

Often, the large populations of Japan and China are used as support for the safety of soy in relation to fertility, but this may not be appropriate for at least two reasons. One, as indicated previously, there could have been some adaptation to isoflavones over the generations and two, if soy adversely affected only a small percentage of Asian women, detecting decreases in overall birth rate among an entire population that consumes soy, would be difficult. So where does all of this leave us?

Should infants be fed soy formula in cases where breast feeding and cow milk based formula are not options? Should children use soy products? Should women attempting to become pregnant use soy products? My answer to all three of these questions is unequivocally yes because overall, the weight of the evidence suggests soy is safe.

But I also believe that more research is needed to definitely resolve the concerns being raised. It would be wrong to embrace the potential health benefits of soy without also considering the possibility of adverse effects in some specific situations. Consequently, I believe that the soy industry should actively facilitate research addressing safety concerns. Only then, can these concerns be alleviated.

To this end, on September 19th, in Brussels, Belgium, as part of a larger symposium, there is going to be a 4 hour session on soy and infant health. I am hopeful this session will help to increase understanding of the issues surrounding soy consumption and stimulate further research. I believe the soy industry is committed to

seeing that the safety issues are resolved in a manner satisfactory to all parties concerned.

Finally, although in the end the critical issue is that safety concerns are resolved as quickly as possible, I find very distasteful the manner in which much of the effort to raise these concerns has taken place. The nasty tone displayed by some is unprofessional, rude and uncalled for, and one that actually hinders the likelihood of the appropriate discussion and research taking place. Nothing can be gained by speaking of people in a disrespectful manner, regardless of what side of the issue one stands.

THE EDITOR RESPONDS

I had hoped that Dr Messina would respond to some of the more critical comments made in SIN Newsletters 1 and 2 about the soy industry's responses to the issues raised in Dr Mike Fitzpatrick's report. In particular I thought that the misleading information on the strength of the soy isoflavones genistein and daidzein emanating from soy interests should have been acknowledged and corrected. However, at the Auckland meeting in March, he told me that, as the Cassidy study showed conclusively that the soy isoflavones were biologically active in humans at dietary levels, he considered that the relative strengths of the soy phytoestrogens and the human estrogen, estradiol, did not warrant further discussion. The Cassidy data were known, of course, in March 1994 when Dr Fitzpatrick finished his report well before the misleading statements from Nestlé's, Wyeth-Ayerst, Sanitarium and Protein Technologies International that were quoted in SIN Newsletter #2. Dr Messina's unfortunate review statement that genistein exerts "an estrogenic effect ranging from approximately 1×10^{-3} to 1×10^5 that of diethylstilbestrol (DES) or estradiol" should have been corrected.

Dr Messina speaks about soy's "hypothesised" beneficial effects against a number of chronic diseases including heart disease, cancer and osteoporosis" and says that the evidence for soy's protective effects against cancer is "speculative". It is thus quite unacceptable that this speculation should be cited over and over again as a major reason why Dr Fitzpatrick's conclusion that soy may have some adverse effects should be dismissed. Dr Messina used this line of argument himself three or four times in his interview with National Radio's Kim Hill on 21 December 1994.

Dr Messina notes that the "critical physiological effects of the isoflavones in relation to chronic disease prevention/treatment may not be at all related to their weak estrogenic activity". It is also possible that the critical physiological actions of the isoflavones in relation to adverse effects may not be related to their estrogenic activity, a point that we have made consistently from very early on. There is, for instance, the immunosuppressive activity of genistein.

There are numbers of studies and case studies in medical and scientific journals citing problems in infants fed with soy formula. Several relate to the functioning of the thyroid gland. These do not identify the component of the soy formula causing

the problem but the Wingspread Workshop scientists note that thyroid dysfunction as an effect of hormone disruptors in animals, including humans.

Regarding fertility, the following quotation is from an article in "Environmental Health Perspectives", a publication of the (US) National Institute of Environmental Health Sciences, in North Carolina. The article addresses the benefits and risks of phytoestrogens.

"Common sense would tell us that soy does not pose a problem for fertility," said Setchell, pointing to the reproductive success of Asians. "However," he added, "that fact could be countered with other similarly logical arguments." One such argument, according to Hughes, is that Asians have been consuming these diets for centuries, and any soy-related fertility problems may have been selectively bred out of the population generations ago. In that case, westerners suddenly switching to a soy-based diet might not have the advantage of that natural selection. Sheehan also adds that, especially with developmental toxicants, there is a long latency period, which makes it difficult to associate an event with a negative outcome. "The fact that there aren't any negative reports can't be taken as an argument that soy diets are safe," he said. [Editor's emphasis]

Finally, I have asked Dr Messina twice to make his criticisms ("distasteful", "nasty tone", "unprofessional, rude and uncalled for") more specific but he has declined to answer me. As Editor I admit to a certain degree of delight following the Little Rock Conference which may have crept into the odd headline of the Newsletter ("Soy Boys get it Wrong Again" etc). This should be seen against a background of 18 months of soy proponents and others ridiculing the concerns we had raised. If the soy industry, regulatory authorities, consultants and others had addressed the questions we asked in a professional manner and had shown an interest in finding out the truth rather than trying to silence us by the spreading of half-truths and unwarranted speculation, that headline might have been out of place. However in context, the Editor stands behind the headlines and the contents of the articles in question.

THE LITTLE ROCK CONFERENCE

Dr Mike Fitzpatrick

During the first three sessions of the conference the principal speakers gave presentations on the detection, mechanisms of action, and benefits of phytoestrogens. I should say, however, that the soy formula issue was raised right at the onset of the conference and much of the discussion that followed the early presentations seemed to find a way back to the baby formula debate. In fact it became quite clear that even those who were sure that our concerns are unfounded had to agree that research into the effects of soy formulas on infants should be commenced immediately.

Steven Barnes and Kenneth Setchell were two conference participants who were quite vocal during these early discussions. Steven Barnes felt that phytoestrogens in soy infant formula would not pose a risk to babies because they were not present in a readily available (bioavailable) form but he had no data to support this theory.

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Kenneth Setchell was more philosophical in his approach and indicated that he was pretty sure that phytoestrogens wouldn't be bad for babies and that he had started a project that he hoped would prove this

However it wasn't until the afternoon session of the second day of the conference that the issue of phytoestrogen risks began to be addressed more formally My interpretation and comments on the presentations are as follows

Michael Boiger reviewed the FDA's methods for assessing risk and noted the difficulties with this methodology when applied to natural substances present in foodstuffs He said that the FDA were not yet able to assess the risks associated with the presence of phytoestrogens in the diet but gave assurances that they were being pro-active in their approach to this and related, difficult problems

Pat Whitten presented data on the effects of coumestrol, a phytoestrogen, on rats when they were exposed to it in their mothers' milk Her results suggested that coumestrol affects developmental processes mediated by either androgen or oestrogen During the discussion session it was noted that similar studies using genistein and daidzein should be carried out

John Anderson's presentation on the effects of genistein on bone tissue was, in many ways, similar in its conclusions to those presentations in the benefits section of the conference He showed that, at low doses, genistein was effective in retention of the spongy lattice-like structure of bone tissue at high doses, however, genistein was less effective, perhaps even having an adverse effect on cells

Wade Welshons' presentation addressed the somewhat controversial issue of the oestrogenic activity of phytoestrogens He presented new data that showed that the oestrogenic activity of phytoestrogens and other oestrogen xenobiotics has been generally underestimated In human serum genistein was shown to be 1/1000 times as potent as oestradiol, however, in the serum of the human fetus, the potency of genistein was much higher, being 1/100 or even 1/10 times as potent as oestradiol

New Zealand's Cliff Irvine was the man everyone was waiting to hear! He presented data on the levels of phytoestrogens in infant foods and in human breast milk and defined the risks that high exposure may have on the developing infant There was considerable discussion following Cliff's presentation Adrian Franke claimed that the phytoestrogen levels in the breast milk of Asian women would be higher than that found by Cliff in NZ women Dan Sheehan called attention to the work of UK researcher Richard Sharpe on decreased male fertility and stressed the importance of following up Cliff's concerns Steven Barnes said that it was important to find out how the phytoestrogens were metabolised by infants, work that Kenneth Setchell said that he was going to do Setchell himself noted that soy infant formulas did not contain cholesterol when it was clear that it was required by infants and was supplied by human milk

The last day of the conference started with a session that mainly focussed on the use of herbal remedies

David Zava reviewed the use of herbal remedies and gave praise to the developments that would see them replace many pharmaceutical hormones He noted that there was a need for control and consumer protection stating that people die in the USA because of misuse of herbal remedies

Dan Sheehan, a senior FDA toxicologist explained some of the difficulties in determining whether or not a compound is toxic He recalled that it wasn't until the 1970s that fetal alcohol syndrome was recognised even though it is, in hindsight, very easy to diagnose He caused a bit of a stir by saying that no one could claim that soy was safe, and added that it would take a very significant population study to get data to prove or disprove the concerns over soy infant formulas

Kenneth Setchell was the last conference speaker He gave an overview of his study of infertility and early deaths in captive cheetahs, noting that there were very clear reasons why the isoflavones were bad news for the cheetah He also presented new data on the levels of isoflavones in human breast milk on the first day after consumption of 30 mg of isoflavones He had found approximately 30 ng/ml (more than 1000 times less than that found in soy infant formulas) Setchell also showed that, contrary to earlier claims, there is significant bioaccumulation of isoflavones in blood and tissue

The conference ended on a high note with the majority of participants certain that phytoestrogen research was still in its infancy

OFFICIAL ADVICE

The following is the background information provided to the Minister of Health on 2 December 1994 prior to the parliamentary question asked by Sandra Lee MP five days later It was prepared by Dr Martin Edwards, Toxicologist in the Food Administration Section of the Ministry of Health and was obtained under the Official Information Act in June 1995

The toxicity of several natural components of raw soybean and soybean products has been well known for years Over time new toxic components have been found in soybean, and knowledge concerning the properties of the other toxicants has expanded The toxicants include trypsin inhibitors, phytic acid, phyto-estrogens, coumarin derivatives, saponins and lectins Potentially (if the dose was high enough over a sufficient length of time) such toxicants could cause significant adverse health effects Possible effects would include growth depression, immunosuppression, abnormal responses to hormone stimulation and cancer

Concerns have recently been raised that control measures (processing during food production and regulatory measures) have not kept pace with knowledge of the identity and toxicology of some of the chemicals present in soybean Widespread traditional use by many cultures may not be relevant experience to modern Western uses Traditional methods of processing and preparing soy-based foods differ in many ways from modern commercial processing and preparation techniques

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The range of foods using soybean has also expanded considerably, using soy raw, and partially or fully processed.

One major focus of concern is on soy-based infant formulae, due to perceived high risk. Soy-based formula is likely to form a significant part of the infant's diet during a sensitive developmental period. The infants are likely to be on soy formula as a consequence of a deliberate choice for an alternative to human milk or cow milk-based formula. Some infants may be given soy-based formula on medical advice after showing allergic reactions to other types of formula. The department of Health advised in 1989 that soy-based formula should not be used as the first choice alternative due to concerns, particularly of high aluminium contents.

The trigger for these new concerns was the independent report compiled by, and on behalf of Mr and Mrs James of Whangarei. The James breed exotic birds and Dick James is a retired lawyer. The James Report is the result of their investigations to find the cause of numerous illnesses and deaths in birds that have occurred in their aviaries. The report has focussed on soybean in the specialised bird feeds they use as the cause of their problems. The research has prompted them to follow their concerns into food for human consumption.

The Ministry is aware that Ross Meurant, as Member of Parliament for Hobson and Associate Minister of Agriculture, has been approached by the James. Officials in other government agencies, the Ministry of Agriculture and Fisheries (stock feed) and the Department of Conservation (endangered species feeding programs) have also been contacted.

The James Report has great potential to cause mischief, particularly in the media, both in New Zealand and overseas. The issues are highly emotive, particularly for parents of infants using soy-based formula (for the health professionals involved with these families by advising the use of such products), vegetarians and other lifestyle groups. (Soybean is also used in the preparation of feed for domestic pets (birds/cats/dogs), domestic animals (horses and other stock). World-wide soybean is big business, particularly in the US, and it is a tradeable item on the international commodity markets.

The Ministry of Health is investigating these concerns. Similarly to regulatory agencies elsewhere in the world, the Ministry is not now in a position to make a valid risk analysis of this issue and recommend measures to control actual risks to consumers. The Ministry, however, is not aware of any unequivocal evidence that soy-based foods are causing any unexpected illness in New Zealand consumers.

Evidence of actual harm to consumers would be very problematical to establish. Toxic effects would tend to manifest over a considerable period of time and also be masked. Symptoms would likely be attributable to several factors, of which soybean consumption would be one. Retrospective epidemiological studies would be time consuming.

The problem is it is relatively easy to show the potential harm that soy-based foods could cause consumers, it will be difficult to establish actual risk and impossible for the Ministry to convince people of the real level of risk that exists even when the risk analysis has been completed.

The situation is further complicated by an increasing volume of literature (popular and scientific) reporting the beneficial effects on health of some of the same components found in raw soybean. (Pharmacologically active chemicals are potentially toxic if the dose is too high or given at inappropriate times. Exposure from soybean in the diet would be to a spectrum of different components and not individual chemicals as used in a research situation.)

The Ministry has already approached the regulatory agencies in the UK, the US, Canada and Australia to determine how they control this issue. Requests for information have also been sent to contacts in the World Health Organisation (WHO).

The Ministry plans to approach manufacturers who use soybean, with our concerns, and seek reassurances that they have adequate systems in place to ensure the safety of their products. Future strategies will be developed as our investigations proceed.

PARLIAMENTARY QUESTIONS AND ANSWERS, 7 Dec 1994

Sandra Lee, MP for Auckland Central:

Mr Speaker, my question is to the Minister of Health and I ask: Has she received a report from the University of Auckland Medical School, recommending that the use of soybean product in infant milk formula be discontinued because of high levels of oestrogenic compounds found in four brands of soya-based infant milk formula available in New Zealand? It's very serious.

Jenny Shipley, Minister of Health:

Mr Speaker, I am aware of the material this member refers to. The Ministry has got a copy of that report and is currently investigating the claims to see if they can be substantiated.

Sandra Lee:

Mr Speaker, will the Minister consider using her powers under Section 40 of the Food Act 1981 to protect the public by directing the recall of soya based infant formula from sale in this country in view of the fact that one of the two reports on the subject has stated that "few mothers would choose to feed a formula containing both active estrogens and immunosuppressant to their baby during the first few months of life if they had an informed choice."

Jenny Shipley:

Mr Speaker, at this stage there is not the evidence to warrant the action the Member suggests that be taken. However, I can tell the member that the Ministry has

approached the manufacturers who use soyabean with their concerns and are seeking assurances that they have adequate systems in place to ensure the safety of their products for infants that will be using that milk. Further strategies will be developed as the Ministry's investigation is completed, or proceeds, if there is evidence to back up the claims that have been made in the paper that she refers to.

Sandra Lee:

I seek the leave of the House to table Dr Woodhams' report and the Robertson report on toxicity of soyabean infant milk powder.

Speaker:

Any objections? There appear to be none.

SUPPORT GROUPS:

Parents of children who may have been adversely affected by soy formula or other soy products may like to contact other parents in their area who have similar problems. If you are one of these parents, please write to the Soy Information Network and we will provide you with a contact name and phone number for a support group near you, if one exists. If you are willing to be the contact name in your area, please let us know.

SUBSCRIPTIONS AND DONATIONS:

If you would like to continue to receive the SIN Newsletter please send a subscription of \$15.00. This subscription may be reduced or waived for parents of affected children for whom it is a hardship. Please apply in writing. Donations to support the work of the Soy Information Network are also requested. Please send a cheque to us at PO Box 100 212, North Shore Mailing Centre. Make out the cheque to the "Soy Information Network".

ENCLOSURES:

We enclose a copy of an NZPA article from the Gisborne Herald covering an address by Dr Pat Tuohy, Medical Director of the Royal NZ Plunket Society which may not have been published in all areas. We also include a copy of Camille Guy's latest magazine article from the NZ Herald. This covers her interview with Dr Reg Morgan of the University of Western Australia, who visited New Zealand during May, and also records a decline in the market share of soy infant formula in NZ from a previously claimed 16% to less than 8%.

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Chapter 2

PHYTOESTROGENS

N. R. Adams

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ewes is linearly related to the degree of estrogenic stimulation.⁴¹ However, this response rapidly becomes refractory after exposure for more than 2 d, limiting its value for studies on isoflavones. Refractoriness may be partially prevented by concurrent administration of progesterone so that the ruminal population of microorganisms is given time to adapt to isoflavone metabolism.³⁴ However, this protection is not complete,⁴² so the assay can normally be considered to be only semiquantitative when applied to isoflavones. On the other hand, if carried out over 2 or 3 d, cervical mucus secretion may be the bioassay of choice for coumestans.

The presence of milky fluid in the mammae of male or young female sheep is often used as an indication of the presence of phytoestrogens, but it cannot be used quantitatively.⁴³ The Allen-Doisy vaginal smear technique has not proven to be a reliable assay in the sheep.

Few attempts have been made to carry out bioassays in cattle, because of the great expense. Smithard, Cole and Kennedy⁴⁴ have described an assay based on changes in the activity of enzymes in the teat.

IV. DISTRIBUTION IN PLANTS

A. Species of Plants

Estrogenic compounds are very widespread in nature. A comprehensive review by Farnsworth et al.⁸ lists 145 species of plants suspected on biological grounds of being estrogenic, 220 species which contain isoflavonoid compounds likely to be estrogenic, and 58 species from which coumestans have been isolated.

Estrogenic steroids have been isolated from plants from a number of different families, many of which appear to have been investigated because of their use in traditional medicine. In contrast, isoflavonoids have been found almost solely in the Leguminosae, with a few other substantiated reports from Iridaceae and Rosaceae.⁸ Isoflavones have most commonly been isolated from species of the genera *Baptisia*, *Genista*, and *Trifolium*, while coumestans have been isolated primarily from the genus *Medicago*.

B. Major Dietary Sources of Phytoestrogens

Most occurrences of estrogenicity have been reported in sheep grazing on pastures of *Trifolium subterraneum* (subterranean clover) or *T. pratense* (red clover) containing formononetin. Problems occur in sheep and cattle exposed to *Medicago sativa* (alfalfa or lucerne) containing coumestrol, sativol, 4'-methoxycoumestrol, and a number of other coumestans.⁴⁵ *T. alexandrinum* (berseem clover) contains genistein and biochanin A and can cause estrogenic signs in animals.⁴⁶ *T. repens* (white clover) may contain trifoliol, coumestrol, and repensol.¹⁴ Coumestans are also found in other species of annual medics used in pasture.⁴⁷ Pastures of all these plants can have an estrogenic effect equivalent to injecting sheep with 5 to 15 µg diethylstilbestrol daily. This amount is roughly equivalent to the total amount of estrogen secreted by a ewe at the peak day of its estrus cycle.

Soybean products may contain up to 0.25% total isoflavones, mainly genistein, daidzein, and glycitein⁴⁸ as well as coumestrol.²⁶ A high concentration of soybeans in the diet can cause signs of estrogenicity in swine.⁴⁹ Soybeans and alfalfa sprouts can contribute detectable amounts of estrogenicity to the human diet.²⁴ Phytoestrogens have also been reported in other human foods such as peas, brussels sprouts, and apples,²⁶ but only in insignificant concentrations.

Moldy corn is the most common cause of estrogenicity in swine, but that is caused by a fungal toxin and will not be covered in this chapter.

C. Factors Affecting the Concentration in Plants

1. Isoflavones

Because of their economic importance, the factors affecting the concentration of isofla-

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Table 2
CONCENTRATIONS OF ISOFLAVONES IN
CULTIVARS OF CLOVER AS A
PERCENTAGE OF DRY MATTER

	Formononet- tin	Gesi- stein	Biochanin A
Subterranean clover ^a			
Yarloop	1.5	2.8	0.5
Dwalganup	1.3	1.6	0.8
Dinninup	1.2	1.1	1.5
Geraldton	0.9	0.6	0.9
Tallarook	0.8	0.7	0.6
Dahak	0.2	0.4	0.2
Clare	0.15	2.8	0.1
Trakkala	0.15	0.9	2.0
Seaton Park	0.12	0.8	1.6
Bacchus Marsh	0.11	1.0	2.3
Red clover ^b			
Broad	0.99	0.01	0.95
Tetraploid	0.90	0.01	0.74
Morocco	0.14	0.01	0.12
White clover ^c			
Ladino	0.02	0.01	0.02

^a Rossiter, 1970.

^b Wong, 1963.

^c Cox, 1978.

vones in subterranean clover have been investigated in detail. Most of the variation is due to the genotype of the plant, so the average concentration in the leaves of any individual cultivar is relatively stable. Table 2 gives average values for a number of genotypes.

Environmental influences on the isoflavone levels in subterranean clover have been examined thoroughly, particularly by Rossiter and colleagues in a series of studies from 1966 to 1973.^{11,12,50-52} The concentrations of isoflavones, particularly formononetin, in the fully expanded leaf were increased markedly by mineral deficiencies of nitrogen, sulfur, and especially phosphorus, though the effects of potassium, zinc, and copper were negligible. Stresses due to water deficit or to excess (waterlogging) also resulted in marked increases in isoflavone levels. On the other hand, both high and low temperatures and very low light intensities usually lowered isoflavone levels. The two herbicides 2,4-D and paraquat also produced lower levels. Interestingly, defoliation stress, for which one symptom is a marked reduction in leaf size, had virtually no influence on isoflavone levels.

Under farming conditions, phosphorus deficiency and possibly also sulfur deficiency have been the most important of the various factors studied. The direct effects via higher formononetin concentrations are likely to be mitigated by decreased pasture growth rates, but this in turn may be associated with decreased stocking rates, the net effect is likely to be an increase in formononetin intake. However, indirect effects of nutrient deficiencies arising from changes in botanical composition⁵³ can be important. For instance, alleviation of phosphorus deficiency not only reduces formononetin concentration in the clover leaves, but (often more importantly) decreases the proportion of clover in established pastures and thus reduces formononetin intake still further.

The concentration of isoflavones varies within the plant, being highest in the leaves, intermediate in flowers, and lowest in petioles, stems, and roots.⁵⁴ The content of isoflavone in the leaf fraction decreases as the plants age, except in the Yarloop cultivar, and the

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proportion of stem to leaf increases. Thus, it should be expected that the amount of isoflavones in pasture should decrease as the plants mature. A tendency to greater activity in winter (when plants were younger) was noted by Bennett et al.,³¹ but Davies and Dudzinski³² were not able to detect seasonal changes in pasture estrogenicity using bioassay in sheep.

The concentration of isoflavones, particularly formononetin, increased in subterranean clover suffering fungal disease,³³ but the effect on total isoflavone available to the animal was unstated.

Less work has been carried out on red clover, but it appears that genetic factors, stage of growth, temperature, and phosphate fertilizer play a role similar to that in subterranean clover.³⁴ The stage of growth is important, and pastures have been reported to be more estrogenic in spring than after flowering in autumn.³⁷

Isoflavones are present in clover only while it is green, and disappear rapidly with wilting. Dry, senescent pastures of subterranean clover are not estrogenic. The concentration of isoflavone can be maintained if the clover is dried rapidly,³⁵ and well-made hay and ensilage³⁶ may retain most of the original estrogenicity.

2 Coumestans

The factors affecting the concentrations of coumestans in plants are very different from those controlling isoflavones. Most studies have been carried out on alfalfa, in which the concentration of coumestans depends primarily on the presence and severity of foliar pathogens; in the absence of infection the concentration of coumestans was very low and was not increased by temperature, stage of growth, or phosphate fertilizer.³⁸ The genotype of the plant was important in so far as plants which were resistant to disease suffered less infection and so had lower coumestan levels.³⁹

Response to infection also appears to be the main factor affecting the concentration of coumestans in white clover.⁴⁰ In medic the situation is less clear. Green material normally contains little coumestan activity.⁴⁰ Fungal infection increased the concentration of coumestans in green *Medicago litoralis*,⁴⁷ but most of the coumestans appeared to accumulate in the leaves between maturity and senescence regardless of infection, and thus the total amount may reflect primarily the duration of this period.⁴⁷

V. PHYSIOLOGICAL AND PHARMACOLOGICAL PROPERTIES

By definition, phytoestrogens are plant substances which have estrogenic properties. At present, there are no other pharmacological activities of the recognized phytoestrogens apart from those mediated by the estrogen system.

A. Molecular Biology of Estrogenic Action

In the mammal, the endogenous estrogens are the steroids estrone, estradiol, and estrinol. These steroids act primarily by binding to the estrogen receptor, a protein which is probably located in the cell nucleus. After binding, the steroid-receptor complex is "transformed", by which it acquires the ability to bind to specific sites on the DNA and activate specific genes which cause the responses observed (See Figure 3).

The ability of a molecule to bind to the estrogen receptor, and thus to cause an estrogenic response, is determined by its shape. Few steroids other than estrone, estradiol, and estrinol bind to the estrogen receptor, but a number of nonsteroidal compounds will. All these substances have a structure with some basic similarities, including a phenolic hydroxyl group and another hydroxyl group attached to a carbon ring in appropriate configuration, with a fixed distance between the hydroxyl groups. The simplest and most potent of these compounds is diethylstilbestrol (Figure 4). Compounds which have methoxy groups at the active site may become active after they have been demethylated. A fuller and more accurate description of the relationship between structure and estrogenic activity is given by Jordan et al.⁴⁰

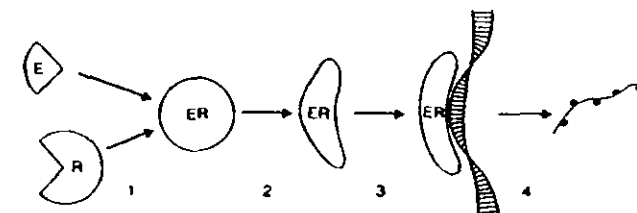


FIGURE 3 Model of estrogenic action. After (1) binding of estrogen (E) to its receptor (R), the estrogen-receptor complex is activated (2) and able to bind to DNA (3), resulting in transcription of specific genes.

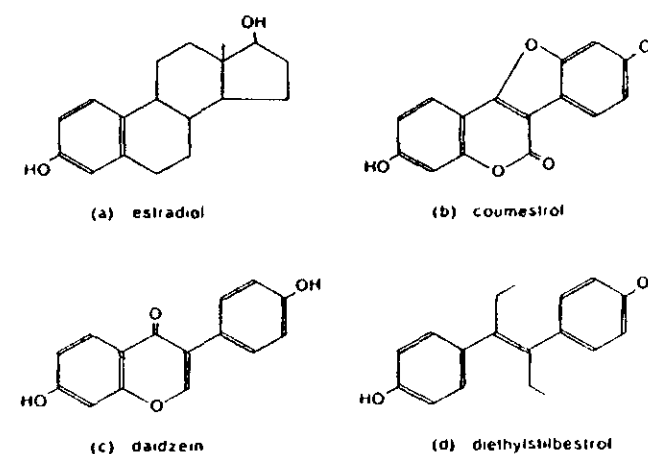


FIGURE 4 Structures of estrogenic compounds (a) steroid, (b) coumestan, (c) isoflavone, and (d) stilbene.

B. Relative Activity of Phytoestrogens

Although all estrogens act by binding to the estrogen receptor, the affinity with which they bind varies greatly. Thus, there are large variations between substances in the concentrations necessary to bring about an estrogenic effect. Furthermore, the binding affinity is not an infallible guide to the potency of a substance. After binding, estrogens vary in their ability to "transform" the receptor and thereby initiate estrogenic action (Figure 3). As reviewed by Jordan et al.,⁴⁰ the structural requirements for transformation are not yet fully understood, so the potency of an estrogen cannot be predicted accurately from its structure.

Plant estrogens have less affinity for the receptor than steroidal estrogens (Table 3), and the ranking is similar across species of animals. Coumestans have around $1/20$ and isoflavones $1/100$ the affinity of estradiol.⁴¹ Not only is the binding affinity less, but the phytoestrogen-receptor complex formed after binding is less able to be transformed to bind to DNA.⁴² The complex is also less stable so that the duration of binding of the complex to DNA is reduced.^{43,44} Thus, the overall estrogenic effect of phytoestrogens is even less than is indicated by the reduced affinity for the receptor (compare Tables 1 and 3).

Comparisons of the estrogenic effects of phytoestrogens with the responses to estradiol depend on many factors, summarized in Section III. In general terms, coumestans have $1/100$ and isoflavones $1/10,000$ the activity of estradiol when given orally (Table 4).

Table 3
RELATIVE ABILITIES OF COMPOUNDS
TO BIND TO THE ESTROGEN
RECEPTOR

	Species*			
	Sheep		Goat	Human
	A	B		
Diethylstilbestrol	230	—	—	—
Estradiol	100	100	100	100
Coumestrol	5	0.43	1.4	10
Genistein	0.9	0.15	0.6	2
Equol	0.4	—	—	—
Daidzein	0.1	—	—	0
Biochanin A	<0.01	—	<0.1	—
Formononetin	<0.01	—	<0.1	0.001

* Data from (Sheep[A]) Shutt and Cox, 1972, (Sheep[B]) Mathieson and Kitts, 1980, (Goat) Shermesh et al., 1972, and (Human) Martin et al., 1978

Table 4
RELATIVE ACTIVITY OF PLANT
ESTROGENS ADMINISTERED TO
SHEEP

	Study*			
	A	B	C	
	i/r	i/m	(i/m)	(per os)
Diethylstilbestrol	—	100,000	100,000	—
Estradiol	—	—	—	1,000
Coumestrol	1.5	200	—	19
Genistein	0.1	2	—	—
Biochanin A	0.1	2	—	—
Formononetin	0.1	0	—	—
Equol	—	—	1.8—3.6	—

* Data from (A) Braden et al., 1967, either intraruminally (i/r) or intramuscularly (i/m) (B) Kozuro et al., 1984, and (C) Newsome and Kitts, 1979

C. Antiestrogenic Effects

Additive effects of coumestrol and estrogenic growth promotants have been reported in cattle.²³ In contrast, because they bind inefficiently to the receptor, phytoestrogens can also impede the action of the animal's own endogenous steroidal estrogen. All plant estrogens can also act as antiestrogens, whether the effect of phytoestrogens are additive or antagonistic to the steroids (i.e., estrogenic or antiestrogenic) depends on the relative amounts of the two substances.⁴³ In general terms, the plant estrogens act additively when steroid levels are low and antagonistically when steroid levels are high. Since endogenous steroid concentrations fluctuate during the estrus cycle, it is possible that phytoestrogens may at different times be both additive and antagonistic in the same animal.

Although there are reports of specific antiestrogens in plants, these have not been sufficiently well characterized to determine whether the effects were simply the results of com-

petition with steroids by weak phytoestrogens. An alternate possibility is that the compounds had toxic effects, reducing the responsiveness of the animal to stimulation by estrogen or, indeed, by other substances. True antiestrogens have been synthesized in the laboratory,⁴⁰ but no well-characterized antiestrogen has yet been isolated from plants. Antiestrogenic effects may also result from laboratory artifacts. The injection of plant extracts into laboratory animals can cause a stress response accompanied by the release of progesterone and corticosterone from the adrenal gland.⁴⁶ These steroids can antagonize the effects of estrogen and thus give an appearance of antiestrogenicity to the substance being injected.

D. Physiological Responses to Estrogen

The three main classes of action of estrogen in the body are the classical effects on reproduction, effects on general body metabolism, and effects on organogenesis during fetal life.

1 Reproductive Effects

Endogenous estrogens are responsible for the expression of mating behavior in the female of most species and for the control of the secretion of the gonadotropic hormones, particularly luteinizing hormone, from the pituitary gland. Plant estrogens seem relatively ineffectual in all of these functions. There is no report of any plant estrogen causing estrous behavior in ovariectomized animals. Furthermore, the effects of plant estrogens on the secretion of luteinizing hormone are relatively minor^{47,48} and can probably be accounted for largely as a result of ovarian suppression (see below). Leavitt⁴⁹ found that coumestrol was only one quarter as effective on the pituitary as on the uterus when compared with estradiol. Perhaps phytoestrogens do not cross the blood-brain barrier readily.

Estrogens also cause hypertrophy and hyperplasia throughout the reproductive tract. After treatment with estrogen, the epithelial and stromal cells of the uterus increase in number and size and the epithelial cells of the cervix manufacture and secrete mucus. The epithelial cells of the vagina increase in number and become cornified. These changes are accompanied by increased blood flow. In every case that has been examined, phytoestrogens produce changes in the reproductive tract which are qualitatively the same as those produced by endogenous estradiol.

Both steroidal and plant estrogens also affect the ovary and the mammary gland. Exogenous estrogen causes dysplasia of the ovarian granulosa cells, thus impeding the maturation of the ovarian follicles. This can reduce the secretion of estradiol by the ovary and thus cause secondary feedback effects on pituitary function. Estrogens cause the mammary glands to secrete milk, and the nipples to grow. It has been suggested that ewes grazed on estrogenic pastures may produce a greater supply of milk.⁵⁰

2 Metabolic Effects

Estrogens influence many pathways of general metabolism, but the effects vary greatly among species. In humans, most interest has been focused on the role of estrogens in lipid metabolism. In ruminants, attention has been paid to the increased growth rate seen in animals treated with estrogen. In the rat, by contrast, estrogens cause loss of body weight. These changes do not seem to be direct effects of estrogen, but result from modulation of other hormones by estrogen. Thus, it is not possible to extrapolate the growth-enhancing activity of estrogens from their potency on the uterus. There has been considerable speculation in the literature about the growth promoting abilities of the plant estrogens for ruminants. This problem has been very difficult to solve because of the unavailability of sufficient amounts of phytoestrogens to carry out adequate studies. Attempts to approach the problem using estrogenic feeds are inconclusive because the feeds invariably differ from the control feed in nutritive value. A number of studies have been carried out using coumestrol. In

reviewing these, Trenkle and Burroughs⁷¹ concluded that coumestans may have a slight growth-promoting effect, while Livingston⁶ concluded that they did not. There are no data for the isoflavones.

3. Morphogenic and Carcinogenic Effects

Estrogens play a role in the organization of the reproductive tract and the central nervous system during fetal life. Most studies on the neural effects have been carried out in rodents, in which early exposure to estrogen affects the hypothalamus in such a way that when the animal reaches adult life its behavioral and gonadotropin responses to estrogens or androgens follow the male, rather than the female pattern.⁷²

In both rodents and humans, exposure to exogenous estrogen during fetal life impairs the normal development of the female genital tract. As a result, uterine-like cells may be found around the vaginal entrance of the cervix, and in time these may become cancerous. This had led to diethylstilbestrol being classified as carcinogenic for humans, although similar effects can be produced in mice with estradiol⁷³ and coumestrol.⁷⁴ It is not clear, however, whether the estrogen itself is the carcinogen or whether the cancer arises because the development abnormality has resulted in the cells being exposed to an unusual environment. Similarly, although it is clear that many cancers of the mammary gland or the uterus grow more rapidly in the presence of estrogen, it is not at all certain that estrogens can cause the initial carcinogenic event.

VI. METABOLISM

Although the isoflavones occur in plants predominantly as glycosides, these are readily hydrolyzed either by plant enzymes during mastication or by acid in the stomach and by bacterial action in the gut. Most phytoestrogens appear to be either absorbed into the body or degraded by intestinal bacteria, and little is excreted unchanged. After absorption, most of the estrogenic material is conjugated by the liver or kidney and excreted in the urine or in the feces.

An understanding of the metabolic pathways by which the isoflavones are broken down is proven to be essential for understanding the relative importance of these compounds to the animal. Most studies have been carried out in the ruminant, but the general principles appear to be applicable to other species.

Metabolism in Ruminants

The metabolism of isoflavones in the rumen of sheep has been reviewed a number of times,^{6,7,75} and an outline only will be presented here. Genistein and biochanin A are converted almost entirely to *p*-ethylphenol and organic acids, while formononetin is mostly methylated and reduced to equol (Figure 5), which is absorbed into the body.³⁰ The pathway by which formononetin is metabolized to equol is unclear, it may proceed through 4-methoxy-equol or via daidzein. Under some conditions 4'-methoxy-equol may be the major excretory product,^{75,76} but the proportion of formononetin excreted in this form is very variable.

The 4'-methoxy-equol cannot bind to the estrogen receptor, but it is estrogenic when injected into mice,⁷⁶ presumably because it can be demethylated in the liver. In the sheep it is probable that the demethylation of formononetin or 4'-methoxy-equol is a rate-limiting step,³⁰ and is thus important in determining the overall estrogenicity of a clover pasture. Salt supplementation of marginally deficient animals increases the concentration of the methylating vitamin B₁₂, and this can produce an enhancement of the estrogenicity of terranean clover pastures if cobalt is limiting.^{55,77} Although most demethylation probably occurs in the rumen, demethylation can also occur in the liver.⁷⁸

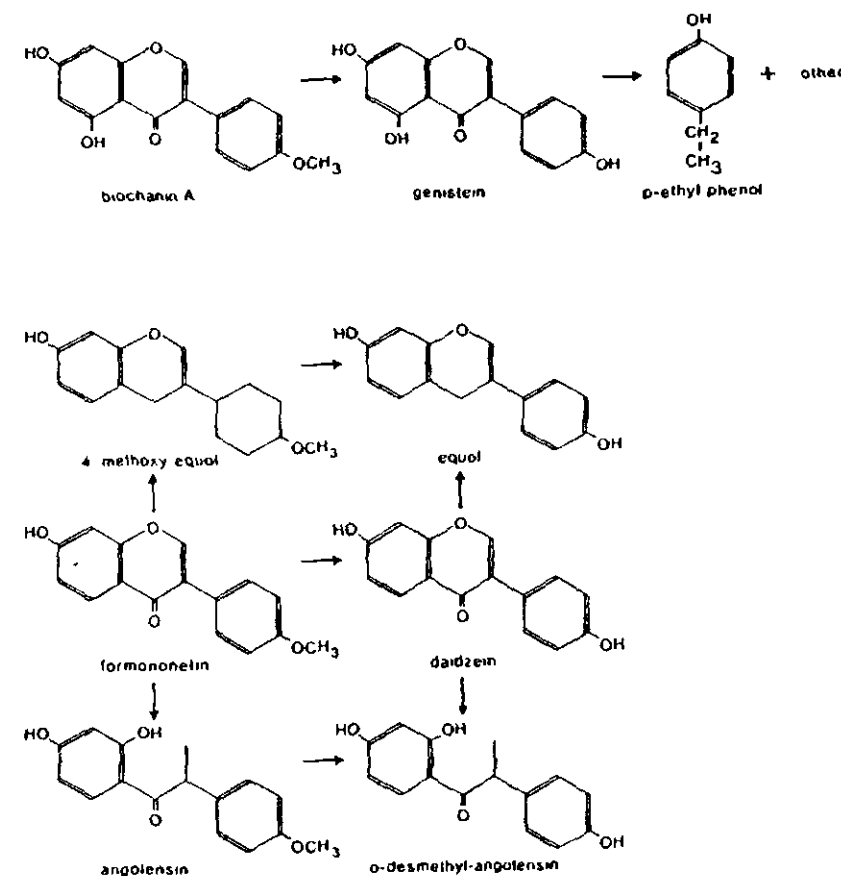


FIGURE 5 Suggested patterns of metabolism of isoflavones in the rumen. Formononetin is normally metabolized to equol, rather than to desmethyl angolensin.

Equol is absorbed rapidly from the rumen, with a mean residence time of 1.7 h,³⁰ and little is excreted in the feces. The equol is rapidly conjugated to glucuronides⁶ in the liver and excreted in the urine. Shutt et al.³⁰ calculated that 67% of the formononetin ingested was excreted in the urine as equol. Excretion of the isoflavones appears to be very rapid, so that any interference with clearance rate (e.g., liver or kidney damage) might be expected to increase the degree of estrogenic effect.⁷⁹

Less is known about the metabolism of coumestans in the sheep. In contrast to the position with isoflavones, adaptive changes by the ruminal microflora have not been found,³⁴ although it is probable that 4'-methoxycoumestrol is demethylated extensively in the rumen. However, coumestrol has much less effect in the sheep if given intraruminally than if given intramuscularly (Table 4).⁷⁹ Some of this difference has been attributed⁷⁹ to a greater rate of conjugation in the liver of material absorbed from the intestinal tract, but breakdown in the gut must also play a role. Coumestrol is conjugated to both sulfates and glucuronides.⁶ The clearance rate of coumestrol from the blood appears to be slower than that of genistein.⁷⁹ The concentrations of coumestrol which have been reported in the plasma of sheep, goats,

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Toxicants of Plant Origin

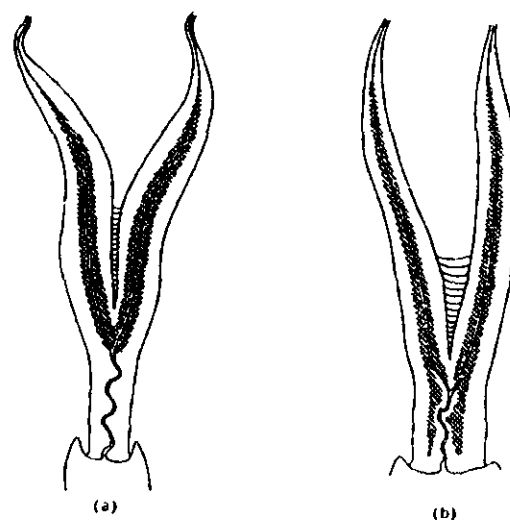


FIGURE 8 Diagram of the cervix and uterus of a normal ewe (a) and a ewe with permanent clover infertility (b) showing the extent of endometrial tissue (cross-hatched)

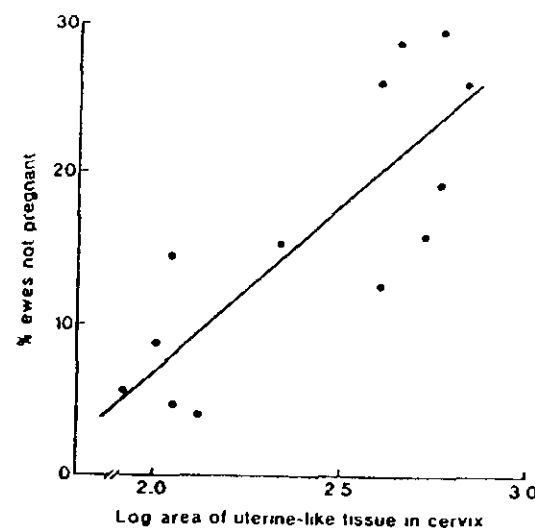


FIGURE 9 Relationship between the mean area of endometrial tissue in the cervix and the proportion of nonpregnant ewes in 13 commercial sheep flocks in Western Australia

Defeminization can be produced in ewes by much lower amounts of estrogen than are required to produce the classical signs of clover disease.¹⁰² It appears that the duration of exposure is more important than the amount of estrogen in the pasture. Reductions in fertility are likely to be important only where ewes graze estrogenic pasture containing >0.2% formononetin for more than 4 months of the year. In Australia many millions of ewes are affected, but only to a mild degree, so the average reduction in the proportion of ewes lambing is about 10%.¹¹⁰

There is no cure for defeminization. The main preventative treatment available is to maintain phosphatic fertilizer and encourage grasses in the pasture¹¹¹ and to replace the cultivars of clover in the pasture with strains containing less formononetin. Possible management options, including changes to husbandry, have been discussed by Lightfoot.³

2 Other Effects

Sheep grazed on highly estrogenic pastures consisting of relatively pure stands of subterranean clover or red clover containing high concentrations (>0.8%) of the isoflavone formononetin may suffer marked clinical signs. Ewes may be affected by severe infertility accompanied by gross pathological changes in the uterus, including *hydrops uteri*, *pyometron*, cystic hyperplastic endometritis, and massive adhesions resulting from metritis.¹¹² There may be outbreaks of uterine prolapse unassociated with parturition, while up to 70% of pregnant ewes may suffer dystocia because of uterine inertia and failure of the cervix to dilate.^{112,113} The epithelium of the bulbourethral glands of wethers undergoes stratified squamous metaplasia, and the glands may enlarge and fill with fluid, so as to be visible externally.¹¹² This enlargement, together with precipitates of isoflavone metabolic products,⁷⁶ can cause blockage of the urethra and death from rupture of the bladder. Nonpregnant ewes, wethers, and even rams may develop mammary glands and lactate. These gross clinical signs have been termed "clover disease"¹¹⁴ and are now relatively rare even in Western Australia where they were originally described.

The spectacular clinical signs make diagnosis relatively easy. Because the problem develops only in animals exposed to large amounts of estrogen for several months, it is seen only on red clover or cultivars of subterranean clover containing large amounts of formononetin. These include cultivars Dwalganup, Dinninup, Yarloop, and, to a lesser extent, Geraldton. The cultivar Tallarook also contains high concentrations of formononetin and is potentially dangerous. The condition is most severe on newly sown pastures, partly because these are more clover dominant and phosphate deficient. However, there are also other unknown factors which influence the severity of the estrogenic effect.

The losses in wethers may be prevented by treatment with testosterone.¹¹⁵ There is no preventive treatment or cure for ewes, but the problem may be ameliorated by husbandry.³ If the severe form of this condition occurs on a farm, it is usually best to stop trying to breed the ewes. Most ewes in the flock become infertile, so there is little point in culling those which fail to bear a lamb.³

B. Cattle

Infertility has been reported in cattle ingesting estrogenic feed and is thus comparable with the "temporary infertility" described for sheep. There are no reports of permanent infertility in cattle. In all cases, clinical signs such as mammary development, discharge of cervical mucus, and swelling of the vulva and uterus have been observed. Most cases have been reported in cows in intensively managed dairy herds being fed supplements of alfalfa which contained coumestans. Despite the high levels of isoflavones in pastures in Western Australia, there have been no confirmed reports of estrogenic problems in cattle. It is probable that isoflavones do not usually cause problems in cattle, although estrogenic infertility has been suspected in cattle grazed on both subterranean clover¹¹⁶ and red clover.¹¹⁷

The infertility results from abnormal function of the pituitary/ovarian axis. The reproductive problem frequently resembles the abnormalities associated with cystic ovaries, including prolonged but irregular estrus, nymphomania, or even the development of masculine sexual characteristics.^{118, 119} Cysts may be palpable on the ovaries, while ovarian hypoplasia and anestrus have also been observed after prolonged exposure.¹²⁰ After removal from the estrogenic feed, the infertility may take several months to resolve, presumably because the ovarian cysts are slow to regress.

Outbreaks of abortion in cows have been attributed to plant estrogens,¹²¹ although the evidence is not very strong. Abortion in cattle after ingestion of pine needles was originally ascribed to an antiestrogen,¹²² and in the field, may be accompanied by signs of hyperestrogenism. However, these have not been reproduced experimentally and it is more likely that abortion is due to a fetotoxic agent.¹²³

Subclinical temporary infertility has not been reported in cattle. However, it is possible that unexplained outbreaks of cystic ovaries, or even aspects of the seasonal fluctuations in the prevalence of cystic ovaries in cow herds, may be due to phytoestrogens in the feed.¹¹⁶

Other Species

Outbreaks of hyperestrogenism are not uncommon in swine, but almost invariably they can be attributed to the feeding of moldy corn. The fungal estrogen zearalenone produces clinical signs including a swollen vulva and enlargement of the mammary glands and tests. The reproductive process is impaired at a number of sites in the sow, including anestrus, failure to conceive, and embryonic mortality.

Pigs fed large amounts of soybean containing genistein have shown swelling of the vulva, but there were no other effects.⁴⁹

There are clinical reports of hyperestrogenism in both goat does and mares run on estrogenic pasture, but these have not been fully investigated.

Impairment of the reproductive process has been examined in laboratory animals fed plant estrogens. Mice fed diets containing between 50 and 900 ppm coumestrol had a reduced litter rate, an increased proportion of abnormal embryos, and slightly reduced fertility.¹²⁴ In pigs fed estrogenic subterranean clover had reduced conception rates, but continued to show estrus.¹²⁵

Persistent morphogenic effects of coumestrol have also been demonstrated in laboratory animals. Rats injected with 1 mg coumestrol on day 5 of life had persistent estrus in adult life,¹²⁶ apparently because masculinization of the hypothalamus resulted in dysfunction of the ovary. Mice injected with 100 µg coumestrol on days 1 to 5 of life also had ovarian abnormalities, but in addition had abnormal development of the vagina.⁷⁴

IX. MODIFICATION BY PLANT BREEDING AND SELECTION

Plant estrogens are found in many of the major forage legumes, for which extensive investigations are being carried out into the breeding and selection of new varieties. For alfalfa and white clover, the production of cultivars which are resistant to pests and diseases is of prime importance for productivity. The content of phytoestrogens in alfalfa¹²⁷ and white clover¹²⁸ is also dependent of the presence and degree of infection, so the goals of economic production and low estrogenicity coincide for these two species. Therefore, breeding for low estrogenicity imposes no cost on these programs and is likely to proceed readily under present conditions.

In red clover and subterranean clover, the position is different. In these plants, the content of phytoestrogen is determined genetically, so the plant breeder needs to pay specific attention to the problem if progress is to be made. Furthermore, for both of these clovers the selection of low-estrogen cultivars is a major tool in combatting the losses in animals.

Most work on subterranean clover has been carried out in Australia where it has been coordinated in the National Subterranean Clover Improvement Program.¹²⁹ This work aims to produce strains of clover with good persistency and disease resistance, adapted to different environmental and management regimes. A low content of formononetin (<0.1%) is a primary selection criterion, and because of the abundance of strains available, this is relatively easy to attain. Where possible, low concentrations of genistein and biochanin A are also sought. Whilst it is generally thought that these levels should be safe for sheep, the recent observations of reduced lambing performance in the absence of clinical signs (see Section VIII.A.1) indicates that this assumption may need to be studied further.

Some of the old, highly estrogenic cultivars are well adapted to particular environments, and replacement of these can present a problem. It is likely that old pastures which have been resown will continue as a mixture of cultivars, and therefore attention is also paid to determine those factors which permit a cultivar to be successful in mixtures with the cultivars which they are designed to replace.

Although plant breeding is also the main technique available for the control of estrogens in red clover, plant breeders have been less aware of potential estrogenic problems than breeders of subterranean clover. The Welsh plant breeding program has screened red clover for formononetin, but the breeding studies on red clover in New Zealand and the U.S. have largely ignored the potential problem.¹³⁰ As a result of this approach, the development of the Grasslands Pawera strain of red clover in New Zealand has resulted in the production of plant material containing 1% formononetin, and the plant would not be recommended for use by breeding stock.¹³⁰

X. FUTURE PERSPECTIVES

Although plant estrogens were first isolated shortly after the initial isolation of estrogens from animals in the late 1920s, they did not become the subject of extensive research until the mid 1940s. The recognition of serious clinical problems in sheep grazing estrogenic subterranean clover in Western Australia was soon followed by the observation of lesser problems elsewhere. Over the subsequent 25 years, the estrogenic compounds were isolated, their relative estrogenicity determined, and programs were established to minimize their occurrence in animal fodder. Once these programs were put in place, it was generally assumed that the problem had been solved. In the last 15 years, relatively little research has been carried out on plant estrogens and their effects.

These initiatives have been successful in controlling clinical problems from plant estrogens. Strains of red clover and subterranean clover with a low content of formononetin are now available for most of the potential environments. Some further work on economic methods for placing the new cultivars into established pastures, and factors affecting the subsequent competition between cultivars, is desirable. The continued development of cultivars of alfalfa and white clover resistant to most pathogens should maintain the concentrations of coumestans at relatively low levels. There appears little need to change the initiatives established by the plant researchers in the 1960s, except to seek greater production of digestible organic matter.

Animal scientists have fewer grounds for complacency. In recent times the existence of subclinical losses caused by plant estrogens has become apparent. These losses can be detected only by measurement of phytoestrogens or estrogenic damage and of associated suboptimal reproductive performance in exposed animals. Preliminary studies indicate that defeminizing subfertility and temporary infertility in sheep are widespread in Australia and New Zealand, but the actual extent of the problem is unknown. Although cultivars of subterranean clover low in formononetin are available, it is probable that they will never completely replace existing strains. The occurrence of interbreeding between cultivars also

suggests that clover pastures will eventually reach an equilibrium with low but detectable levels of formononetin. The concentration of formononetin in pasture which is completely "safe" for sheep is unknown. The extent of problems caused by plant estrogens in other parts of the world is also unclear. In cattle, there are no data on the role of phytoestrogens in the relatively common "cystic ovaries" syndrome.

Future progress in animal studies may occur in two areas. First, the application of currently available techniques to measure the extent of subclinical reproductive losses in ruminants should lead to a greater awareness and understanding of the problem. There appears to be substantial variability between flocks in the severity of estrogenic problems, apart from differences in the estrogenicity of the pasture. An improved knowledge of the conditions under which reproductive losses occur may reveal the sources of this variability and enable them to be exploited. Secondly, the current techniques for reducing the phytoestrogenic content of plants need to be complemented by techniques for reducing the estrogenic effects in animals. Animals may be immunized against plant estrogens.^{6,11,12} The metabolic degradation of plant estrogens in the rumen might be enhanced.⁷ In the case of permanent infertility in ewes, other hormones which may play a role in the development of lesions might be manipulated.¹³

Studies on the possible effects of plant estrogens on humans are at a very early stage. It has been shown that humans ingest and excrete plant estrogens. However, this will remain of academic interest unless estrogenic effects are demonstrated in humans ingesting plant estrogens as part of a relatively normal diet.

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Metabolism of Estrogenic Isoflavones in Domestic Animals (43828)

TORBJÖRN LUNDH¹

Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences,
S-750 07 Uppsala, Sweden

Abstract. The metabolism of estrogenic isoflavones in cattle and sheep is reviewed. Results from *in vitro* and *in vivo* studies are discussed, mainly regarding whether differences in sensitivity to phytoestrogens between cattle and sheep depend on differences in metabolism, particularly in conjugative capacity. Results from a feeding experiment with pigs fed red clover meal are presented. Levels of phytoestrogens in plasma from the pig are compared with those found in plasma from ruminants fed red clover silage. Some aspects relating to the possibility of pigs being exposed to risks when fed with feed containing estrogenic isoflavones are briefly discussed.

[P S E B M 1995, Vol 208]

A substantial work about the metabolism and estrogenic effects of isoflavones on sheep has been described since a massive outbreak of permanent and temporary infertility in sheep grazing on certain cultivars of subterranean clover (*Trifolium subterraneum*, L.) in Western Australia was reported in the 1940s (1). Since then, several different herbs have been shown to contain estrogenic substances that are capable of affecting the reproduction when consumed in large amounts (2). In Sweden, red clover (*Trifolium pratense*) is quantitatively the most important source of phytoestrogens, with a normal concentration of 0.5%–2.5% of dry matter (3). Dairy cows are allowed to graze on red clover pastures during the summer. During the indoor season, lasting about 7 months, the cattle are often fed silage with high contents of red clover. Thus, daily consumption of phytoestrogens may reach 50–100 g. Despite this, there have been no reports of permanent infertility in cattle, and, consequently, the estrogenic effects have been suggested to be generally weaker on cattle than on sheep (4, 5). Even if cattle are not affected to the same extent as sheep, the syndromes of infertility occurring in the former have been associated with consumption

of estrogenic pasturage (6, 7, 8, 9, 10). The suggested differences in sensitivity to phytoestrogens between cattle and sheep have been proposed to depend on differences in metabolism, especially differences in detoxication capacity between the two species, cattle and sheep (11).

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Ruminants

Rumen Metabolism. Metabolism in sheep has been reviewed a number of times (12, 13, 14), whereas very little has been described about metabolism in other species. However, rumen metabolism of isoflavones in other species has been suggested to be qualitatively similar to that in sheep (15).

The isoflavones occur in intact plants predominantly as glycosides (16) and are readily hydrolysed by plant enzymes or by microorganisms in the rumen. The major metabolic transformation of isoflavones is performed by microorganisms in the rumen (17). Biochanin A is demethylated to genistein and via ring cleavage to p-ethyl phenol and organic acids (Fig. 1). In contrast, formononetin, which is indirectly responsible for estrogenic disturbances in sheep (18), is mainly demethylated to daidzein and further via hydrogenation and ring fission predominantly to equol (Fig. 2). However, many metabolic interconversions are not completely defined. Intermediary compounds from ring cleavage of genistein to simpler phenol compounds have not yet been isolated. Furthermore, the metabolic pathway of formononetin may proceed, under some circumstances, via an alternative route involving reduction without prior demethylation, where

¹ To whom requests for reprints should be addressed at Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, P.O. Box 7024, S-750 07 Uppsala, Sweden.

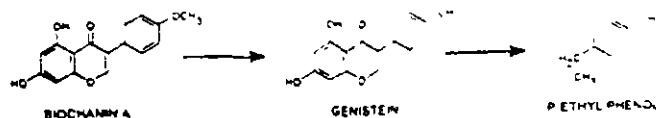


Figure 1. Rumen metabolism of biochanin A in sheep, suggested by Cox and Davies (13).

O-methyl-equol is the major excretory product of formononetin (12). The proportion of formononetin excreted in this form is very variable and factors that affect the extent to which formononetin is metabolized by this route have yet to be elucidated.

Hepatic Metabolism. The liver is generally regarded as the major organ for metabolism and detoxification of foreign substances. Therefore, the apparently lower susceptibility to phytoestrogens of cattle in comparison with sheep has been suggested to depend on differences in liver metabolism (11, 12). We have investigated the demethylating and conjugative rate of formononetin and daidzein in microsome preparations from sheep and cow livers (19).

Demethylating of formononetin to daidzein in liver microsomes was exceedingly small in both species. No further reduction of daidzein to equol appears to occur in the liver or is very small, since no equol was detected with the HPLC technique used (3). Furthermore, the results indicate that the liver contributes very little to the total degradation of phytoestrogens in ruminants, and rumen microorganisms probably account for most of the demethylation and reduction activity (17, 20).

The conjugative activity was much higher than the demethylating activity in both species. Both formononetin and daidzein was about 1.5 times more efficiently conjugated by the ovine liver than the bovine liver when uridine 5'-diphosphoglucuronic acid (UDPGA) was present (Table I).

In order to achieve a maximum glucuronidation rate, two different effectors, uridine 5'-diphospho-N-acetylglucosamine (UDPAG) and Lubrol PX were included in some incubations. When the effectors were added to the test tubes together with microsomes UDPGA and the β -glucuronidase inhibitor, Saccharic lactone (*S. lactone*), the conjugation pattern changed: total activity increased, but no significant difference between the two species was observed.

Metabolism of Isoflavones in the Gastrointestinal Epithelium. No explanation of the suggested differences in sensitivity to phytoestrogens between cattle and sheep could be established in the experiment with liver microsomes. Other tissues capable of detoxifying these substances must occur if the differences in sensitivity between cattle and sheep depend on their capacity to detoxify the estrogenic isoflavones. It is reasonable to believe that different dietary substances should be detoxicated before they reach the blood circulation. Therefore, the gastrointestinal epithelium

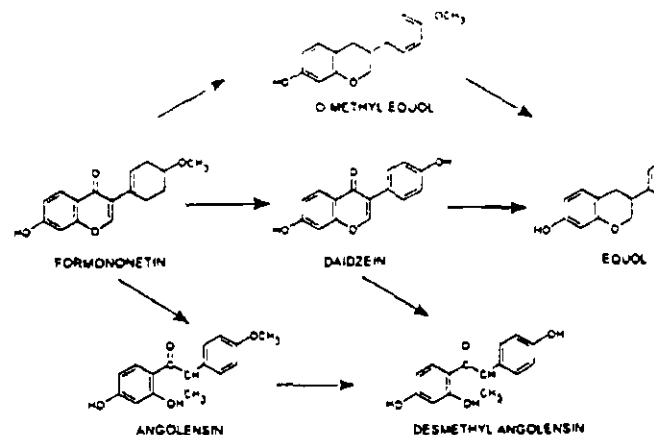


Figure 2. Rumen metabolism of formononetin in sheep, suggested by Cox and Davies (13). The major metabolic pathway of formononetin is via daidzein to equol, rather than via O-methyl-equol and to desmethyl angolensin.

should probably be the most important site for detoxifying phytoestrogens and other harmful dietary substances before they enter the blood circulation.

In order to evaluate if differences in extrahepatic metabolism may explain the differences in susceptibility to phytoestrogens between cattle and sheep, the glucuronidation activity in the epithelium from the four compartments of the complex stomach and the small intestine have been investigated (21). This study was performed in a similar way to the liver study, but homogenates were used instead of microsomes. The results in Figure 3 show that a considerable conjugation activity takes place in the gastrointestinal epithelium, which cannot be ignored. The conjugation activity was 3 to 20 times greater in sheep than in cattle in all parts of the gastrointestinal tract, except in the intestinal mucosa, where the activity was about 10 times higher in cattle than in sheep.

Even though the conjugation rate is almost the same in rumen, reticulum, and omasum, the rumen is probably the most important tissue in the gastrointestinal tract as regards detoxification of these substances. The reason for this supposition is that ingested food stays in the rumen for a relatively long period (i.e., 1-3 days, depending upon the nature of the diet and animal species). The absorption of phytoestrogens takes place mainly in the rumen (22, 23); therefore, the higher bovine conjugation in the small intestine compared with the rumen and other organs was unexpected.

The conjugative activity differed depending on which substrate was used. The highest activity was observed when equol was used as substrate, about 3 to 10 times higher than when daidzein and formononetin were used. The large variations in conjugation rate between formononetin, daidzein, and equol are unexpected as the differences in chemical structure are very small. The differences in glucuronidation activity

Table 1. Conjugation of Formononetin and Daidzein by Cow and Sheep Liver Microsomes*

Additions	Formononetin disappeared ($\mu\text{g}/120\text{ min}$)		Daidzein disappeared ($\mu\text{g}/120\text{ min}$)	
	Cow	Sheep	Cow	Sheep
UDPGA + β -Glu	0.6 ± 0.8	1.1 ± 1.0	0.4 ± 1.6	0.1 ± 0.1
UDPGA	3.7 ± 1.4	6.6 ± 0.8^b	7.1 ± 0.9	10.0 ± 2.1
UDPGA + (UDPAG + S. lactone)	7.4 ± 1.1	7.4 ± 2.6	13.0 ± 1.1	12.0 ± 2.1
UDPGA + (Lubrol + S. lactone)	7.9 ± 0.6	7.6 ± 1.8	12.7 ± 0.9	12.2 ± 1.1

* The figures are mean values \pm SD of five to eight experiments.^b $P < 0.05$.

(From Lundh et al., J Agric Food Chem., 36:22, 1988. With permission)

between formononetin and daidzein may be explained by differences in their chemical structure where formononetin has two methyl groups and daidzein only one, which can bind to glucuronic acid. The difference in chemical structure between daidzein and equol is small: equol has lost a keto group in Position 4 and has a single bond between C-2 and C-3 (Fig. 2). It is therefore more difficult to explain why these two substances differ so much in conjugation activity. However, it is well known that the glucuronosyltransferase exists in multiple forms. Whether the higher conjugation rate of equol in comparison with daidzein is due to different isoenzymes or merely to steric hindrance cannot be explained without further investigation. On the other hand, it seems rather logical that the conjugation rate of equol is higher than for formononetin and daidzein, because equol is the major substance absorbed from the rumen to the blood circulation.

Metabolism In Vivo. *In vivo* metabolism in ruminants of substances present in the feed is influenced by a complex pattern of variables. Even if the liver, gastrointestinal tissue, and to a certain extent, the kidney may be regarded as detoxification centers, other phenomena such as absorption, excretion, degree of conjugation, etc. may cooperate in an *in vivo* situation, which cannot be anticipated by means of only *in vitro* studies. Being able to determine the concentrations of phytoestrogens in blood samples taken from animals during feeding makes it possible to illuminate the complex interactions which occur *in vivo*.

In a comparative feeding study (23), five dairy cattle from a herd normally fed on red clover/grass silage (50%/50%) were used as experimental animals. The feed ratio for the cows was about 13–14 kg of silage, offered twice a day, corresponding to a daily mean intake of about 14–15 g of formononetin and 0.3 g of daidzein. The sheep were offered the same silage as the cows, and blood samples were collected after a 10-day period of prefeeding. To compare the plasma concentrations of the phytoestrogens in sheep with those in cattle, the sheep were offered the same amount of red clover silage, with consideration to the basal metabolic rate (which represents 0.247 kg of si-

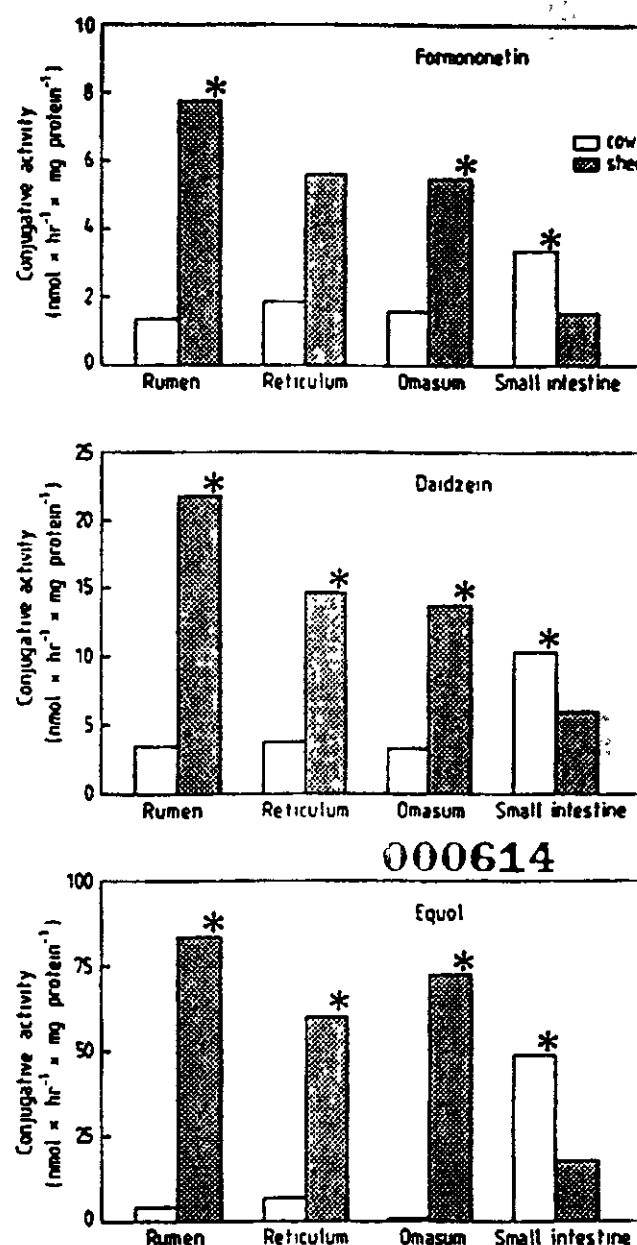


Figure 3. Conjugative activity toward formononetin, daidzein and equol in various gastrointestinal tissue homogenates from cattle and sheep. The columns represent mean values of data from three animals of each species. The asterisks mark significantly greater activity than that in the other species. (From Lundh, J Agric Food Chem 38:1012, 1990. With permission).

METABOLISM OF ESTROGENIC ISOFLAVONES

lage/liveweight^{0.75}) corresponding to a mean intake of about 3.2 g formononetin per day per sheep. Such high daily intake of formononetin is enough to cause apparent estrogenic symptoms in ovariectomized ewes (24) and adversely affect fertility in sheep (25). The analysis of total (conjugated and free) and free (unconjugated) phytoestrogens in blood plasma was performed by a HPLC-method previously described (26).

Formononetin and daidzein were absorbed very rapidly in cattle and reached a total (free and conjugated) maximum level within 1 hr after feeding. At this time, the plasma concentration was about three times higher in the cow than in the sheep, but declined to the same level as in sheep after about 2-3 hr (Fig. 4, A and B). The high value after 7 hr in bovine plasma, presented as a dotted curve in Figure 4 and 5, depends on the cows being accidentally fed with their other half of the daily silage ration about 15-20 min before the last blood sample was taken. However, this result confirms that absorption of formononetin and daidzein is very rapid in the cow and take place already in the rumen.

The concentration of total equol in bovine plasma was almost constant during the whole sampling period. In the sheep, the initial equol concentration increased from about 80 µg/100 ml plasma, and did not reach the same level as in the cow until after 2-3 hr (Fig. 4C). These results show that the cattle are exposed to a higher concentration of equol for a longer time than the sheep since the plasma concentration of equol in the sheep blood decreases by about 50% during the night (14-16 hr after food intake), whereas the concentration remains fairly constant in cows. The differences in plasma concentration of total equol at the beginning of the sample time indicates that the clearance of equol is faster in the sheep. Concentrations of total equol in sheep plasma, similar to those presented in Figure 4C, are high enough to cause estrogenic symptoms in sheep (11, 27).

Glucuronidation is the major detoxication system for several potentially toxic endogenous and exogenous substances, including phytoestrogens. Formononetin, daidzein, and equol are mainly found as conjugates in blood. Not more than about 5% of the total amount was found as unconjugated substances in plasma at any time and the concentration of free formononetin was very low (Fig. 5A) (daidzein was detected in even lower concentrations, just around the detection limit). The free amount of equol constitutes about 5% of the total amount in cows and about 1% in sheep. The concentration of free equol was, however, about 10 times higher in plasma from cows than from sheep, whereas no differences in plasma levels of unconjugated formononetin and daidzein occurred (Fig. 5B).

In contrast to Braden *et al.* (11), who suggested that the weaker effects of phytoestrogens on cattle de-

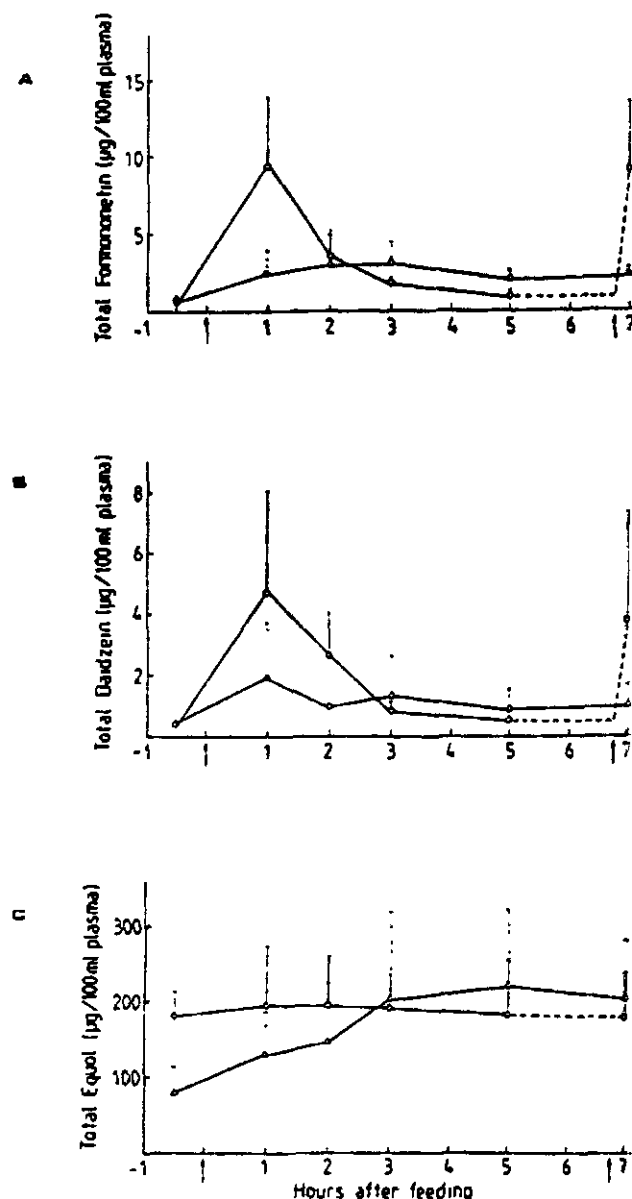


Figure 4. Concentrations of total (free and conjugated) phytoestrogens (A) formononetin, (B) daidzein, and their metabol (C) equol in bovine (O—O) and (Δ—Δ) ovine blood plasma different times after feeding on red clover/grass silage. Data represent mean \pm SD from five animals. The first arrow indicates when the feed was offered to the cows and sheep. The line dotted between 5 and 7 hr since the cows were offered the other half of their daily feed ration just before the last blood sample was taken which is indicated by the second arrow. (From Lun *et al.*, *J Agr Food Chem* 38:1530, 1990. With permission).

pend on the circulating phytoestrogens and their metabolites being more efficiently conjugated in cattle than in sheep, our results suggest that sheep have higher capacity to conjugate phytoestrogens. According to Shutt and Braden (27), equol is the main substance responsible for reproductive disturbances in sheep, and the prevailing opinion is that biological active forms consist of free, unconjugated, and, to certain degree, sulfonated substances. With this mind and from the results presented here, the she-

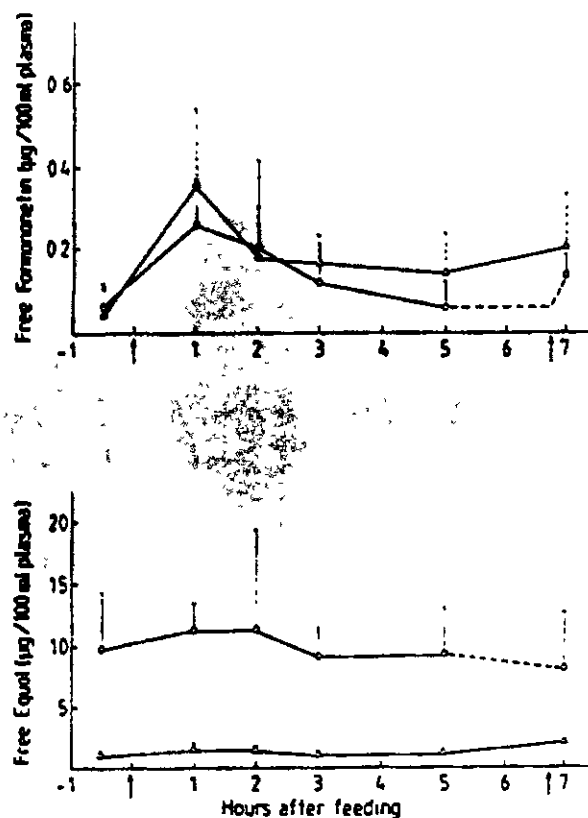


Figure 5. Concentrations of free (A) formononetin and (B) equol in bovine (○—○) and (△—△) ovine blood plasma at different times after feeding on red clover/grass silage. Explanations to the different signs is given in Figure 4. (From Lundh et al., J Ag Food Chem 38:1530, 1990. With permission).

seems to be better equipped than the cow for detoxifying and eliminating phytoestrogens. Based on our *in vitro* studies with liver microsomes (Table I, cf.), gastrointestinal homogenates (Fig. 3, cf.) and in *in vivo* experiments (Fig. 4 and 5, cf.), we assume that the sheep conjugates phytoestrogens more efficiently than the cow. Furthermore, the large amount of free equol in bovine plasma indicates that other factors than differences in conjugation rate between cattle and sheep explain the differences in sensitivity to phytoestrogens. Such factors might be on the receptor level. The concentration of estrogen receptors in uterus was about two to four times higher in sheep than in cattle (28, 29, 30).

Monogastric Animals

Metabolism In Pigs. The metabolism of phytoestrogens in pigs is not well documented and to my knowledge no studies on that subject have been described before. However, metabolic studies on such monogastric animals as rat (31), guinea pig (27), fowl (32), and humans (33) have been described in the literature. The major metabolic transformation of phytoestrogens in humans is performed by the intestinal microorganisms which probably is also the case for monogastric animals (33).

The pig may be exposed to high levels of phytoestrogens since their food might contain large amounts of legumes (such as red clover or soya products) containing estrogenic isoflavones. Furthermore, in Sweden, the usage of red clover products as food for pigs is predicted to increase in the future.

In a feeding experiment, two pigs were fed on a mixture containing 20% of red clover meal corresponding to a daily mean intake of 866, 88, 97, and 378 mg of formononetin, daidzein, genistein, and biochanin A respectively. The animals were prefed with the experimental diet 7 days before the blood samples were collected via permanent vein catheters, at different times after feeding. Formononetin was absorbed very rapidly and reached a total maximum level (free and conjugated) within 1 hr and remained at a rather stable concentration of 80–100 µg/100 ml plasma, which is about 10 times higher than the highest level found in bovine plasma. The high concentration of the formononetin in blood plasma immediately after feeding indicates that the absorption has already taken place in the stomach. Since the food stays in the stomach only for about 2 hr, absorption from the intestine is also most probable. Daidzein and equol show similar patterns to formononetin, but the concentrations were about 5–15 times lower (Fig. 6). Even total genistein was found in about similar concentrations as equol.

Very little formononetin occurred in unconjugated form, 0.2–0.6 µg/100 ml, which represents about 0.5% of the total amount (Fig. 7). Daidzein occurred in even lower concentrations (not shown). The free amount of equol in porcine plasma was about 13 times higher than that for formononetin (Fig. 7). The free amount of equol constitutes about 30%–50% of the total amount in pigs. The corresponding values for sheep and cow were 1% and 5% which suggest fundamental differences in conjugation of equol between the pig and ruminants. The plasma concentration of total equol in the pig was about 10 to 15 times lower than in plasma from the sheep and the cow but the free amount of equol being at the same level as for the ruminants (about 2 and 10 µg/100 ml for sheep and cow, respectively).

Urine samples taken continuously for 8 hr via a ureter catheter show that the isoflavones are mainly excreted via the urine. About 55% of the ingested formononetin and daidzein, originated from the morning feed, was excreted within 8 hr feeding. The proportion of these excreted isoflavones were 72% of formononetin, 6% daidzein and the remaining 22% was identified as equol. Thus, the major part of the formononetin ingested by the pig was excreted unchanged without prior degradation. Furthermore, only trace amounts of the phytoestrogens were detected in blood plasma 3 days after the red clover feed was withdrawn.

Pigs are probably the most sensitive animals to the

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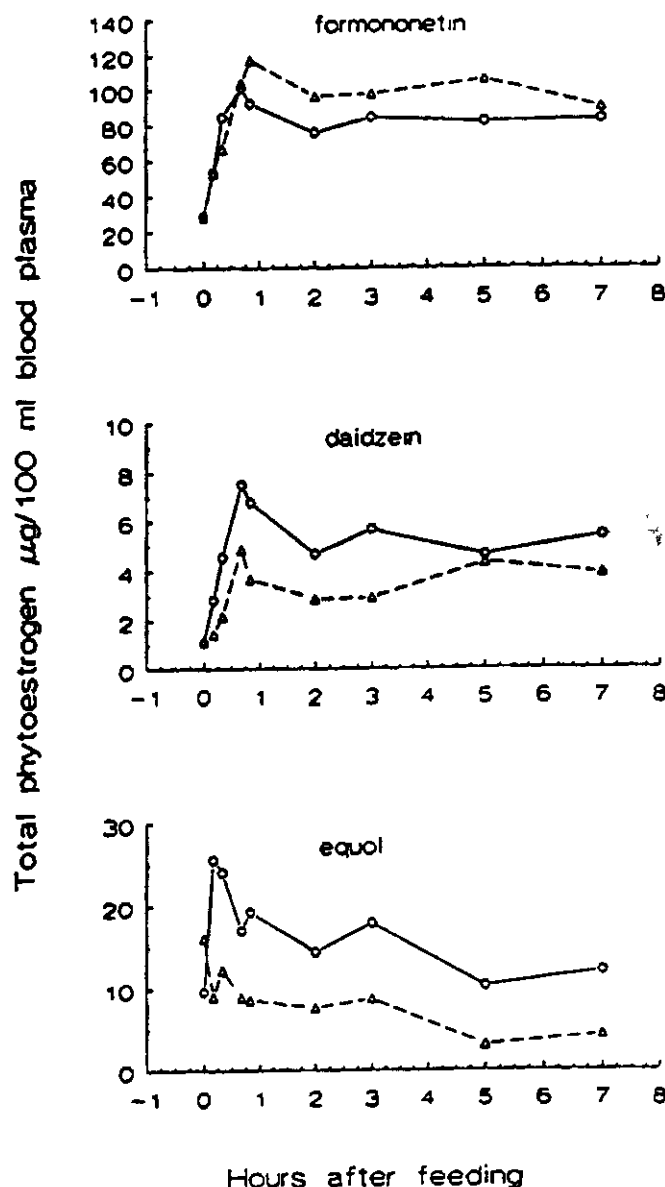


Figure 6. Concentrations of total (free and conjugated) phytoestrogens in blood plasma from two pigs (Pig 1, \circ — \circ ; Pig 2, Δ — Δ) at different times after feeding on 20% red clover meal.

"mycoestrogen" zearalenone (34), which is produced by the mold fungus *Fusarium sp.* Hyperestrogenism is not uncommon in pigs fed on fusarium-infected feed as corn, barely or hay. Because of the high concentration of unconjugated equol shown in Figure 7, it would be interesting to conduct a more extensive study on the effects of phytoestrogens on reproduction in the pig.

Implication

As mentioned earlier, most of the phytoestrogens occur in the intact plant as glucosides, which are hydrolyzed in the rumen and further demethylated and reduced by the microorganisms (17). A very minor part of these hydrolyzed phytoestrogens is absorbed very quickly from the rumen, and reaches the blood circulation unconjugated. The bulk are, however, con-

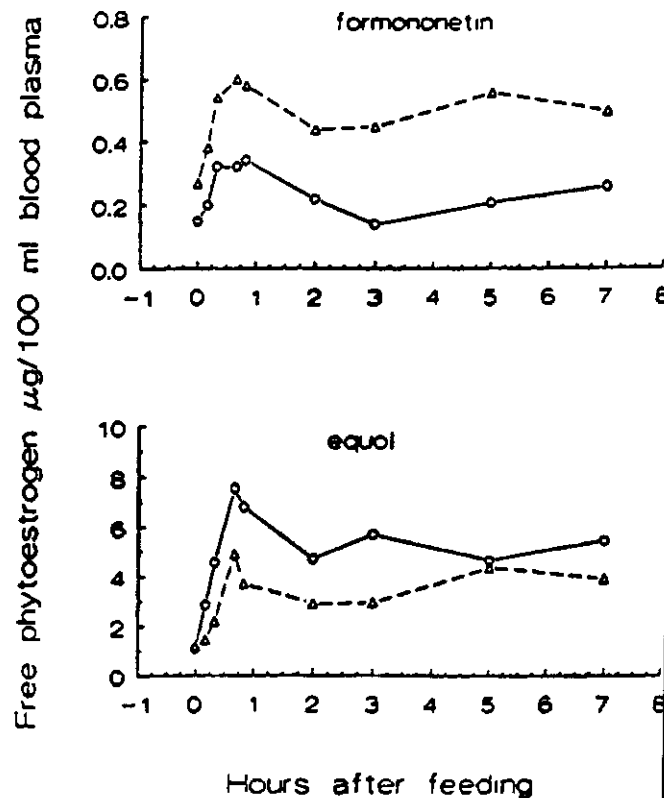


Figure 7. Concentrations of free formononetin and equol in blood plasma from two pigs (Pig 1, \circ — \circ ; and Pig 2, Δ — Δ) at different times after feeding on 20% red clover meal.

jugated with glucuronic acid already in gastrointestinal epithelium. The substances remaining unconjugated when entering the blood circulation are mainly conjugated by the liver and perhaps also by other extrahepatic tissues as the kidney. The high conjugative activities of isoflavones in gastrointestinal epithelium from cattle and sheep implicate that the liver's role as the predominantly detoxifying organ probably has been overestimated in ruminants. Detoxification by the means of conjugation in different tissues probably explains why no more than about 5% of the isoflavones are found in unconjugated form in blood plasma from sheep and cattle. The metabolite equol is excreted mainly via the urine, and 70% of the daily intake of formononetin was recovered as equol in urine from sheep fed on red clover (22).

According to Markiewicz *et al.* (35), the relative potency of equol is 0.061% of estradiol-17 β . If we consider that during feeding with silage the concentrations of unconjugated equol in ovine plasma is about 20,000 pg/ml, the potency can be approximately 100 times higher than the activity of estradiol-17 β during estrus (36).

The pig seems to differ markedly in comparison with ruminants regarding conjugation of equol. Only 50%–70% of total equol was found in conjugated form whereas the corresponding figure for conjugated equol in plasma from cow and sheep is 95%–99%. The sig-

Hormone Studies on Ewes Grazing an Oestrogenic (Yarloop Clover) Pasture During the Reproductive Cycle

J. M. Obor^a and R. F. Seamark

Department of Animal Physiology and Department of Obstetrics and Gynaecology,
University of Adelaide, Adelaide, S.A. 5001.

^a Present address: Department of Agriculture, Straus Research Centre, Naracoorte, S.A. 3271.

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Abstract

The endocrine function of Merino and Corriedale ewes grazing an oestrogenic (Yarloop clover) pasture has been studied during the oestrous cycle, pregnancy and parturition, and the results compared with those from a study of similar ewes grazing a neighbouring grass pasture. Plasma progesterone, oestrogen and corticoids were measured using competitive protein binding assay procedures.

During the oestrous cycle clearly anomalous patterns in hormone content were evident in ewes grazing Yarloop, and this related to their significantly poorer ($P < 0.001$) fertility.

The first mating, when ewes were 1½ years of age, was particularly affected. Successful conception took place in only 27% of ewes mated on Yarloop, compared with 95% on grass. Evidence of disturbance in the normal patterns of both plasma oestrogen and progesterone was found in infertile ewes, including a shortened period of luteal function.

Disturbance of endocrine function caused by Yarloop clover ingestion was also found in pregnant ewes, with the mean plasma progesterone concentrations during the latter half of pregnancy reduced ($P < 0.05$) and the plasma oestrogen and corticoid levels tending to be higher in these animals.

In detailed hormone studies in the periparturient period, both groups showed a similar fall in plasma progesterone and rise in plasma oestrogen prior to parturition. Where excessive time was taken for parturition (more than 30 min) this was reflected in higher plasma corticoid levels ($P < 0.05$) within 8 h of birth.

Introduction

Reproductive difficulties in sheep grazing subterranean clover pastures were first described by Bennetts (1944) and Bennetts *et al.* (1946). Characteristic signs were ewe infertility, dystocia and uterine prolapse resulting in high ewe and lamb mortality. This syndrome of aberrant reproductive functions on clover pastures is now commonly referred to as 'clover disease' and its occurrence has been widely recognized in association with many different clover species throughout the world (Moule 1961; Moule *et al.* 1963; Bickoff 1968).

The results of extensive studies have revealed that the aetiology relates to oestrogenic isoflavones present in the plants (Batterham *et al.* 1965). However, the exact way in which these substances exert their adverse effects on reproductive function is as yet unresolved. In the present study, an investigation has been carried out to examine the possibility that the clover acts by causing disturbances in the endocrinology of reproduction.

Preliminary investigations (Obor *et al.* 1971a) suggested that the reproductive endocrine function for grazing flock sheep could be monitored by relatively simple radio-ligand assays for plasma hormone levels. These indices throughout the reproductive cycle was therefore

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sheep grazing an oestrogenic pasture (*Trifolium subterranean* cv. Yarloop) and a non-oestrogenic grass pasture.

Preliminary reports on this study have been published previously (Obst and Seamark 1970, 1972; Obst *et al.* 1971b; Obst 1972).

Materials and Methods

Merino ewes and wethers (Koonoon strain) were obtained from flocks of the Agricultural Research Station at Wanbi, S.A., and were born and reared on non-clover pastures. Corriedale ewes and wethers were obtained from flocks at the Kangaroo Island Research Centre (KIRC), maintained on mixed 50/50 pastures of perennial rye grass and clover (cv. Mt. Barker, Yarloop and Woogenellup). The Merino rams (Bungaree strain) and Corriedale rams were also from KIRC flocks.

The Yarloop clover pasture of 17.2 ha (area A) was cleared and sown with Yarloop in 1959. Visual estimations of species composition of the pasture during the period of study (1969–1971) indicated that more than 70% of available feedstuff was Yarloop, with annual grasses and weeds constituting the remainder of the pasture. The grass pasture of 35 ha (area B) had a history similar to the Yarloop pasture until 1967 when a renovation program successfully established a pasture dominant in perennial rye grass. Contamination with Yarloop regrowth after renovation was less than 5% of the total pasture in 1969, but this increased to about 15 and 20% in 1970 and 1971 respectively.

Procedures

The investigation was initiated in February 1969 when ewes were 1½ years of age. Two groups of animals were formed each of 50 Merino and 50 Corriedale ewes and 40 wethers. One group was then maintained on the Yarloop clover pasture and the other on grass pasture throughout the period of the experiment. Ewes and wethers were weighed monthly. To maintain approximately equivalent liveweights in the two groups during and between years the stocking rates were adjusted with additional KIRC wethers. From January to April 1970, when dry Yarloop residues were limited, the Yarloop group was supplemented with Yarloop clover hay.

Study I. Two fertile rams were introduced into the flock grazing Yarloop in February 1969 and the first six Merino and six Corriedale ewes mated were used for blood sampling and hormone measurement. During this period the pasture consisted mainly of green germinating Yarloop clover.

The ewes on the grass pasture were first joined with a vasectomized ram and the first six ewes mated were kept separately with the teaser ram for blood sampling and hormone determination. The remaining ewes in each group were mated to four fertile rams. Venous blood samples of 10 ml were collected into heparinized containers every second day commencing on the day of mating (day 0) or the first day after mating (day 1). Blood sampling continued for a period of 42 days.

Study II. Four vasectomized rams fitted with Sire-line harnesses and crayons were introduced on 2 October 1969 to the ewes which had grazed the Yarloop pasture since its germination in February 1969. The identity of the animals mated was recorded daily. Of the ewes which did not produce a lamb in study I, the first 20 to mate (13 Merinos and 7 Corriedales) were confined to a smaller area (C) of green Yarloop clover within the same area (A) to aid the collection of blood samples.

Blood samples of 20 ml for hormone determination were collected daily at 0900 h from the 20 ewes commencing on 20 October until 21 November 1969. Plasma progesterone determinations were made on all samples collected, but plasma oestrogen concentrations were determined only on samples selected from ewes which exhibited specific patterns in the function of their corpora lutea. Corpus luteum function was classified on the basis of peripheral plasma progesterone concentrations as follows.

- (1) Shortened—progesterone levels declined from day 12 to values less than 1.0 ng/ml on day 14 and remained below 1.0 ng/ml until oestrus.
- (2) Normal—progesterone levels were maintained above 1.0 ng/ml from day 12 to day 14 inclusive and then declined to below 1.0 ng/ml by day 19.
- (3) Prolonged—progesterone levels were maintained above 1.0 ng/ml from day 12 to beyond day 19.

Study III. Merino and Corriedale ewes on each pasture were mated separately for 6 weeks from mid-February to the end of March 1970 with two rams of the same breed per group. Ewes were

then 2½ years old. Twenty ewes (5 Merino and 5 Corriedale from each pasture group) which did not return to service during the 6-week mating period were selected for hormone measurement.

To facilitate blood collection, the selected ewes were confined to smaller areas on the same pastures and jugular vein blood samples of 20 ml were taken daily at 0800 h from day 40 of gestation to 2 days post-partum. Additional blood samples were taken at 1600 and 2400 h from day 137 of gestation onwards to follow periparturient changes in more detail.

Lambing Observations

Lambing ewes (10) on the Yarloop pasture whose blood was being sampled were continuously observed with the aid of spotlights and binoculars to allow the recording of time of birth, duration of parturition and signs of dystocia. Duration of parturition (in minutes) was defined as the time from first appearance of the lamb's front feet at the vulva to complete delivery of the lamb. The 10 lambing ewes on grass pasture were observed every 30 min, and time of birth, incidence of dystocia and approximate length of parturition were recorded.

General lambing observations on all other ewes were performed daily at approximately 0800 h when each lamb born was identified with its mother, ear-tagged and weighed. The incidence of dystocia was recorded and a post-mortem examination made on ewes which died near term, or were unable to lamb even with manual assistance, to ascertain the number of dead lambs *in utero*.

Hormone Assay Procedures

(i) **Progesterone assay.** The competitive protein binding (CPB) procedure used was essentially the same as that described by Thorburn *et al.* (1969), employing light petroleum as the plasma extractant and dog or hen plasma as a source of corticosteroid binding globulin with [³H]cortisol as the competing tracer. Free and bound steroids were separated on small Sephadex G25 (fine) columns (Bassett and Hinks 1969).

Progesterone standards containing 0, 0.2, 0.4, 1.0, 2.0 and 4.0 ng added to 0.2 or 0.5 ml of oophorectomized ewe plasma were run with each assay and results are expressed as progesterone equivalents.

(ii) **Oestrogen assay.** An index of the level of plasma oestrogen was determined using a CPB method based on that described by Korenman (1968, 1969). Uteri of 6-day-pregnant rabbits were used as a source of binding protein and the cytosol was prepared as described by Korenman (1968).

For assay, plasma (2–4 ml) was extracted with 2 volumes of ethyl acetate and the residue, after removal of the solvent, was reacted with the uterine cytosol. The competing tracer was [³H]oestra-3,17β-diol. Competition for binding sites was allowed to occur for at least 6 h, usually overnight, at 4°C and the free and bound fractions separated by small Sephadex columns using 4 ml of 0.01M tris buffer (pH 8) as eluent.

Oestrogen standards containing 0, 20, 50, 100, 200 and 400 pg of oestra-3,17β-diol (E₂-17β) were run with each assay. As a check on procedure, samples of oophorectomized ewe plasma and water (2 or 4 ml) were also included as blanks.

(iii) **Cortisol assay.** Plasma cortisol concentrations were determined by the method of Bassett and Hinks (1969). Results expressed as cortisol equivalents (ng/ml) were corrected to allow for interference by progesterone present in the ethanol extract.

Assessment of Oestrogenicity of the Pastures

(i) **Isoflavone content.** Samples from Yarloop pasture taken in July–August 1970 were kindly analysed for the isoflavones, genistein, formononetin and biochanin A by Dr R. C. Rossiter of the Division of Land Resources Management, CSIRO, Wembley, Perth, W. A.

(ii) **Bioassay.** At various times during 1969 and 1970, the size of the bulbo-urethral gland of wethers grazing the two pastures was assessed by digital palpation through the rectum, and the size and state of the glands were scored as follows to assess any accumulative effects of the oestrogenic activity of the pasture:

Score	Diameter (mm)	Description
1	5	Normal, soft palpable
2	5–15	Slightly enlarged, hard
3	15–25	Enlarged, hard
4	25–35	Very enlarged, hard
5	35	Grossly enlarged with presence of a calcareous mass

(iii) *Analysis of results.* Comparisons of reproductive performance between pastures were made using the χ^2 test (Snedecor and Cochran 1968) and the significance of differences in the mean plasma hormone concentrations assessed using Student's *t*-test (Snedecor and Cochran 1968). Attempts were also made to relate individual ewe hormone levels with lambing observations.

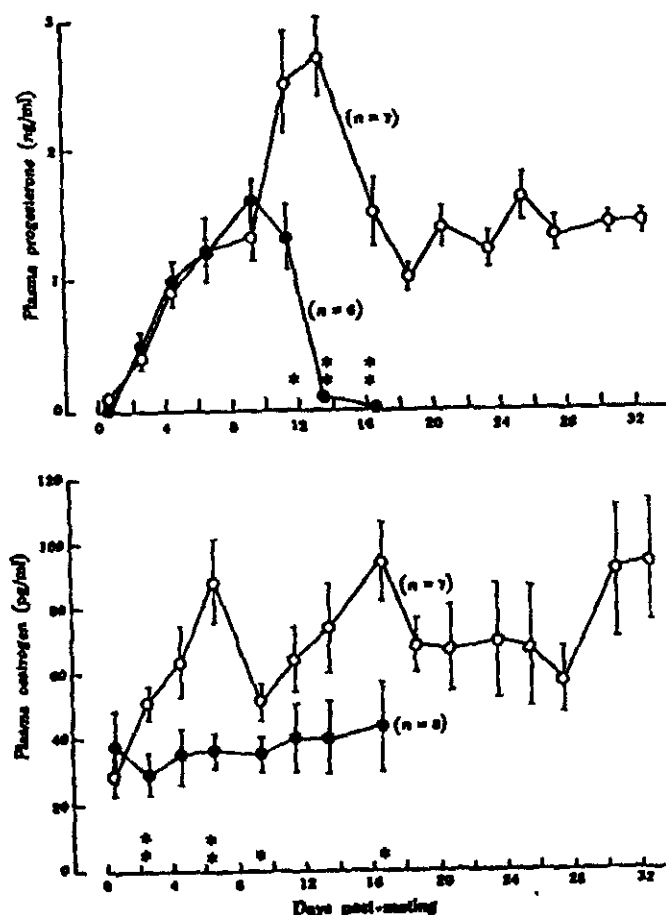


Fig. 1. (a) Plasma progesterone and (b) oestrogen following mating in ewes grazing green Yarloop clover (study 1). Vertical bars represent \pm S.E.M. for fertile (O) and infertile (●) ewes. The numbers of ewes in each group are shown in brackets. Statistically significant differences are denoted thus: * $P < 0.05$, ** $P < 0.01$.

Results

Pasture Oestrogenicity

The mean content of the isoflavones formononetin, genistein and biochanin A in the Yarloop clover during July and August 1970 was 1.2, 2.5, and 0.54% respectively, of the dry leaf weight.

The bulbo-urethral glands of the wethers were palpated at intervals of approximately 2 months from February 1969 to July 1970, and during this time 34 out of 40 wethers grazing Yarloop pasture and 13 out of 40 wethers grazing grass showed evidence of gland enlargement. On Yarloop, 24 of the 34 were given a score of 3 or more for at least two of the six palpations, whereas only 5 of the 13 on grass pastures received a score of 3 or more.

Fertility and Reproductive Performance

The overall fertility of ewes grazing Yarloop pastures (Table 1) was severely reduced compared with ewes on grass ($P < 0.001$). The largest difference in fertility between groups of animals on the different pastures occurred at the first mating in 1969. Following this mating only 14% of the Corriedales and 40% of the Merino ewes produced lambs while grazing green Yarloop pasture compared with over 90% of Corriedales and Merinos grazing the grass pasture. The percentages of Merino and Corriedale ewes which lambed 0, 1, 2 or 3 times during the 3 years whilst grazing on Yarloop were 22.2, 37.4, 28.3 and 12.1%, respectively, whereas the comparable values for ewes on the grass pastures were 3.1, 9.5, 34.7, and 52.6%.

Lamb survival was lower ($P < 0.01$) on the Yarloop pasture (55%) than on the grass pasture (71%). The differences between pastures in numbers of lambs born and in lamb and ewe mortality were most marked in the Corriedales. Uterine prolapse following parturition was observed only on the Yarloop pasture.

Endocrine Investigations: Assay Assessment

For the progesterone assay, estimates of the coefficient of variation at different points on the standard curve ranged from 2.6 to 6.5%. The mean recovery \pm S.E.M. of added amounts of progesterone (0.2–8.0 ng) was $90 \pm 2\%$ ($n = 12$) with no plasma and $86 \pm 2.5\%$ ($n = 12$) with oophorectomized ewe plasma.

For the oestrogen assay the coefficient of variation of determinations of various amounts of $E_2-17\beta$ over the useful range (20–400 pg) of the standard curve varied from 2.9 to 6.1%. Recovery estimates of non-labelled $E_2-17\beta$ (0–200 pg) added to ewe plasma were 93–96%.

Of the isoflavones tested, genistein in 10-ng amounts assayed as equivalent to 35 pg of $E_2-17\beta$. However, 200 ng or more of diadzein, biochanin A or formononetin was required to be present in samples before significant interference was recorded from these compounds. No equol was available during these investigations but the results of Shutt and Cox (1972) indicate that equol may react similarly to genistein under the conditions of assay employed.

Study 1. All of the ewes on Yarloop selected for hormone assessment remated at least once during the 42-day period of study (17 matings total) and five lambs resulted, two to the second mating. The mean \pm S.E.M. plasma progesterone and oestrogen concentrations following these infertile and fertile matings of animals on Yarloop are presented in Fig. 1. Plasma progesterone concentrations were similar up to days 9–10 in both groups. At days 11–12, however, progesterone concentrations of the infertile group began to fall to reach oestrous levels at days 13–14, indicating a shortened period of corpus luteum (CL) function. Infertile matings were also characterized by lower plasma oestrogen levels than were found in fertile matings (Fig. 1).

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Study II. Following 9 months of grazing on green Yarloop pastures, nearly all ewes which were infertile at their first mating and showed evidence of shortened periods of CL function had normal luteal phases. Of the 32 luteal phases studied, 24 were of normal length and were followed by oestrus, three had normal levels of plasma progesterone but there was no subsequent oestrus in the period of study, two luteal phases were shortened and the remaining three were prolonged. Patterns of plasma progesterone and oestrogen that are representative of each type of CL function are presented in Figs 2 and 3. Plasma progesterone concentrations (Fig. 4) on days 6–14 post-oestrus were significantly ($P < 0.05$) higher in ewes grazing Yarloop in October 1969 (study II), than in those grazing Yarloop or grass in February–March 1969 (study I).

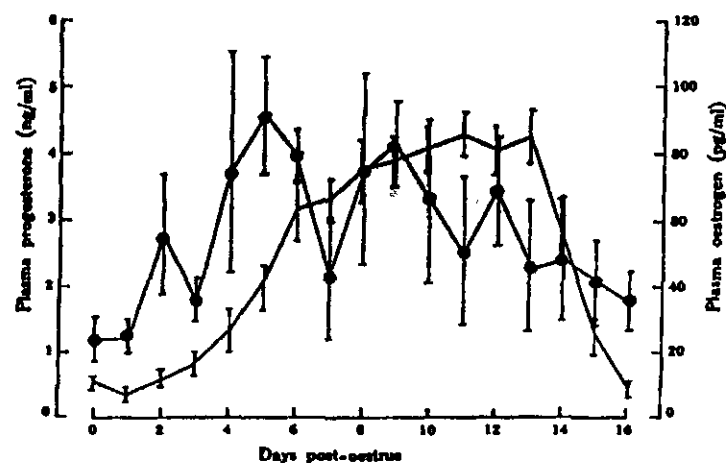


Fig. 2. Plasma progesterone (—) and oestrogen (●) in five ewes exhibiting normal CL function while grazing Yarloop clover pastures (study II). Vertical bars represent \pm S.E.M.

The higher percentage (84%) of normal luteal phases among the selected ewes in study I compared with study II is reflected in the improved fertility of the experimental flock during the mating in February and March 1970 (Table 1).

Study III. The mean plasma progesterone concentration of pregnant ewes grazing Yarloop was significantly ($P < 0.05$) lower than in those grazing grass pasture from 90 days gestation to term (Fig. 5a). Plasma corticoid concentrations for ewes on both pastures increased significantly ($P < 0.001$) from 40 days gestation to 100–120 days gestation (Fig. 5a). Thereafter, concentrations declined towards term but the mean values for ewes on Yarloop remained higher ($P < 0.05$) from 120 to 140 days gestation than the mean values for those on grass pasture.

Significantly ($P < 0.05$) higher concentrations of plasma oestrogen were observed from 110 to 120 days and at 140 and 145 days gestation (Fig. 5a) in the ewes grazing Yarloop pasture compared with the ewes grazing grass pasture throughout pregnancy. No differences, however, were apparent between the two groups in the characteristic rise in plasma oestrogen occurring within 24 h of birth (Fig. 5b).

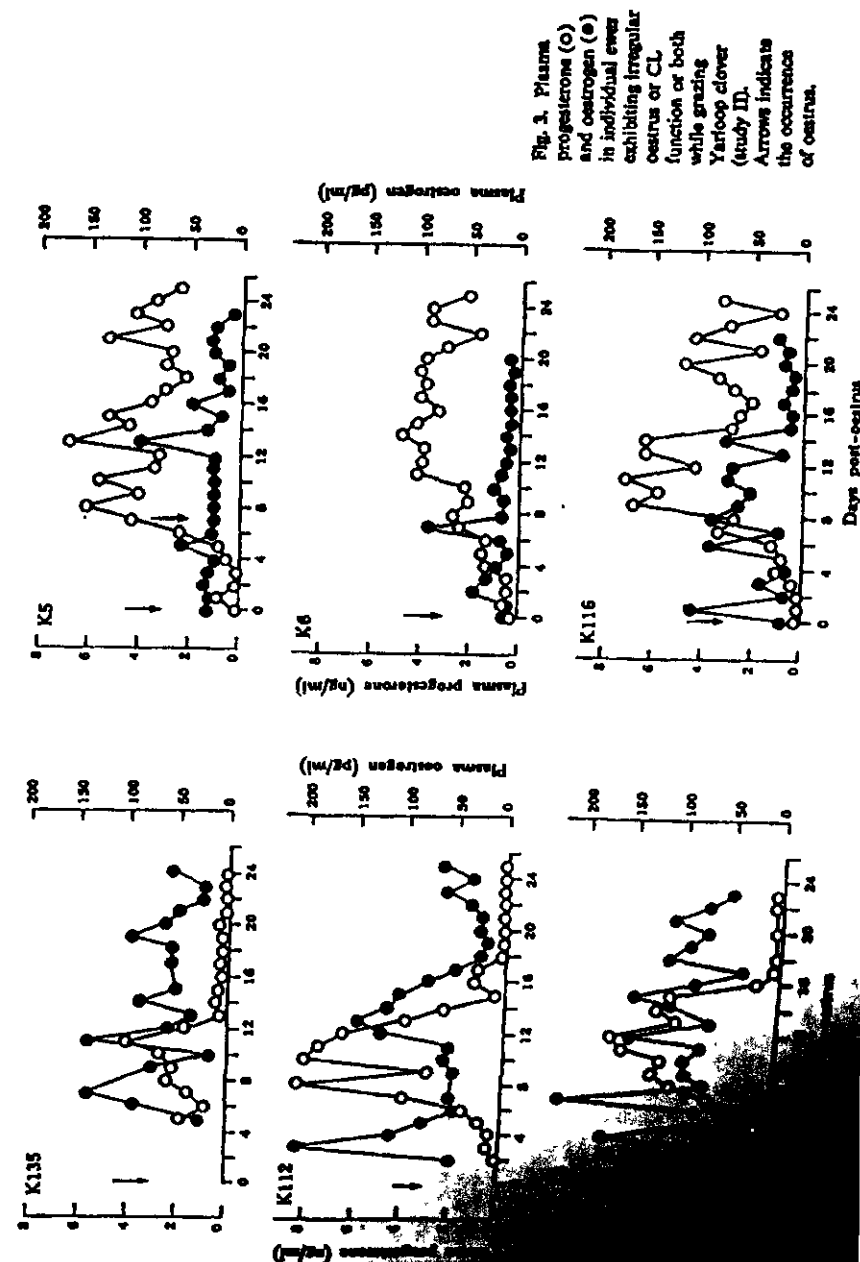


Fig. 3. Plasma progesterone (O) and oestrogen (●) in individual ewes exhibiting irregular oestrus or CL function or both while grazing Yarloop clover (study II). Arrows indicate the occurrence of oestrus.

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The mean (\pm S.E.M.) gestation lengths and birth weights of lambs were similar between the groups selected from Yarloop (151.4 ± 1.70 days, 4.15 ± 0.25 kg) and grass (150 ± 1.06 days, 4.05 ± 0.15 kg) pastures. However, four ewes on the Yarloop pasture experienced lambing difficulties compared with only one on grass. In these ewes the mean plasma corticoid concentration within 8 h of birth was higher ($P < 0.05$) than in the other ewes where parturition took less than 30 min. No relationship was apparent, however, between plasma progesterone or oestrogen concentration and duration of parturition.

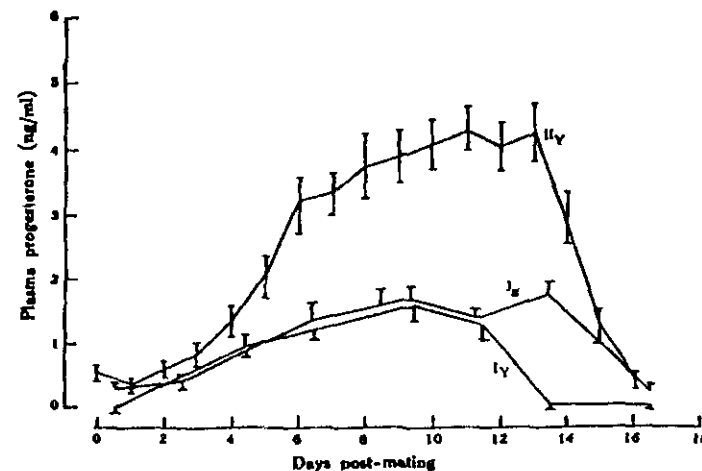


Fig. 4. Plasma progesterone in ewes grazing Yarloop clover in study I (I_Y) and study II (II_Y) compared with ewes grazing grass pasture in study I (I_G). Vertical bars represent \pm S.E.M. $n = 5$. Progesterone concentrations in study II were significantly higher ($P < 0.05$) from day 6 to day 14 post-mating.

Discussion

The study reveals that the poor reproductive performance of ewes grazing Yarloop is associated with marked disturbances in reproductive endocrine function. That hormonal disturbances may occur in ewes grazing oestrogenic pasture was suggested by the anomalies in both the incidence of oestrous and inter-oestrous intervals observed by previous workers (Underwood and Shier 1952; Turnbull *et al.* 1966; Fels and Neil 1968).

The cause of the particularly low fertility of ewes grazing Yarloop in 1969 remains unresolved. It appears to have been a temporary effect, as the majority of the ewes had improved fertility in study II despite continued grazing of the Yarloop in the intervening 9-month period. The improvement in fertility was accompanied by a higher incidence of normal periods of luteal function and, interestingly, higher plasma progesterone levels than found in study I. These changes probably relate to age of the ewe and seasonal factors, but it is possible, as suggested by Morely *et al.* (1969), that some ewes adapted to adverse effects of ingesting the large amounts of

oestrogenic isoflavones present in the pasture. Certainly, the pastures remained oestrogenic as indicated by the effects on the bulbo-urethral gland of the wethers.

Table 1. Reproductive performance of Merino and Corriedale ewes grazing Yarloop or grass pasture during the years 1969, 1970 and 1971

	Merinos			Total or mean	Corriedales			Total or mean	Merinos and Corriedales 1969-1971
	1969	1970	1971		1969	1970	1971		
Yarloop pasture									
Ewes mated	50	43	44	137	50	44	45	139	276
% Ewes fertile	40	67	59	55	14	50	44	38	46
Lambs born									
Singles	20	24	21	65	7	17	20	44	109
Multiples	0	10	10	20	0	10	8	18	38
Total (% of total ewes)	40	79	70	62	14	61	62	45	53
Lamb deaths									
Singles	11	7	7	25	3	11	9	23	48
Multiples	0	5	3	8	0	7	3	10	18
Total (% of lambs born)	55	35	32	39	43	67	43	53	45
Ewe deaths	0	3	5 ^a	8	0	4	6 ^a	10	18
Lamb deaths in utero	0	4	4	8	0	6	5	11	19
Lambs marked (% of ewes mated)	18	51	48	38	8	20	36	21	29
Grass pasture									
Ewes mated	52	46	47	145	48	38	43	129	274
% Ewes fertile	90	74	74	80	100	87	74	78	84
Lambs born									
Singles	44	24	21	89	47	25	24	96	185
Multiples	6	20	28	54	2	16	17	35	89
Total (% of total ewes)	96	96	104	99	102	108	95	102	100
Lamb deaths									
Single	12	4	2	18	11	5	4	20	38
Multiples	4	13	17	34	2	2	3	7	41
Total (% of lambs born)	32	39	39	36	27	17	17	21	29
Ewe deaths	0	2	2	4	0	0	0	0	4
Lamb deaths in utero	0	4	3	7	0	0	0	0	7
Lambs marked (% of ewes mated)	65	59	64	63	75	89	79	81	71

^a Includes two ewes with prolapsed uterus.

^a Includes one ewe with prolapsed uterus.

The basis for the endocrine disturbances caused by Yarloop in pregnancy is also not understood. Whether the different responses in ewes grazing Yarloop reflect changes in the rates of hormone clearance or both is unknown.

Recent studies on the origin of plasma progesterone in pregnancy to 110 days gestation the CL is a major contributor.

the ovarian contribution is small (Edgar and Ronaldson 1958; Mattner and Thorburn 1971). Albeit, bilateral oophorectomy at 110-124 days gestation resulted in a 50% reduction of peripheral plasma progesterone concentrations in late pregnancy (Fylling 1970), a difference similar to that seen between the ewes on Yarloop and those on grass pasture.

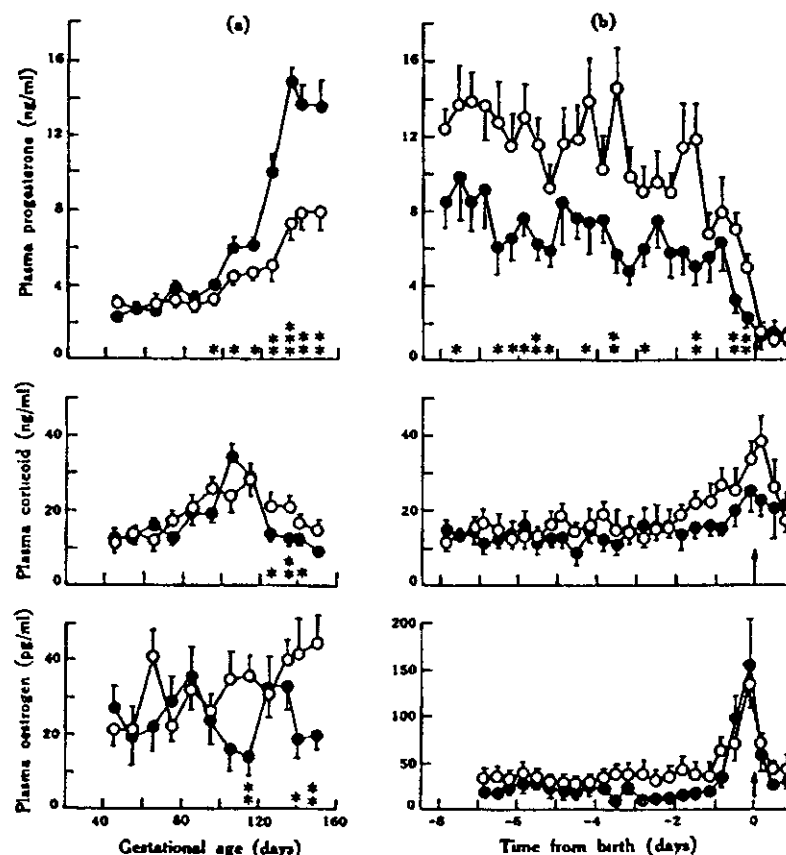


Fig. 5. Plasma progesterone, corticoid and oestrogen (a) during pregnancy and (b) about parturition in nine ewes grazing grass (●) and nine ewes grazing Yarloop clover (○). Vertical bars represent \pm S.E.M. Statistically significant differences are denoted thus: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The pattern of plasma corticoid found in ewes on grass pastures is very similar to that reported by Basson *et al.* (1969). The reduction in plasma corticoid concentration occurring after 120 days gestation has been attributed to an expansion in plasma volume or, alternatively, to increase in the metabolic clearance rate of cortisol (Paterson and Harrison 1968). Thus the higher than normal concentration of maternal

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corticoid in the Yarloop group may reflect disturbances in the factors regulating these functions.

Undernourishment combined with cold stress can also cause higher than normal maternal plasma corticoid concentrations in the latter part of pregnancy (Lindner 1959; Saba 1965). However, because both the ewes on Yarloop and those on grass produced lambs with similar birth weights, and because both groups were exposed to the same climate and handling regimen, it is unlikely that these factors would explain the higher plasma corticoid levels in ewes grazing Yarloop pasture.

The apparent association between duration of parturition and plasma corticoid concentrations in the 8 h prior to birth may simply relate to the fact that the ewes with dystocia were stressed. It is known, however, that a functioning foetal pituitary-adrenal axis is necessary for successful parturition (Liggins *et al.* 1967; Drost and Holm 1968), and it is feasible that on account of high maternal corticoid concentrations some corticoid could cross the placenta (Dixon *et al.* 1970) and affect the development of this axis resulting in dystocia and the poor post-partum survival.

The pattern of plasma oestrogen concentration during pregnancy was similar to that reported by Challis (1971). No evidence was obtained in the present study to suggest that the characteristic pre-partum oestrogen rise was related to the length of parturition. Oestrogen peaks were observed in ewes which showed signs of dystocia as well as in ewes which were able to deliver their lambs unaided, both on Yarloop and on grass pasture. However, it is possible that in ewes exhibiting dystocia, the normal response by tissues was mollified by competition or previous effects of the high levels of circulating isoflavones in the plasma. Alternatively, the lambing difficulties may have been due to disturbances in the oestrogen/progesterone ratio seen in the ewes grazing Yarloop. Such an imbalance may have prevented not only normal myometrial activity, but also the normal preparation or relaxation of the uterus, cervix and vulva (Hindson *et al.* 1967, Csapo 1969).

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(CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL CHEMISTRY, PURDUE UNIVERSITY AGRICULTURAL EXPERIMENT STATION, IN COOPERATION WITH U. S. REGIONAL SOYBEAN INDUSTRIAL PRODUCTS LABORATORY)¹

Genistin (an Isoflavone Glucoside) and its Aglucone, Genistein, from Soybeans

BY E. D. WALTER

Genistein, which is now known to occur in soybeans as the aglucone of genistin, was isolated from Dyer's Broom (*Genista tinctoria*) in 1899 by Perkin and Newbury.² The isoflavone nucleus was established for genistein by Baker and Robinson³ in 1926, when they found it to be identical with prunetol, and in 1928 they synthesized genistein. The constitution of genistein was thereby established as 5,7,4'-trihydroxyisoflavone.

In 1931 Walz⁴ isolated genistin from a 90% methanol extract of soybean meal. He found that hydrolysis of genistin with hydrochloric acid gave one mole of genistein and one mole of glucose. The glucose was identified by optical rotation and by a Bertrand determination of the reducing sugar after its separation from the aglucone. From degradative experiments he found that the glucose was bound to genistein at position seven and that the constitution of genistin was 5,7,4'-trihydroxyisoflavone-7-glucoside.

In 1939 Okano and Beppu⁵ reported four isoflavones from soybeans. One of these was named isogenistein, but apparently none of them had the same physical properties shown by genistin or genistein.

During the course of a study of the carbohydrates of soybeans, an attempt was made to isolate certain sugars from a methanol extract of oil-free soybean meal. After the removal of some of the phosphatides from a concentrated methanol solution by the addition of acetone, the solution was concentrated and upon the addition of water, white, granular aggregates crystallized. These crystals, when dissolved in hot ethanol, gave a reddish-violet coloration with an ethanol solution of ferric chloride. The properties of the purified material agreed with those reported by Walz for genistin.

This paper confirms some of Walz's work and

presents further evidence that the sugar hydrolyzed from genistin is *D*-glucose. Additional physical and chemical properties of genistin and its aglucone, genistein, are also presented. The melting points, optical rotation and carbon and hydrogen analyses of the compounds presented are in agreement with the data presented by Walz for the corresponding substances.

A small amount of some other crystalline material was obtained from the mother liquors, which gave qualitative evidence for another flavone. Work is in progress in this Laboratory on the isolation and identification of this material.

Experimental

Isolation and Properties of Genistin.—Commercial soybean flakes, which had been extracted with hexane, were placed in a cloth bag in 10-kg. quantities and put into the extraction chamber of a Lloyds extractor. Sufficient methanol (about 24 liters) was added to cover the bag of flakes. The valves in the apparatus were so adjusted that the extractor would operate as a reflux. Steam was passed through the coil surrounding the extraction chamber and the mixture was refluxed for twenty-four hours. The extract was drained off, a fresh supply of methanol was added to the flakes and the mixture was again refluxed for twenty-four hours. The extracts were combined and concentrated to a volume of about 1.5 liters. Acetone was added to the concentrate until precipitation ceased. The acetone precipitated some of the phosphatides, which carried with them carbohydrates, saponin and other impurities. After the supernatant liquid became clear, it was decanted and concentrated on the steam-bath to a thin syrup. About two volumes of water was added to this syrup and on standing for a day at room temperature the genistin crystallized in yellowish-white granular aggregates.

The crude genistin was removed from the mother liquor by centrifuging. It was then dissolved in approximately 90% hot ethanol, treated with Nuchar and filtered. Crystallization was effected by concentrating the filtrate and cooling it to room temperature. At this point the crystals were contaminated with saponin. This was detected readily by adding a few crystals to concentrated sulfuric acid. When saponin is present the clear yellow color due to genistin soon changes to red and later to purple. With pure genistin, however, the yellow color with sulfuric acid is permanent. After several recrystallizations from 90% ethanol the genistin was obtained in pale-yellow thin rectangular plates of m.p. 216° (Walz recorded 25--236°). Further treatment of the mother liquors with Nuchar, followed by concentration, yielded additional genistin. The total yield was 0.10% of the dry hexane-extracted soybean flakes.

(1) The U. S. Regional Soybean Industrial Products Laboratory is a cooperative organization participated in by the Bureau of Agricultural Chemistry and Engineering and Plant Industry of the United States Department of Agriculture and the Agricultural Experiment Stations of the North Central States of Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota and Wisconsin.

(2) A. O. Perkin and F. G. Newbury, *J. Chem. Soc.*, 75, 519 (1919).

(3) W. Baker and R. Robinson, *ibid.*, 2715 (1926); *ibid.*, 3115 (1928).

(4) H. Walz, *Ann.*, 489, 113 (1931).

(5) K. Okano and I. Beppu, *Bull. Agr. Chem. Soc. Japan*, 13, 110 (1939).

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4. Carter, M.W.; Matrone, G.; Smart, W.W.G., Jr. 1955. Effect of genistin on reproduction of the mouse. *J. of Nutrition* 55(4):639-45 April. [15 ref]

Summary: In a control diet containing 0.2% added genistin, the authors found little effect on reproduction in mice, although soybeans were found to elicit an estrogenic response in mice. An estrogen is a substance capable of stimulating the growth of female reproductive organs and the development of female secondary characteristics in animals. "Reproductive disturbances have been reported to occur in sheep and in rabbits fed the soybean plant as a large part of the diet by Hunt (1935), Kendall et al. (1950), and Matrone (1953). That these reproductive disturbances might have been caused in part by the presence of an estrogenic-like substance can be inferred from data in the literature. ... Both commercial soybean oil meal and isolated genistin (an isoflavone) significantly lowered the age at which the vaginal opening was opened."

"The principal effect on reproduction of 0.2% genistin in the diet was a decrease in the number of litters born, whereas litter size was not affected. The amount of commercial soybean oil meal (80% of dry wt.) fed to mice could produce a dietary level of 0.12% genistin; the number of litters born was not statistically significant but the number of litters obtained was less than that from the group of females on the control diet." Address: Animal Nutrition Section, Dep. of Animal Industry, North Carolina Agric. Exp. Station, Raleigh.

5. East, June. 1955. The effect of genistein on the fertility of mice. *J. of Endocrinology* 13(1):94-100. Oct. [15 ref]

Summary: Synthetic genistein (5,7:4'-trihydroxy isoflavone) proved to be oestrogenic (oestrogenic, i.e. produced vaginal cornification) when included in the normal diet of immature, spayed, and intact female mice in amounts calculate to give daily intakes of 2, 10, and 15 mg respectively. Consumption of genistein also precipitated vaginal opening in immature mice.

"The fertility of adult male mice fed 15 mg genistein daily for 22-25 days was more markedly affected than that of adult females similarly treated for 31-55 days. Of ten males, five were rendered sterile and the fertility of three others was impaired. Two of ten females did not mate and abnormal numbers of still-born young were produced by the remaining animals. Four males and one female did recover fertility when transferred to normal diet." Address: Medical Res. for Medical Research, Hill Hill, London, N.W. 7 (England).

6. Kitts, D.D.; Krishnamurti, C.R.; Kitts, W.D. 1980. Uterine weight changes and tritium labeled uridine uptake in rats treated with phytoestrogens. *Canadian J. of Animal Science* 60(2):531-34. June. [9 ref]

Summary: Note: This is a fairly early report showing that soybeans have estrogenic activity. Biological tests in rodents and ruminants suggest that plant estrogens (phytoestrogens) resemble natural occurring internal (endogenous) estrogens in their mode of action. Estradiol-17 β , diethylstilbestrol (DES) and certain phytoestrogens extracted from soybean meal and alfalfa hay were administered intraperitoneally [injected into the abdomen] of immature female rats. The major estrogenic compounds in soybean meal were genistein and daidzein, while those in alfalfa hay were genistein, formononetin and coumestrol. Coumestrol and genistein exhibited uterine responses (changes in uterine weight and moisture content) similar to but much weaker than those of estradiol 17 β and DES; the dose required to produce the effects was approximately 100 to 1000 times higher than in the latter two compounds. Address: Dep. of Animal Science, Univ. of British Columbia, Vancouver, BC V6T 2A2, Canada.

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EFFECT OF GENISTIN ON REPRODUCTION OF THE MOUSE^{1,2}

M. W. CARTER, GERRARD MATRONE AND W. W. G. SMART, JR.
*Animal Nutrition Section of Department of Animal Industry, North Carolina
Agricultural Experiment Station, Raleigh*

(Received for publication November 1, 1954)

Reproductive disturbances have been reported to occur in sheep and in rabbits fed the soybean plant as a large part of the diet (Hunt, '35; Kendall et al., '30; Matrone, '52). That these reproductive disturbances might have been caused in part by the presence of an estrogenic-like substance can be inferred from data in the literature. The evidence is as follows: first, these reproductive disturbances are similar to those reported to occur in sheep grazing on the estrogenically active subterranean clover pastures in Australia (Bennetts and Underwood, '49; Underwood and Shier, '51); and second, the compound, genistein ($\frac{1}{2}$ 5, 7-trihydroxyisoflavone), responsible for the estrogenic activity of subterranean clover (Bradbury and White, '51) also is present in soybean oil meal as the glucoside of genistein, genistin (Walz, '31; Walter, '41).

Injections of genistin have shown it to be estrogenically active (Cheng et al., '53). By means of the mouse uterine weight assay, Carter and associates ('53) have shown that commercial soybean oil meal also is estrogenically active but that commercial soybean oil meal residue from which genistin has been extracted is inactive. The present report, a part of

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an investigation of factors in the soybean plant that affect reproduction, deals with the dietary effect of soybean-genistin on the reproduction of the mouse.

EXPERIMENTAL

The dietary variables studied were soybean oil meal and the genistin extracted from commercial soybean oil meal. The diets formulated for this purpose are shown in table 1.

TABLE 1
Ingredients of test diets fed mice during growth and reproduction

CONSTITUENTS	AMOUNT PER KILOGRAM OF DIET		
	Diet I	Diet II	Diet III
	gm	gm	gm
Commercial soybean oil meal			500.0
Extracted soybean oil meal ¹	500.0	500.0	..
Glucose ²	100.0	95.0	100.0
Wesson oil ³	60.0	60.0	60.0
Mineral mix ⁴	40.0	40.0	40.0
Genistin ⁵	..	2.0	..

¹ Commercial soybean oil meal extracted with hot methanol.

² "Cereose," Corn Products Refining Company, New York, N. Y.

³ Water soluble vitamins and methionine added replaced an equal weight of glucose.

⁴ Wesson Oil and Snowdrift Sales Company, New Orleans, La.

⁵ Oleum percomorphum and alpha-tocopherol were dissolved in Wesson oil.

⁶ Wesson's modification of the Osborne and Mendel salt mixture.

⁷ Genistin isolated from commercial soybean oil meal.

Diet I was considered as the control since it was made up chiefly of soybean oil meal from which genistin had been extracted by Walter's method (Walter, '41). Meal thus extracted has been shown to be estrogenically inactive (Carter et al., '53). Diet II consisted of the same constituents as diet I except that genistin had been added to represent 0.2% of the diet. Diet III was made up chiefly of commercial soybean oil meal containing approximately 0.1% genistin (Walter, '41). Vitamins were added to provide, per kilogram of diet: 0.02 mg B₁₂, 0.2 mg biotin, 1 mg folic acid, 5 mg thiamine.

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5 mg pyridoxine, 6 mg alpha-tocopherol acetate, 10 mg riboflavin, 10 mg nicotinic acid, 10 mg 2-methyl-1,4-naphthoquinone, 30 mg Ca-pantothenate, 1 gm para-aminobenzoic acid, 1 gm inositol, 1.5 gm choline chloride, and 24 drops of oleum percomorphum. The vitamins added to the diets were essentially the same as those added to synthetic diets for normal reproduction and lactation in the mouse (Fenton and Cowgill, '47), except for the addition of vitamin B₁₂ and the substitution of oleum percomorphum for cod liver oil concentrate. In addition 6 gm of DL-methionine per kilogram of diet was added as a safety measure since soybeans are low in this amino acid. The salt mixture used was that devised by Wesson ('32).

The experiment, involving a total of 108 female Swiss albino mice, was a randomized block design in which the female mice were assigned to blocks on the basis of uniformity in weight. A block consisted of three cages to which the three experimental diets were assigned at random. Each cage housed three female mice.

In an effort to increase the probability of revealing any possible effect of dietary genistin and soybean oil meal on reproduction, female mice were placed on the experimental diets as soon as they were weaned, at approximately three weeks of age.¹ Observations on weight gain, feed intake, and time of vaginal opening were made over a period of 4 weeks, preliminary to the reproduction study. Since the time of the vaginal opening is indicative of estrogenic activity (Emmons, '50), this criterion was used as an index of the estrogenic activity of the diets.

The males used for breeding were raised on Purina Laboratory Chow. Following the growth study with the females, one male was assigned at random to each cage of females and left there for one 21-day period. The criteria for repro-

¹Mice were furnished by M. P. Bailey of the North Carolina State Laboratory of Hygiene. Since no birth dates are kept at the State Laboratory of Hygiene, the mice are weaned according to weight. The weights of the mice selected for this study ranged from 9.4 to 12.1 gm.

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duction were the number of young born alive per female on each diet, the number of litters dropped, and the number of young per litter.

RESULTS AND DISCUSSION

The average number of days from the time the females were placed on the experimental diets until the vaginas opened was: Diet I (control) 16.3; diet II (genistin) 6.4; diet III (commercial soybean oil meal) 5.9. An analysis of these data indicated that the difference in days required for vaginal opening was significantly higher ($P \leq 0.01$) for the mice fed diet I as compared to those fed diets II and III. A further evaluation of the estrogenic activity of the diets can be obtained by comparing the age at which vaginal openings occurred with the normal age of 35 days reported in the literature (Snell, '41). Since the mice in this experiment were approximately 21 days old when they were started on the diets, it is apparent that diet I is an adequate control because the vaginal opening of the mice on this diet occurred at a normal age. The mice on diets II and III, however, were less than 35 days old at the time of vaginal opening thus demonstrating the estrogenic activity of these diets.

The average food intake and weight gain, respectively, in grams per cage for the first 4-week period of the experiment were as follows: diet I, 253, 21.7; diet II, 256, 21.5; and diet III, 252, 21.2. An analysis of these data indicated no significant differences in either weight gains or food intake. The possibility remains, however, that genistin may have either beneficial or detrimental effects on growth under different conditions or at other levels.

The group average of the number of young born (per female on each diet), percentage of females dropping litters, average number of young per litter, and average weight per litter are presented in table 2. The group average of the number of young born for the control diet (diet I) was 4.9 and for the genistin diet (diet II), 3.2. A statistical analysis of these data indicated that the difference was significant.

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($P \leq 0.05$). On the per litter basis, the average for the mice on diet I (control) was 6.0, diet II (genistin) 5.4, diet III (commercial soybean oil meal) 5.8. Although the value for the genistin diet was lower than that of the other two diets, the difference was not significant, and all of the values were within the average range of 5 to 6 young per litter reported by Snell ('41). However, only 59% of the females fed diet II bore a litter, whereas 82% of the females fed diet I, and 77% of the females fed diet III bore litters. These data in-

TABLE 2
Summary of data from reproduction study

CRITERIA	DIET I ¹ (control)	DIET II ¹ (genistin)	DIET III ² (commercial soybean oil meal)
Total no. of females	33	34	35
Group average of young born per female	4.9	3.2	4.5
Percentage of females dropping litters	82	59	77
Av. no. young per litter	6.0	5.4	5.8
Av. wt. per litter, gm	8.5	7.9	9.7

¹ Of 36 animals placed on diet one died during reproduction study, the remainder died during the growth study.

² One animal died during reproduction study.

dicates that the effect of genistin in the diet was on the number of litters dropped rather than on the number of young per litter.

Although the percentage of females bearing a litter was lower for the group on the soybean oil meal diet than either the reported normal for mice of 80 to 90% (Snell, '41), or the group of mice on the control diet, the difference was not marked enough to be considered significant. It is possible that the failure of the soybean oil meal diet (diet III) to give the same results as the genistin diet (diet II) was due to the fact that diet II contained more genistin than did diet III. This point deserves further investigation, particularly in

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view of the fact that soybean oil meal *per se* has been shown to be estrogenically active (Carter et al., '53).

Clinical symptoms, such as dystocia, stillbirths and fetal resorption, which have been reported to occur in sheep on the estrogenically active subterranean clover pastures (Underwood, '52) and in rabbits on soybean hay (Kendall et al., '50) were not observed in this study with mice. The fact that the main effect of genistin was on the number of litters born and not on the size of the litter is further evidence that neither resorption nor intrauterine deaths occurred. Since a histological study of uteri, ovaries or other reproductive glands was not made, the reproductive disturbances cannot be attributed to the effect of genistin on any specific gland or tissue.

Since the males received the test diets during the 21-day period they were housed with the females, the possibility exists that the effect of genistin was mediated through the males. Each male, however, sired one or more litters, indicating that regardless of the dietary regime all males were fertile at some time, if not at all times, during the 21-day breeding period.

SUMMARY

A study was conducted to determine the effects of soybean oil meal and of genistin extracted from it on growth and reproduction of the mouse. Growth of the mice from three to 7 weeks of age was not affected by the treatments used in this experiment. Both commercial soybean oil meal and isolated genistin significantly lowered the age at which the vaginas of immature mice opened. The principal effect on reproduction of 0.2% genistin in the diet was a decrease in the number of litters born, whereas litter size was not affected. The effect of commercial soybean oil meal (50% of the diet) on the number of litters born was not statistically significant but the number of litters obtained was less than that from the group of females on the control diet.

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EFFECT OF GENISTIN ON GROWTH AND
DEVELOPMENT OF THE MALE
MOUSE^{1,2}

G. MATRONE, W. W. G. SHARP, JR., M. W. CARTER
AND V. W. SHARP

Animal Nutrition Section, Department of Animal Industry

AND H. W. GAPPEN

University of Tennessee, Knoxville, Tennessee 37996-0001
Submitted for publication, December 26, 1965

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The discovery of the estrogenic activity of genistein, 4,5,7 trihydroxyisoflavone (Bradbury and White, '51) isolated from subterranean clover focused attention on the distribution and physiological effects of estrogenic-like compounds in feeds. Carter et al. ('53) and Cheng et al. ('53) found that the estrogenic activity of soybean oil meal, as measured by the uterine weight of immature female mice, is due to the presence of genistin, the glucoside of genistein. Work in our laboratory (Carter et al. '55) also has shown that when genistin, isolated from soybean oil meal, was fed to mice at a level of 0.2% of the diet fewer litters were born.

The present study is concerned with the further exploration of some of the effects produced by genistin isolated from soybean oil meal. The first objective was to determine the effect of genistin on growth of the male mouse and on the testicular development as measured by testes weights and spermatogenesis. A second objective was to compare the

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effects of genistin with those of a known estrogen to aid in differentiating between estrogenic effects and non-estrogenic effects.

EXPERIMENTAL

Diethylstilbestrol was used for the comparative study since it had been used as the standard of reference in previous experiments (Carter et al., '53). Several levels of genistin and of diethylstilbestrol were studied in order to describe the response curve of each substance.

TABLE 1
Basal diet used in growth study

CONSTITUENTS	AMOUNT PER KILOGRAM OF DIET
	gms
Casem (Vitamins test)	180.0
Starch (corn)	630.0
Vegetable oil ¹	100.0
Mineral mix ²	40.0
Celulose ³	20.0
Cod liver oil ⁴	10.0

¹ Aroclor water-soluble vitamins and methionine replaced an equal weight of starch.

² Wesson Oil and Snowdrift Sales Co., New Orleans.

³ Wesson's modification of the Osborne and Mendel salt mixture.

⁴ Alphacel.

⁵ U.S.P. cod liver oil (850 units A/gm, 85 units D/gm).

The design of the experiment was a randomized block having 10 treatments (4 levels of genistin, 5 levels of diethylstilbestrol and one control, table 2) and 10 replications. The levels of genistin and the first 4 levels of diethylstilbestrol selected for study were equilibrated on the basis of their relative estrogenic potency (9 mg genistin \approx 0.04 μ g diethylstilbestrol) as calculated from data of a previous study (Carter et al., '53). One hundred male mice⁵ approximately three weeks of age were stratified into 10 uniform weight

⁵ Supplied by M. P. Bailey of the North Carolina Laboratory of Hygiene, Raleigh, North Carolina.

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groups of 10 animals each and then each animal within a group was assigned at random to one of the 10 treatments until a complete replication had been formed. This process was repeated for each of the remaining weight groups.

The mice were housed individually in wire cages with screen floors.

The composition of the basal diet is given in table 1. Vitamins and methionine⁴ were provided as reported by Carter et al. ('55). Each animal was fed its assigned daily dose of genistin⁵ or stilbestrol premixed in 1 gm of the basal diet. After this was consumed, untreated basal diet was fed ad libitum. This procedure was repeated daily throughout the experimental period of 6 weeks.

Body weights were recorded weekly. At the termination of the experiment the mice were sacrificed; fresh weights were determined and histological studies were made on the testes, adrenals, spleen and kidneys.

RESULTS

Ten mice died during the experiment; 4 were receiving the 4th level (highest) of genistin, two were receiving the third level of genistin and 4 were scattered, singly, among some of the other levels of the two test substances (table 2).

A regression analysis of the weight gains as the dependent variable and the logarithm of the dosage level as the independent variable indicated that there was a significant ($P \leq 0.01$) linear decrease in growth rate associated with increasing levels of genistin in the diet. In fact, on the average, the mice receiving the 4th level of genistin lost weight. On the other hand all mice receiving stilbestrol gained weight; only the group receiving the highest level of stilbestrol gained significantly less ($P \leq 0.01$) than the control group (table 2).

⁴ Vitamins and methionine were contributed by Marez and Company.

⁵ Genistin was isolated from commercial soybean meal as described by Carter et al. ('51). The soybean meal was contributed by members of the North Carolina Feed Manufacturers' Association.

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Genistin also had a depressing effect on testes weight (table 2). The linear decrease in testes weight associated with the logarithm of the dose levels of genistin, moreover, remained significant when the data were adjusted by covariance to a mean body weight, indicating that genistin depressed testes weight directly. The depressing effect of stilbestrol on testes weight was manifested by the mice on the third level of stil-

TABLE 2

Results of growth study with mice

DOSE	NO DIED	FINAL BODY WEIGHT	GAIN	MEAN WT. AT END OF EXPERIMENTAL PERIOD			
				Testes	Adrenal	Kidney	Spleen
		g	g	mg	mg	mg	mg
Control							
0	0	24.5	13.5	175.4	4.7	350.3	96.5
Genistin, mg							
0	0	22.5	9.3	163.4	5.3	324.9	90.7
13	1	15.5	4.3	140.4	5.6	231.9	50.2
36	2	12.0	0.5	57.8	4.4	192.5	49.3
72	4	10.0	-1.5	16.4	4.5	192.9	36.0
Diethylstilbestrol, μ g							
.04	0	24.1	12.7	173.5	5.4	360.7	93.8
.05	1	24.2	12.7	173.8	5.0	339.1	85.7
.10	1	24.3	13.1	134.6	5.0	396.7	100.6
.32	0	22.3	12.6	146.8	5.9	359.1	40.3
.64	1	21.2	9.7	122.3	5.5	334.5	36.0
L.S.D. 0.05 ¹		2.3	2.6	32.7	0.8	52.5	55.2
L.S.D. 0.01		3.6	3.4	43.5	1.1	69.7	77.3

¹ Died before completion of experiment.

² Least significant difference at specified probability levels.

bestrol although the body weight of this group of animals was not significantly different from that of the controls. The histological examination of the testicular tissue showed that no spermatozoa were present in the testes of the groups receiving the two higher levels of genistin. Spermatozoa, though present in the testes of the group receiving the highest level of diethylstilbestrol, were fewer in number than in testes of control animals.

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As is shown in table 2, there was an increase in adrenal weights associated with the first two levels of genistin, followed by a drop in adrenal weights in animals receiving the two highest levels of genistin. All the groups of mice receiving stilbestrol showed an increase in adrenal weight as compared to the control group. There was only a slight correlation between body weight and adrenal weight ($r = +0.027$).

The differences observed in kidney weights could be explained by differences in body weight. The correlation between body weight and kidney weight was $r = +0.769$. Necrotic areas were observed on the kidneys of animals receiving the three higher levels of genistin. 4

The spleen weights, table 2, were quite variable. There were no significant differences in weight of the organ that could be attributed to differences in treatment of the animal.

DISCUSSION

The evidence presented indicates that genistin at certain dose levels has a detrimental effect on survival, growth rate and spermatogenesis in mice. Undoubtedly the effects observed could be partially explained by the probable coincident decrease in food intake but it is unlikely that taste *per se* was a significant factor, because of the feeding procedure used. It appears, therefore, that the results obtained were due, in part at least, to an effect of genistin other than nutrient intake. 4

One of the questions remaining is whether or not the effects of genistin are associated with its estrogenic properties. The depressing effects of exogenous estrogens on growth and testicular development are considered to be mediated via the pituitary. All the naturally-occurring estrogens and stilbestrol apparently decrease the output of gonadotrophin and growth hormone of the pituitary (Richards and Kuster, '41; and Emmens and Parks, '47). /

A comparison of the results obtained on growth and testicular development indicate that the physiological action of genistin is different from that of stilbestrol. As is shown in

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table 2, a significant ($P \leq 0.01$) effect of genistin on growth is manifested at a lower dose (9 mg/day) than it is on testes weight (18 mg/day), whereas the effect of stilbestrol is in the reverse order (0.64 μ g to depress growth and 0.16 μ g to depress testes weight). Furthermore, if a comparison is made between the group of mice on the highest dose level of stilbestrol (0.64 μ g/day) and the group on the lowest dose level of genistin (9 mg/day), two groups which are comparable in body weight, it is observed that the testes weight of the genistin-treated mice is significantly greater ($P \leq 0.05$) than that of those of the stilbestrol-treated group. The kidney weights of these two groups, however, are not significantly different. These data indicate, therefore, that the action of genistin on testes weight is different from that of stilbestrol.

Morrell and Hart ('41) reported that immature female rats continued to grow, although at a lower rate than controls, despite the administration of relatively enormous doses of stilbestrol (1.07 mg/day). These authors also reported there was no direct quantitative relationship between size of dose and decrease in growth. Richards and Kueter ('41) reported that immature male and female rats administered 2 mg daily of stilbestrol continued to grow for approximately two weeks before growth was arrested. Deaths attributable to massive doses of stilbestrol were not reported in either of the two papers mentioned. In contrast to these results reported in the literature and to those obtained with stilbestrol in this study, a direct relationship was found between the dose level of genistin and the retardation in growth. Moreover, the higher dose level of genistin appeared to be lethal. It appears from these results, therefore, that genistin, in relation to its estrogenic activity, has a greater depressing effect on growth than does stilbestrol.

The differences in the results between genistin and stilbestrol on growth and testicular development indicate that the effects obtained with genistin in this study may be primarily non-estrogenic.

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SUMMARY

When genistin was fed at levels of 0, 15, 30 and 72 mg per day per mouse the following results were obtained:

1. An inverse linear relationship was found between the logarithm of dose levels of genistin and growth rate.
2. Genistin appeared to have a depressing effect on testes weight beyond that attributable to differences in body weight.
3. No spermatozoa were present in testes of the mice given the two higher levels of genistin.

The effects of genistin on growth and testicular development differed, both qualitatively and quantitatively from those of stilbestrol.

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ADAMS

1 INTRODUCTION

Plant products have been used in folk medicine from ancient times as aphrodisiacs, aids in childbirth, abortifacients, and promoters of fertility. Both increases and decreases in fertility in animals have also been attributed to specific compounds in plants. Estrogenic compounds were isolated from plants in 1928, only a few years after they were isolated from animals, and since that time plant estrogens have been found to be very widespread. This historical background has led to considerable confusion. On the one hand, there is a tendency to attribute to estrogens all of the pharmacological effects of plants on reproduction, while on the other, there is the problem of explaining the apparent lack of clinical signs resulting from plant estrogens which appear to be almost ubiquitous in the environment.

Part of the confusion arises from the failure to recognize that plants may contain compounds other than estrogens which can affect reproductive performance. Many early studies need to be reevaluated with the benefit of hindsight to determine whether the reproductive abnormalities described could in truth be attributed solely to estrogenic compounds in the plants. A good example of this is the recent isolation of 6-methoxybenzoxazolinone from sprouting wheat.¹ This compound is not estrogenic itself, but it can stimulate the secretion of estradiol from an animal's ovaries, and this causes the occurrence of estrogenic signs. Similar exacting studies remain to be done for many of the early reports of estrogens from a variety of plant sources.

Later research has concentrated on a relatively small number of plant species, but even for these, confusion remains over the importance of phytoestrogens to animals. Clinical effects of phytoestrogens in domestic animals have been observed in many countries and on all continents.² However, the amounts of phytoestrogen in the environment are rarely sufficient to totally disrupt the reproductive process, so that subclinical impairment is more common. Furthermore, because phytoestrogens are so widespread, whole populations may be affected and an impaired performance in individuals becomes difficult to detect. For example, in Australia plant estrogens cause at least 1,000,000 ewes to fail to lamb each year,³ but the loss occurs without clinical signs and is so widespread that many farmers accept their lambing rates as "normal". The extent of subclinical effects of phytoestrogens in other species, including humans, is unmeasured.

Much of the interest in phytoestrogens was stimulated by the severe clinical effects seen in sheep in Western Australia in the 1940s. Early work was reviewed by Bradbury and White,⁴ and subsequently by Moule et al.⁵ Many reviews since that time have covered more recent aspects of the work, the most general being by Livingston,⁶ Cox,⁶ and Collins and

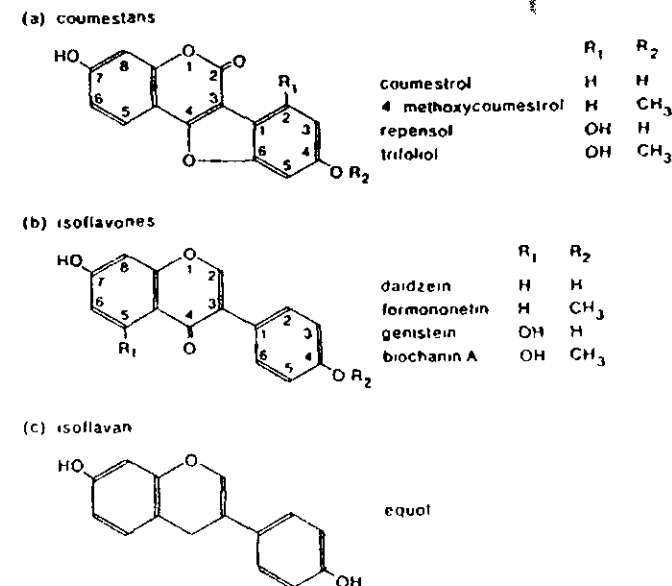


FIGURE 1 Structures of the common plant estrogens

Cox.⁷ The present work attempts to summarize current knowledge, and not to cover any area in depth. The work examines only estrogens found in plants, and not fungal estrogens such as the resorcylic acid lactones (e.g., zearalenone).

II CHEMISTRY AND BIOSYNTHESIS

A. Chemical Compounds

The earliest estrogens to be isolated from plants were the steroids estrone and estradiol, isolated from palm kernels and willow catkins, respectively. Since the 1940s, attention has focused on isoflavonoid compounds, particularly the isoflavones and coumestans. A review by Farnsworth et al.⁸ lists 29 plant compounds shown to be estrogenic. The most widespread and important of these are shown in Figure 1. Among the isoflavones, the most important compounds are genistein, biochanin A, formononetin, and the isoflavan equol. Coumestrol, 4'-methoxycoumestrol, sativol, trifolol, and repensol are important coumestans. The structures of less common compounds are given in the review by Farnsworth et al.⁸ These compounds are relatively insoluble in water, and to increase their solubility and stability they occur in the plant as glycosides. The isoflavones occur as 7-O-glucosides, and normally the sugar has malonate or methylmalonate hemiesters.⁹

The compounds vary greatly in their relative estrogenicity. As discussed in Sections III C and V B, the estimate of estrogenicity is partly influenced by the technique used for measurement. Compounds may be metabolized by the animal before binding to the estrogen receptor, and even after binding the effectiveness of the phytoestrogen-receptor complex in stimulating a gene response varies with the compound. For these reasons, the most generally used comparison of intrinsic estrogenicity is based on bioassay by measuring uterine weight after feeding to mice. Results from such studies are summarized in Table 1. Although the plant estrogens are relatively inactive, they frequently occur in such high concentrations in plants that they are able to exert effects on animals.

Table 1
POTENCY OF THE PLANT ESTROGENS
RELATIVE TO DIETHYLSTILBESTROL

	Study ^a			
	A	B	C	D
Diethylstilbestrol	100,000	100,000	100,000	—
Estrone	6,900	—	—	—
Coumestrol	35	—	25	—
Genistein	1	1.35	—	—
Daidzein	0.75	0.35	—	—
Biochanin A	0.46	0.9	—	—
Formononetin	0.26	<0.01	—	—
Equol	—	—	—	0.25

^a Data from (A) Buckoff et al., 1962, (B) Wong and Flux, 1962, (C) Buckoff et al., 1959, and (D) Shutt and Braden, 1968.

B. Synthetic Pathways

The synthesis of the plant estrogens has been well reviewed by Wong.¹⁰ The isoflavones and the coumestans are both based on the same isoflavonoid carbon skeleton, being distinguished by the degree of oxidation of the central three carbon atoms (Figure 2). The synthesis of the basic skeleton requires the formation of condensation of phosphoenol-pyruvate with erythrose-4-phosphate, via intermediate compounds and a further addition of phosphoenol-pyruvate, to form phenylalanine and then cinnamic acid (the "shikimic acid pathway"). The A ring, on the other hand, is formed by acetate condensation using acetyl-CoA in a variation of fatty acid biosynthesis. It is probably cyclized before condensation with cinnamic acid to form chalcone, the precursor of flavone compounds.

The formation of isoflavones from flavones results from B ring migration, a reaction which appears limited to the Leguminosae and which is probably rate limiting. Coumestans are then probably formed from isoflavones via dehydropterocarpan, and other intermediates (Figure 2).

C. Physiology of Synthesis

Rossiter and Beck¹¹ suggested that the shoot apex is a major site of isoflavone synthesis in subterranean clover. About half the final content of isoflavone is present at leaf emergence, the rest being produced during leaf and cell expansion. Synthesis is normally halted at full expansion, although some turnover of the isoflavones continues. Daidzein, which is absent from younger leaves, appears in expanded leaves. Under exceptional conditions, e.g., in detached expanded leaves supplied with light and sucrose, net isoflavone accumulation may continue.

Rossiter¹² showed that conditions which cause the accumulation of starch or soluble sugar also increased the concentration of isoflavones. A close curvilinear relationship was obtained between isoflavones and soluble sugars per cell at the stage of full leaf expansion (when isoflavone synthesis normally ceases). Thus, soluble sugar content provided a good index of the level of carbon substrates used for isoflavone synthesis.

D. Significance to Plant Physiology

The most probable role for the coumestans is an action as phytoalexins, a group of substances which assist the plant to resist pathogens. Coumestrol itself is an alexin, and the concentration of both coumestans^{13,14} and isoflavones¹⁵ increases in plants during attack by phytopathogens.

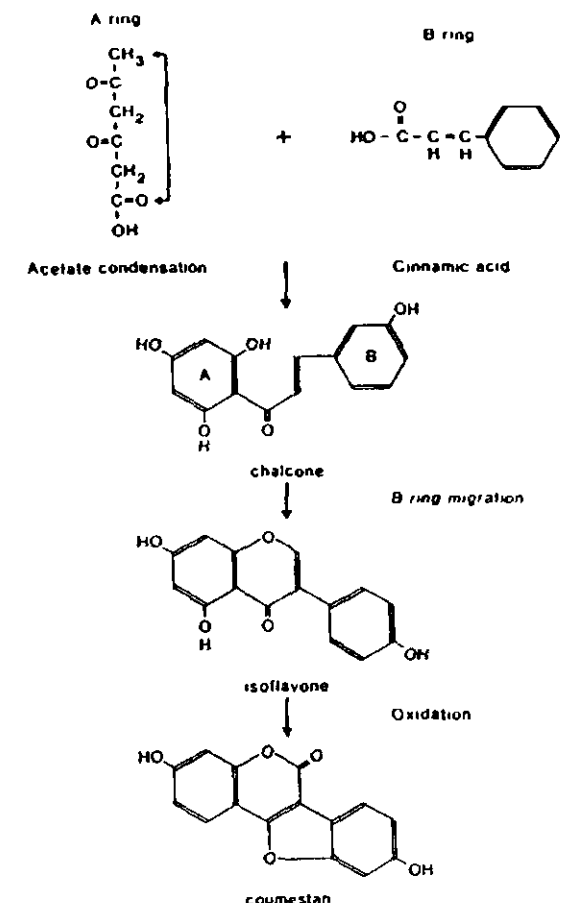


FIGURE 2 Biosynthesis of isoflavonoids

The role of isoflavones is not obvious. They frequently form a substantial proportion of the dry matter of the plant, and in view of their apparent metabolic inertness, it has been suggested that the isoflavones are simply storage compounds. However, this seems unlikely because they are synthesized primarily during active growth, when demand for carbon is greatest, and not at maturity when carbon is available for storage.

The isoflavones from red clover may inhibit the germination of its own seed.¹⁶ Isoflavones genistein and daidzein are also reported to inhibit the ability of *Rhizobium* bacteria to induce root nodules.¹⁷

It has been suggested that plant estrogens have an ecological role in controlling populations of herbivores which graze estrogenic swards. The subtlety of this hypothesis is attractive, but it is difficult to believe it has a major ecological effect. It has also been claimed that isoflavone glycosides render plants unpalatable¹⁸ and thereby reduce intake by grazing animals. This notion accords with field evidence that there are differences between cultivars of clover in voluntary intake by sheep.

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III. ASSAY AND BIOASSAY

A. Chemical Assay of Plant Material

Similar techniques are used for the extraction, isolation, and measurement of the isoflavones and the coumestans. The standard technique for the assay of isoflavones was described by Beck.¹⁹ Fresh plant material was first ground and left for some time to allow plant enzymes to hydrolyze the isoflavone glycosides. The isoflavones were extracted into hot ethanol, purified in further extraction steps and separated by thin-layer chromatography. More recent assays have utilized high-pressure liquid chromatography (HPLC) to speed up analyses and improve precision.²⁰ Acid hydrolysis may be used to free the aglycones.²¹ Chemical analyses have been used mainly by plant breeders, who have modified the original technique to increase the throughput at the expense of precision. Francis and Millington²² modified Beck's technique for use on single leaves. Formononetin is the main isoflavone of interest to the plant breeder, and Gosden and Jones²³ were able to omit the chromatographic separation steps by taking advantage of the relatively low fluorescence of biochanin A and genistein when compared with formononetin.

The standard method for the assay of coumestans described by Livingston et al.²⁴ was very similar to that described above for isoflavones. Currently, HPLC is the separation method of choice for most uses.²⁵ Extraction procedures have been improved by extracting at pH 9.5,²⁶ and in the case of soybeans, by first extracting with petroleum ether.²⁷

B. Chemical Assay of Animal Material

Methods for the assay of isoflavones and coumestrol in animal tissues were first described in detail by Lindner.²⁸ After extraction with ethyl acetate, the compounds were purified by partition between solvents and separated by liquid-gas chromatography. The limitation to this and subsequent studies has been the detection of the compounds once they are isolated. Coumestrol (2 ng/ml) and formononetin, daidzein, and equol (20 to 50 ng/ml) may be detected by fluorescence,²⁹ but genistein and biochanin A cannot and have been detected by UV absorbance at 1 µg/ml. These latter compounds are more readily detected in a protein binding assay using estrogen receptors.²⁹ Present detection methods are adequate for studies on digesta, but are extended to their limits to detect the concentrations of the free compounds normally found circulating in plasma.³⁰ Further progress in studies on concentrations within the animal will probably be dependent on the application of mass spectrometry or other more sensitive methods of detection.

C. Bioassays

Bioassays have proven more useful to animal scientists than chemical assays because they reflect all of the phytoestrogens present in the feed and also reflect the results of metabolic processes within the animal. These features make it important to choose the appropriate bioassay. When this is done, it has usually been found that the correlation between chemical assays and bioassay is relatively good.³¹

Many different bioassays have been described, and it is important to understand the reasons for differences between assays. In particular, the metabolism of isoflavones varies considerably among animal species, so that the assays should be carried out in the species being investigated. Reliance on chemical assay, and extrapolation of bioassay results from monogastric to ruminant animals, has led to severe errors in the past. On the other hand, the use of bioassays has promoted the discovery of new plant estrogens in white clover.³⁴

1. Bioassay in Monogastric Animals

Many screening studies are carried out in immature (16 to 25 d old) rats or mice using increased uterine weight as an end point. As the animals become more mature, it is possible

for the ovaries to contribute to the final result, and the use of adult ovariectomized animals may be more accurate. It is advisable to use uterine weight as the end point to permit comparison with other published data. Early studies used the cornification of vaginal cells recovered by a vaginal smear (the Allen-Doisy technique), but this is less easy to quantify. An increase in uterine weight does not prove in itself that the compound is estrogenic. Demonstration that the compound binds to the estrogen receptor is a more specific test, but is less useful as a bioassay for the reasons described in Section V.

Rats and mice do not eat green material readily, so the test substance may be administered by stomach tube, or extracts may be impregnated into the animals' feed. Guinea pigs may be used if it is desired to feed green material. Extracts may also be given by intramuscular injection, thereby avoiding the metabolism of substances in the animals' gut. However, the injection of large amounts of the relatively insoluble isoflavones may cause them to precipitate at the injection site.³² It is also likely that the conjugated isoflavones would be less effectively hydrolyzed if given parenterally.

The activity of the standards used in the assay will also depend on the route of administration. If steroidal estrogen is used as a standard, injection may be the preferred route, because estradiol is metabolized in the gut to a much greater extent than is diethylstilbestrol (Table 1) and comparisons of test substances with oral doses of estradiol can be excessively variable.

Hyperestrogenicity has been described in swine and suspected in horses, but these species have not been used in bioassays. There are no reports of toxicity or the bioassay of phytoestrogens in humans. If bioassays were required in humans, measurement of changes in sex hormone binding globulin or cortisol binding globulin in men or postmenopausal women may be a useful approach.

2. Bioassay in Ruminants

The isoflavones genistein and biochanin A are converted in the rumen to nonestrogenic products, while formononetin is reduced and demethylated to the more estrogenic compound equol (see Section IV). Thus, the same plant material gives very different results depending on whether the bioassay is carried out in mice or in sheep.³³ The metabolic patterns of the ruminal microbial population usually take 7 to 12 d to adapt to the new substrates, so bioassays of isoflavones in ruminants should run for at least this period. If plants contain significant amounts of genistein or biochanin A, the estrogenicity decreases dramatically after the first few days of exposure.^{34,35} In contrast, the pattern of metabolism of coumestans in the rumen does not appear to change with time.³⁶ Thus, bioassay of the coumestans may be considerably simpler.

The increase in uterine weight of ovariectomized ewes after exposure to estrogen provides an assay which has been well defined and validated.³¹ The results of this assay correlated well with the degree of temporary infertility observed in ewes grazed on the pasture.³⁷ The major disadvantage of the assay is its expense, but this may be reduced by examining the ratio of RNA to DNA in uterine biopsies.³⁸ This latter assay is sensitive and precise, but has not yet been used widely enough for its accuracy to be fully validated.

An increase in the teat length of wethers grazed on suspected pasture has been used to measure estrogenicity.³⁹ The precision of the assay is increased by using a reference mark on the base of the teat before measurement.⁴⁰ This assay is valid for pasture with a relatively low estrogenicity,³⁹ and furthermore it tends to overestimate the potency of the pasture.³⁷ Within these limitations, the assay is simple and cheap, and repeated use of the same animals. The test may also be used in a nonquantitative way to indicate fluctuations in the estrogenicity of the pasture in continuously grazed pastures, and so may indicate the effects of changes in pasture composition over time.

The weight of secretions recovered by a cotton swab from the vagina of ovariectomized

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Table 5
CONCENTRATIONS OF COUMESTROL IN
THE PLASMA AFTER FEEDING

Study*	Intake of coumestrol (mg/24 h)	Concentration (ng/ml)	
		Free	Conjugated
Sheep			
A	952	8.1	28.1
	514	3.7	14.5
B	270	7	12-40
	350	9	58
Goat			
C	12	2-4	—
Cow			
D	0.4	1.2	0.5

* Study (A) Kelly and Lindsay, 1978, (B) Shutt et al., 1969, (C) Shemesh et al., 1972, and (D) Mannov et al., 1969

and cattle are given in Table 5. The relatively similar concentrations from greatly differing intakes may reflect either variable excretion rates or difficulties with assays at very low concentrations of coumestrol.

The metabolism of both isoflavones and coumestans in cattle is believed to follow a pattern similar to that of sheep.⁶⁰ Cattle may conjugate and excrete the isoflavones more rapidly than sheep, and this could explain why clinical reports of problems in cattle are less common.

B. Metabolism in Monogastric Animals

The excretion of isoflavones by the rat and the guinea pig appears to occur primarily via the feces, rather than the urine.^{61, 62} It is probable that excretion in the bile is followed by metabolic transformations of the isoflavones by the intestinal microflora and enteric/hepatic recirculation. The metabolic transformations of the isoflavones appear different in monogastric animals than in ruminants. In the fowl, genistein and biochanin A, as well as formononetin, can be metabolized to equol.⁶³ Similarly, in the human, genistein and daidzein can be metabolized by the intestinal microflora to equol, which was excreted in the urine.⁶⁴ The extent of breakdown of the isoflavones to nonestrogenic phenolic compounds in monogastric animals is unknown.

The amounts of isoflavones excreted by vegetarian humans can be relatively large.⁶⁵ However, there are no data on the concentrations of these compounds in human plasma. It is probable that the clearance rate is high, because plant estrogens do not bind to human cortisol binding globulin, and are only 14 (coumestrol) to 25% (isoflavones) as efficient as estradiol in binding to sex hormone binding globulin.⁶² On the other hand, this failure to bind to carrier proteins would make the compounds more available to human cells than the equivalent amount of estradiol.

VII EFFECTS ON HUMANS

There are no unequivocal descriptions of plant estrogens affecting human beings. Plants have been used since prehistoric times for their medicinal properties, and many effects are reported on the reproductive system. For example, in European traditional medicine, both fennel and anise have been reputed to increase milk secretion, promote menstruation, facilitate childbirth and increase libido.⁶⁶ Other cultures have used different plants for similar results. Plant estrogens have also been suspected of causing failure of menstruation in women

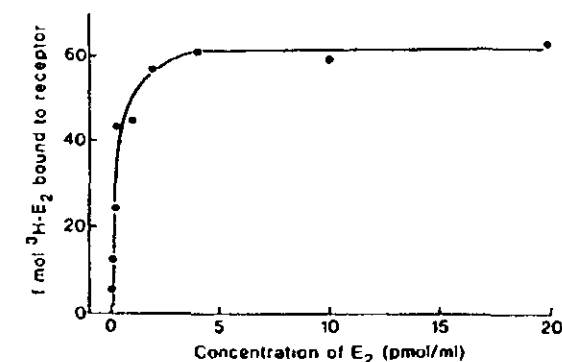


FIGURE 6 Effect of increasing the concentration of [³H]-estradiol on the amount bound to the estrogen receptor

subsisting on tulip bulbs.⁶⁷ However, none of these effects is specific for estrogen, and it is possible that other pharmacological agents were also present in the plants. There have been no controlled dietary studies carried out on humans to observe estrogenic effects.

Most humans have a more varied diet than their domestic animals, reducing the potential to consume large amounts of phytoestrogen from any particular plant source. Furthermore, unlike some proposed environmental hazards, there is a threshold level for estrogen, below which the estrogen receptor is unable to bind to estrogen (Figure 6). However, it is probable that humans would be affected by plant estrogens if they consumed a sufficient amount. Human breast cancer cells (MCF7 cells) have been shown to bind coumestrol and genistein similarly to animal cells and to respond *in vitro*.⁶²

Phytoestrogens are present in human urine⁶⁸ and can reach particularly high levels in the urine of vegetarians.^{69, 70} However, humans produce more endogenous steroidal estrogen than most species, and the concentration of estradiol and estrone in the circulation of humans is at least tenfold greater than that found in most species of domestic animals. Thus, plant estrogens are more likely to be antiestrogenic in humans than in other species.⁶⁵ It has been suggested that the lower level of breast cancer in women from vegetarian cultures may occur because they have a higher intake of phytoestrogens which antagonize the cancer promoting effects of their endogenous estrogen.⁶⁵

Recently, attention has been focused on the phenolic compound enterolactone, which can be isolated from human urine.^{71, 72} This substance is not an estrogen and does not bind to the estrogen receptor.⁷³ It may not even be of dietary origin.⁷⁴ However, it does appear to have some effects *in vitro*,⁷⁵ and its significance remains to be determined.

VIII EFFECTS ON ANIMALS

Plant estrogens have been suspected to cause problems in most species of domestic animals, but the majority of the reports in the literature deal with sheep.

A. Sheep

Phytoestrogens have a variety of deleterious effects on the sheep. Initially, a number of obvious clinical problems were observed in sheep grazed on highly estrogenic clover pastures. These included dystocia, prolapse of the uterus and severe infertility in ewes, and enlargement of the bulbo-urethral glands and death in wethers. This obvious clinical problem was called "clover disease".

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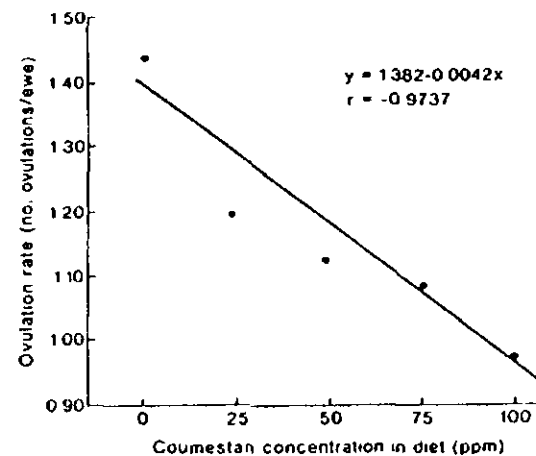


FIGURE 7 Effect of increasing concentrations of coumestrol in the feed on the ovulation rate of ewes. (From Smith, J. F., Jagusch, K. T., Brunswick, L. F. C., and Kelly, R. W., *N Z J Agric Res.*, 22, 411, 1979. With permission.)

With the availability of cultivars of clover low in formononetin, and with improvements in husbandry, this clinical problem diminished. It then became apparent that plant estrogens could reduce the fertility of ewes in the absence of any obvious clinical signs. In Australia at least, low concentrations of phytoestrogens are still very widespread in pastures and cause a mild reduction in fertility in many millions of ewes each year.

The reaction of the ewe to estrogen is more complicated than in most other species. Plant estrogens can reduce the fertility of ewes by two distinct and independent mechanisms. Firstly, there is the usual impairment in reproduction which occurs in all species of animals due to the ingestion of estrogen around the time of mating ("temporary infertility"). Secondly, prolonged exposure to estrogen causes a slow loss of sexual differentiation and an irreversible decline in fertility in the ewe ("permanent infertility", or "defeminization"). These two effects may occur simultaneously in the same ewe, and are also seen in ewes with clinical clover disease.

1 Effects on Fertility

a. Temporary Infertility

Sheep grazed on estrogenic pasture around mating time can have reduced fertility or fecundity. Ewes are very sensitive to phytoestrogens, but rams are relatively resistant.⁹⁰ Concentrations of coumestrols in alfalfa as low as 25 ppm reduce the ovulation rate and thus the twinning rate, of ewes,⁹¹ and the ovulation rate declines linearly as the concentration of coumestrols in the diet increases (Figure 7). High concentrations of coumestrols or isoflavones in the diet may affect the reproductive process in a number of other ways, including failure to ovulate, failure to conceive, and increased embryonic mortality.⁹²⁻⁹⁴ Reduced twinning is a feature of most, but not all of the reports.⁹⁵

After exposure to phytoestrogens for several weeks, ewes may show mammary development and swelling of the vulva.⁹⁶ However, sheep often suffer temporary infertility without any obvious clinical signs. Attention is therefore rarely focused on the problem, and there are no accurate estimates of its frequency or importance in the field. The problem is further complicated by the fact that the most sensitive indicator, the ovulation rate, is also strongly

influenced by nutritional status. Pastures which differ in estrogenicity may also differ in nutritional value, so that in the field there is frequently no knowledge of the potential ovulation rate which might have been achieved by the flock in the absence of phytoestrogens.

Circumstantial evidence suggests that temporary infertility is extremely widespread among sheep. Studies in both Australia and New Zealand have shown that the ovulation rate of sheep grazed on alfalfa is usually lower than that of sheep on comparable grass pastures.⁹²⁻⁹⁷ In California, four of eight samples of alfalfa contained >25 ppm coumestrol,⁹⁸ the concentration found by Smith et al.⁹¹ to be sufficient to reduce ovulation rate. In Australia, most alfalfa crops grown in the moist coastal regions have sufficient foliar disease to produce >25 ppm coumestrol, but alfalfa grown in the drier inland areas is usually nonestrogenic.⁹⁹ Morley et al.¹⁰⁰ have shown that injection with 8 µg diethylstilbestrol daily is sufficient to affect reproduction, and Adams and Atkinson¹⁰⁰ have reduced ovulation rates in ewes with doses of estradiol as low as 1 µg/d. This degree of estrogenicity is attained by many legume-based pastures, particularly if the ewes are freshly placed on the pasture for mating and the rumen does not have time to adapt its metabolic pattern.

There is no treatment for temporary infertility, except to remove the ewes to a nonestrogenic pasture. The ovulation rate recovers quickly, but an impaired ability to conceive remains for 3 to 5 weeks of removal from the estrogenic pasture.¹⁰¹

b. Permanent Infertility (Defeminization)

If ewes are exposed to estrogenic pasture for more than two seasons, they may suffer a decline in fertility from which they never recover. Being permanent, the infertility is also cumulative, becoming worse with each year of exposure. The condition does not normally lead to total sterility, but rather it reduces the chance that the ewe will conceive after mating. Therefore, it may be accurate to call it permanent subfertility. Ewes with permanent subfertility may also suffer additional temporary infertility during concurrent exposure to plant estrogens or they may also show the signs of classical clover disease. However, permanent subfertility usually occurs in the absence of obvious clinical signs.

Affected ewes have subtle abnormalities throughout the reproductive system. The uterine cervix becomes less responsive to the ewe's own endogenous estrogen, and as a result, the spinnbarkeit of the cervical mucus is reduced.¹⁰²⁻¹⁰³ The histological structure of the cervix also changes to take on the appearance of a uterus (Figure 8).¹⁰⁴ Other morphological changes include the presence of small cysts in the endometrium and slight masculinization of the external genitalia. After ovariectomy and challenge with estrogen or testosterone, the ewes display reduced feminine sexual behavior and more masculine sexual behavior when compared with controls.¹⁰⁵ Their ability to give a surge of luteinizing hormone after stimulation with estrogen is also reduced.¹⁰⁶ All of these abnormalities indicate that the problem results from morphogenic, or developmental, actions of estrogen (see Section V D 3). The ewes become defeminized in a process analogous to that which occurs in male animals of all species during organogenesis.¹⁰⁷ However, in contrast to all other mammals, in the ewe this process can occur, albeit slowly, during adult life after organogenesis is completed.

In the field, the estrous cycle of affected ewes is essentially normal.¹⁰⁸ The reduced fertility is due primarily to impaired sperm transport through the cervix¹⁰⁹ because of changes in the cervical architecture and cervical mucus. This leads to a lower chance of the eggs being fertilized. In affected flocks, an increased proportion of ewes fail to conceive and thus return to service. Permanent and temporary infertility therefore affect different stages of the reproductive process. Because it is permanent, the subfertility is expressed even in ewes grazing dry, nonestrogenic pasture. There are frequently no obvious clinical signs, and diagnosis must depend on the measurement of the permanent damage in the reproductive tract of the ewe. Both the spinnbarkeit of cervical mucus and the abnormalities in the cervical histology can be used to estimate the degree of defeminization in commercial flocks.¹¹⁰⁻¹¹¹ (Figure 9)

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Phytoestrogen Content and Estrogenic Effect of Legume Fodder (43825)

HANNU SALONIEMI,*¹ KRISTIINA WÄHALÄ,[†] PÄIVI NYKANEN-KURKI,[‡] KAARLO KALLELA,* AND ILKKA SAASTAMOINEN*

Department of Basic Veterinary Medicine,* College of Veterinary Medicine, Section of Animal Hygiene, FIN-00581 Helsinki; Department of Chemistry,[†] University of Helsinki, FIN-00014 Helsinki; and Agricultural Research Center,[‡] Southern Savo Research Station, FIN-50600 Mikkeli, Finland

Abstract. This study is a summary of Finnish investigations of the phytoestrogen content of legume plants, red clover, white clover, alfalfa, and goat's rue. In addition to the chemical analyses, biological studies were performed. Uterine weight of immature rats was used as an indicator of the estrogenic effect of the fodder used. All red clover varieties studied contained estrogenic isoflavones, especially formononetin and biochanin-A. The phytoestrogen content varied from 1.0% to 2.5% of dry matter. The biological study of white clover showed a clear estrogenic effect not visible through chemical analysis. Alfalfa contains small quantities of formononetin and biochanin-A, but 25–65 ppm coumestrol in dry matter. The estrogenic effect of alfalfa was obvious in the biological study. Goat's rue did not contain any known phytoestrogens, and the biological study was completely negative. (P S E B M 1995, Vol 208)

Studies of the effects of phytoestrogens have been conducted since the early 1940s as a result of widely spread fertility problems observed in Australian sheep (1). In Finland, studies started in the 1960s were aimed primarily at determining whether phytoestrogens were involved in cow fertility disorders which frequently occurred in the spring at the beginning of the grazing season (2). Interest in phytoestrogens has generally been aroused by their adverse properties. They may, however, also be beneficial by increasing the growth rate of animals and the milk yield of cows (3, 4). According to recent studies, they may also have a prophylactic effect against some hormone-related human malignancies (5, 6). The phytoestrogenic effects of legume fodder need to be understood because of increasing consumer interest in so-called organic farming products. This type of farming, which uses no artificial fertilizers, relies heavily on

legume fodder which because of the plant's ability to bind nitrogen from air for its use.

The known phytoestrogens are either isoflavonoids or coumarins. Of the isoflavonoids, biochanin-A and genistein, which in monogastric animals have an estrogenic effect, are broken down in the rumen of ruminants into the inactive para-ethylphenol (3). Two other phytoestrogens, daidzein and formononetin, are converted by ruminal microbia into the active equol. Coumestrol, which is related to the coumarins, is absorbed and is active as such (3).

Many Finnish fodder and pasture plants contain small amounts of phytoestrogens. The highest concentrations of phytoestrogens occur in red clover, where all varieties contain phytoestrogens. Abundant feeding of a diet based on red clover silage has been shown to cause fertility problems in cattle (7).

Attempts have been made to experiment with new legume varieties suitable for the Finnish climate which might have a positive effect on the fodder quality and palatability. These plants include white clover (*Trifolium repens*), alfalfa (*Medicago sativa*), and goat's rue (*Galega orientalis*). At present, their adaptability to Finnish conditions is being studied at agricultural research stations.

In this paper we summarize the Finnish investiga-

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PHYTOESTROGENS IN LEGUME FODDER

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¹ To whom requests for reprints should be addressed at College of Veterinary Medicine, Department of Basic Veterinary Medicine, Section of Animal Hygiene, Box 6, FIN-00581 Helsinki, Finland.

tions of the phytoestrogen content of some legume plants and their estrogenic effects on rats.

Materials and Methods

Legume species studied and the date and place of sample collection are summarized in Table I. Details for cultivation of sampled fodder have been described in earlier reports (8, 9).

The samples consisted of the entire aboveground part of the plant. The samples were ground in a meat chopper immediately after cutting. Thereafter, they were allowed to stand for 30 min at 37°C for the conjugated phytoestrogens to hydrolyse (10) before mixing the samples in absolute ethanol. The samples were then stored in a refrigerator for closer chemical analyses and biological studies.

Studies conducted on subterranean clover have revealed that crushing the leaf tissue release free isoflavones (aglucones) from glucosides through enzymatic hydrolysis (11, 12). Accordingly, when different parts of red clover are milled, enough enzyme (β -glucosidase) is released from every part of the plant (stems not included) to allow complete hydrolysis (13). The adequacy of the hydrolysis method (maceration and incubation for 30 min at 37°C) was established in tests with red and white clover, in which the results of this method and acid hydrolysis were compared. It

was found that maceration and incubation for 30 min at 37°C gave the same results as incubation of 4.6 g dry matter for 2 hr at 80°C in a solution of 1 ml of 4 N HCl in 30 ml of absolute ethanol or incubation of 4.6 g dry matter for 2 hr at 80°C in a solution of 10 ml of 4 N HCl in 30 ml alcohol or incubation of 4.6 g dry matter for 1 hr at 75°C in 10 ml of 25% HCl 10 ml in 80 ml alcohol (9).

Chemical Analyses. The method described earlier (14) was adapted for these studies. The plant samples warmed up at room temperature (50 g in 50 ml absolute ethanol) were mixed intensely for 5 min. The procedure was repeated the next day, after which the samples were filtered through a Büchner funnel. The filtrate was evaporated using a vacuum evaporator (40°C) to reach 100 ml. An aliquot was diluted and filtered through an Aerodisc CR filter (Gelman) before high-performance liquid chromatography (HPLC). The conditions for analysis are described in Table II.

Daidzein, genistein, formononetin, and biochanin-A were determined using a UV detector, and coumestrol was determined by fluorometry.

The following commercial preparations were used as standards: daidzein and formononetin (K & K Laboratories ICN, Cleveland, OH), genistein (Sigma Chemical Co., St. Louis, MO), and biochanin-A (Aldrich-Chemie, Germany). Standards were diluted in absolute alcohol to concentrations of 1–20 μ g/ml. The

Table I. Legume Species Studied and the Date and Place of Sample Collection (9, 16)

Legume (variety)	Research station	Growth stage	Date of sample collection
Red clover (Tepa, Venia, Bjursele)	Southern Savo 61°, 40'	Pasture stage	June 18, 1987
		Silage stage	July 2, 1987
		First aftermath	August 14, 1987
		Second aftermath	August 28, 1987
	Northern Ostrobothnia 64°, 40'	Pasture stage	July 2, 1987
		Silage stage	July 15, 1987
		First aftermath	August 20, 1987
		Second aftermath	September 5, 1987
White clover (Jögeva, Sandra, Tammisto, Undrom)	Southern Savo	Pasture stage	June 17, 1991
		Silage stage	July 4, 1991
		First aftermath	August 6, 1991
		Second aftermath	September 2, 1991
White clover (Undrom)	Karelia, 62°, 20' Kainuu, 65°, 50' N. Ostrobothnia	Pasture stage	June 19, 1991
		Silage stage	July 4, 1991
		First aftermath	August 20, 1991
Alfalfa (Jokioinen)	Sata-Häme 61°, 30'	Bud stage	June 17, 1991
		Early blossom	July 27, 1991
		First aftermath	August 1, 1991
		Second aftermath	August 12, 1991
Goat's rue	Karelia, Sata-Häme	Bud stage	June 17, 1991
		Early blossom	June 27, 1991
		First aftermath	August 1, 1991
		Second aftermath	August 12, 1991

Table II. Analytical Conditions in Phytoestrogen Analysis of Red Clover (16) and That of Some Other Legume Fodder (9)

	Red clover analysis	Other legume analysis
Equipment	Perkin-Elmer 1220 liquid chromatograph UV detector Perkin-Elmer LC-55 at 254 nm Sigma 10 lab data system	Hewlett Packard 1050 liquid chromatograph, automatic sampler and UV detector at 254 nm Fluorometer Perkin-Elmer LS-4, Ex. 304, Em. 454 nm HP Chem Station data system
Pre column	3 μ m 3 cm C-18 258-0160	Lichrospher 100 RP-18, 5 μ m, 4 \times 4
Column	5 μ m 10 cm HS-5 HCODS 258-0152 P-E	Lichrospher 100 RP-18, 5 μ m, 250 \times 4 mm Hewlett Packard
Eluent	40% acetonitrile in water	acetonitrile/water 40%, after 5 min; 70%, after 8.5 min; 80%, after 9 min 100% run time 15 min, post time 7 min
Flow rate	1 ml/min	1 ml/min
Temperature	55°C	ambient
Sample size	2 μ l	10–20 μ l

injection volume used was 20 μ l, while on the fluorometer it was 10 μ l or less.

Biological Studies. Uterine weight of immature rats was used as an indicator of the estrogenic effect of the fodder used. The method has been described in details by Kallala (15) and Saloniemi *et al.* (9). For rat experiments, ether was chosen for extraction of fodder samples (due to its high volatility), whereas alcohol extraction was used for ordinary chemical analysis. The concentrations of isoflavones and coumestrol in prepared rat fodder paralleled those observed in chemical studies (9).

Results

Phytoestrogen Content and Estrogenic Effects of Red Clover (*Trifolium pratense*). The most important Finnish legume used for fodder today is red clover. Its estrogenic effect and factors affecting it have been studied in Finland and in other countries. According to several studies (2, 3, 8, 15, 16), all the red clover varieties studied in the Nordic countries con-

tain estrogenic isoflavones, especially formononetin. There are, however, clear differences between varieties. It has also been observed that the growth stage and the temperature affect the quantity of phytoestrogens. They are formed most abundantly in the spring during the rapid growth period or in the autumn in the abundant aftermath (8, 16) (Table III). The uterine weight of immature rats increased from 20 mg to 50–60 mg when rats received 2 g red clover dry matter per day for 5 days (7).

Cool weather during the growing season increases the amounts of phytoestrogens (3, 13). A clear difference was observed in the phytoestrogen concentrations of red clover varieties between North and South Finland (16). The amount of nutrients also have an effect. The formononetin concentration of red clover is higher in a phosphorus-poor soil than in a soil fertilized with phosphorus (13). It has also been observed that with increasing nitrogen doses the phytoestrogen concentration and raw protein concentrations in red clover decrease (8). The possible effect of a number of factors such as soil or rain has not yet been studied as far as we know.

Phytoestrogen Content and Estrogenic Effects of White Clover (*Trifolium repens*). The quantities of estrogenic isoflavones discovered in the white clover varieties were small, only 0.02%–0.06% in dry matter (Table IV). Formononetin was the main component (90%–95% of the total) and a very small amount of genistein (5%–10%) was detected. In most of the material, the amount of daidzein and biochanin-A was below the detection limit. Also small quantities of coumestrol were found, but it was not recovered in all samples. The effect of white clover samples on the

Table III. Phytoestrogen Content of Red Clover as Percentage of Dry Matter at Different Growth Rates (Range of Means, Three Varieties and Two Research Stations) (16)

Growth state	Daidzein	Genistein	Formononetin	Biochanin-A
Pasture stage	n a *	0.04–0.09	0.66–1.05	0.39–1.46
Silage stage	0.02–0.03	0.04–0.08	0.48–0.77	0.32–1.40
First aftermath	n a	0.04–0.14	0.70–1.04	0.55–1.45
Second aftermath	n a.	0.03–0.11	0.60–0.93	0.40–1.17

Note. Variety Tepa had higher values than Venla and Bjursele, but the differences are not statistically significant. Values in Northern Ostrobothnia research station were higher than in Southern Savo ($p < 0.01$).

* n a. = not analyzed.

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Table IV. Phytoestrogen Content of White Clover at Different Growth Rates and the Effects on the Weight of Immature Rat Uterus (Range of Means, Four Varieties and Four Research Stations) (9)

Growth state	Isoflavones (% in DM ^a)	Coumestrol (ppm in DM)	Increase in uterine weight ^b (mg)
Pasture stage	0.01-0.06	<1-5.8	15.5 (8.6-18.9)
Silage stage	0.02-0.03	<1-4.2	17.7 (15.0-21.0)
First aftermath	0.02-0.04	<1-8.7	15.6 (8.6-20.6)
Second aftermath	0.02-0.03	<1-8.9	36.0 (31.1-45.0)

Note. Biological study. Duration of experiment, 5 days; experimental extract, 3 g dry matter/day; rats/test group, 5 rats; rats/control group, 8 rats, uterine weight 21.0 ± 2.0 mg. No statistically significant differences were found between varieties and research stations.

^a DM = dry matter

^b Mean (and range) of samples of different varieties.

weight of the rat uterus was, however, clearly positive (Table IV).

There are only a few observations on the estrogenic disorders caused by white clover in domestic animals (17). As in alfalfa, the most active phytoestrogen in white clover is coumestrol, which may increase considerably as a result of, or be solely attributable to, fungal diseases (18, 19).

According to the present HPLC study, the phytoestrogen concentrations of all white clover varieties were small at all Finnish research stations. The samples contained only 0.02%-0.06% estrogenic isoflavones, whereas red clover may contain more than 2%. Also coumestrol was found in small quantities and inconsistently.

A biological study of the efficacy of the estrogenic effect of white clover varieties was clearly positive, but inconsistent with the results of the chemical study. It is obvious that a small quantity of isoflavones does not explain the increased weight of the uterus, since formononetin is almost inactive in rats. The slightly elevated coumestrol content in the late autumn samples of varieties, Tammisto and Undrom, could have been due to the colder weather in the autumn and may to some extent have affected the results of the biological studies. The cold autumn weather and especially the night frosts may increase coumestrol concentrations considerably, as they do phytoestrogen concentrations in general (3). Small and inconsistent coumestrol concentrations do not, however, explain the obvious discrepancy between the results of the biological and chemical studies. In fact, there were apparently other substances increasing the estrogenic potency which were shown in the biological studies but not in

the chemical studies. These might be substances of the coumarin group, which might explain the inconsistent results of alfalfa and especially of white clover. Their contribution should be studied further in the future.

Phytoestrogen Content and Estrogenic Effects of Alfalfa (*Medicago sativa*). The estrogenic efficacy of alfalfa was studied in the early days of phytoestrogen research in Finland. The criteria then were the changes in the weight of the uterus and vaginal smear of ovariectomized rats (2). Of the varieties then studied, Rhizoma had an obvious effect, Normad a smaller effect.

According to the present HPLC study, the estrogenic effect of alfalfa is most obviously attributable to coumestrol. The samples contained very small quantities of estrogenic isoflavones (formononetin and biochanin-A), whereas the coumestrol content was remarkably high in the spring and especially in the autumn (Table V). According to the present biological study, the estrogenic effect of alfalfa was also quite obvious. However, no seasonal changes in phytoestrogen content or biological activity were seen in alfalfa, unlike in red clover where there are high estrogen concentrations in early spring, a clear decrease by mid-summer, and an increase again in aftermath (8).

Coumestrol is known to be about 30 times more effective than genistein in mice (11), and to cause estrogen-related disorders in animals (20). In addition, coumestrol seems to have a cumulative effect. Consequently, even quite small amounts (25 ppm) in the fodder may have an adverse effect and reduce the fertility of sheep (21). Whitten *et al.* (22) have found that coumestrol induces uterine growth in rats over a 90-hr period at a dietary concentration of 0.01%-0.1%. Lower doses not active over this period were active when provided over a longer period. A coumestrol concentration as low as 0.005% induced uterine enlargement if the diet was provided for over 180 hr.

Table V. Phytoestrogen Content of Alfalfa (variety Jokioinen) and the Effects on the Weight of Immature Rat Uterus (Sata-Hame Research Station) (9)

Growth stage	Coumestrol (ppm in DM ^a)	Increase in uterine weight (mg)
Bud stage ^b	33.7	21.2
Early blossom	64.8	57.9
First aftermath	25.3	19.3
Second aftermath	63.0 ^c	46.2

Note. Biological study. Duration of experiment, 5 days; experimental extract, 3 g dry matter/day; rats/test group, 5 rats; rats/control group, 8 rats, uterine weight 21.0 ± 2.0 mg.

^a DM = dry matter.

^b Small amount of formononetin and signs of biochanin-A in samples of bud stage.

^c HPLC result of ether extract.

Coumestrol has been observed to accumulate in alfalfa following insect or fungal attack (23). According to some studies, the coumestrol concentration of alfalfa is a consequence of fungal diseases (21). Healthy plants contain only very small quantities of coumestrol (19). There are, however, differences between varieties (19, 24). In the samples investigated here, there were no indications of fungal or other diseases.

Phytoestrogen Content and Estrogenic Effects of Goat's Rue (*Galega orientalis*). Of the plants investigated, goat's rue did not contain any known phytoestrogens. Only traces of isoflavones (Karelia Research Station) and coumestrol (Sata-Hame Research Station) were observed in the plant samples of early spring and late autumn. The biological study of the effect of phytoestrogens on the weight of the rat uterus was completely negative. As far as we know, there are no studies or information in the literature concerning the estrogenic properties of goat's rue (9).

Discussion

Red clover has the highest phytoestrogen content of Finnish legume fodder plants, varying from 1% to 2.5% of dry matter. Formononetin and biochanin-A concentrations are highest, 0.3%–1.5% of dry matter. The amount of genistein is at the level 0.03%–0.15%, but the daidzein concentration is less than 0.03% of dry matter. The biological effect on the uterus of immature rats is large.

All white clover varieties contain very small quantities of estrogenic isoflavonoids (0.01%–0.06% of dry matter) and coumestrol (less than 10 ppm). However, in biological studies white clover still caused an increase in uterine weight which was about half of that of red clover. Until now, we have not found the chemical substance causing this biological effect.

Alfalfa contains 25–65 ppm coumestrol and has as large an effect as red clover on uterine weight of immature rats.

Goat's rue does not contain any known phytoestrogens. Even in biological studies, it has no estrogenic effect.

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Clinical Changes in Ovariectomized Ewes Exposed to Phytoestrogens and 17 β -Estradiol Implants (43838)

AGNES I. NWANNENNA,^{*1} T. J-O. LUNDH,[†] A. MADEJ,^{‡2} G. FREDRIKSSON,^{*3} AND G. BJÖRNHAG[§]
Departments of Obstetrics and Gynaecology, Animal Nutrition and Management,† Physiology,‡ and Animal Physiology,§ Swedish University of Agricultural Sciences S-75007 Uppsala, Sweden*

Abstract. Eight Swedish Finewool Landrace ewes, ovariectomized 5 months earlier and kept on nonestrogenic hay, were each fed 3.5 kg red clover silage, corresponding to 6.1 g phytoestrogens (of which 3.5 g was formononetin) per day, for 14 days in November (short days). In January (short days), two groups (3 each) of these ewes received one or two 17 β -estradiol ac implants. In May (long days), one of two new groups (4 each) of these ewes was reexposed to phytoestrogens for another 14 days while the other served as a control. Physical examination of ewes for changes in reproductive organs was carried out two or three times per week during each feeding/treatment, and continued until observed changes disappeared. Clinically significant changes occurred in the reproductive organs of ewes fed red clover. Vulva color changed from pale to pink and red, and there were enlargements of the vulva, uterus, and udder. In addition, teat length and circumference increased, and secretion of milky fluid began. These changes were similar, but more pronounced during treatment with 17 β -estradiol, particularly teat circumference. The changes in vulva were more dramatic in May than in November and resembled those observed in ewes treated with estradiol. Our data show that a daily intake of 3.5 g formononetin for 14 days caused the increase of teat size and changes in the color of the vulva and in uterus weight in ovariectomized ewes. [P S E B M 1995, Vol 208]

The presence of estrogenic substances in plants was first demonstrated in the 1920s (1, 2). Later, such substances were reported to cause reproductive disorders in sheep grazing subterranean clover in Australia (3). Genistein and other isoflavones, daidzein, biochanin-A, and formononetin were isolated from clover and proved to be the cause of the disorders (1). The estrogenic activities of forage plants have been reported from various parts of the world (4, 5, 6, 7). It is known that the relative activity of individual isoflavones may vary depending on the strain as well as the species of animals (8) owing to inter- (2) and

intraspecific variation in the metabolism of isoflavones (9). Formononetin is the most important isoflavone causing reproductive disorders in sheep (10) through its main stable metabolite, equol.

This paper presents the clinical responses of ovariectomized Swedish Finewool Landrace ewes exposed either to red clover silage or to 17 β -estradiol.

Materials and Methods

Experimental Animals. Eight Swedish Finewool Landrace ewes, all of which had lambed in January, were ovariectomized in May through a mid-ventral laparotomy under general anaesthesia. The ewes were kept on pasture until September. They were then moved indoors and kept in individual, adjacent boxes under natural light conditions until the end of the experiment. The ewes were given *ad libitum* access to nonestrogenic hay (mainly Timothy grass) and 100 g of concentrate (39% barley, 39% oats, 11% soya, 7% rapeseed, and 4% other additions) per day except during experimental feeding periods. They had free access to mineral licks and good drinking water at all times.

Phytoestrogens. Red clover (*Trifolium pratense*)

¹ Permanent address: Ahmadu Bello University, Faculty of Veterinary Medicine, Department of Surgery and Medicine, Zaria, Nigeria

² To whom requests for reprints should be addressed at Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, Department of Physiology, P.O. Box 7045, S-750 07 Uppsala, Sweden

³ Deceased.

silage was used as the source of plant estrogens. High performance liquid chromatography was used to determine the estrogen content of the feed samples (11).

17 β -Estradiol Implants. Silastic tubes (6 cm long, 0.335 cm i.d., 0.465 cm o.d.; Dow Corning Medical, Midland, MI) were filled with 17 β -estradiol crystals (Sigma Chemical Co., St. Louis, MO) and sealed at both ends with silastic Medical adhesive type A (Dow Corning, Midland, MI). It has previously been reported that the release rate of 17 β -estradiol from this size implants is 45 μ g/day (12). Prior to use, implants were incubated in 100 ml normal saline for 24 hr at room temperature, under constant agitation to avoid a transient peak in plasma estradiol after insertion. Implants were introduced subcutaneously in the axillary region of ewes while anaesthetized by xylazine (Rompun[®] vet.; Bayer Sverige AB, Göteborg, Sweden) and removed after 14 days.

Experimental Protocol. At the end of October, all ewes (mean body weight of 64 kg) were pretreated for 6 days with feed consisting of incremental increases (20%, 30%, 50%, 50%, 75%, 75%) of red clover silage, to allow the rumen microbial populations to adapt. After the final pretreatment, 24 hr later (Day 0), the ewes were fed 3.5 kg of 100% red clover silage for 14 days (about 8 hr of daylight). Test samples were taken, the ewes were accustomed to a nonestrogenic diet by gradually reducing the red clover silage content over a 6-day period.

On the 7-8 of January (6.3 hr of daylight), six of the eight ewes, in which all clinical signs of red clover effects had disappeared, were randomly assigned to two groups (3 each) which received one or two implants (a daily dose of 45 or 90 μ g 17 β -estradiol). The implants were removed after 14 days.

Five months after the initial treatment, the ewes were randomly redistributed to another two groups (4 each). Group I (mean body weight of 74 kg) was reexposed to red clover as previously described. Group II (mean body weight of 75 kg) was kept on hay and served as controls. All ewes were terminated at the end of this experiment and reproductive organs were collected, weighed, and examined for gross changes. In addition, blood samples were collected for subsequent progesterone and 17 β -estradiol analysis. At the time of termination these animals were exposed to approximately 16 hr daylight.

Clinical Examinations. All ewes were clinically examined throughout the treatment to assess the physical condition of the reproductive organs. These examinations were carried out two or three times per week during the control periods, each exposure to red clover and 17 β -estradiol. Udder growth and milky fluid secretion were noted, and the teat lengths and circumferences were measured. Averages for left and right teat measurements were recorded. Teat length

was measured as previously described (13), except that permanent markers were used instead of tattoos. To measure teat circumference, we used thin, long nylon strings calibrated (mm) in the middle along a distance of 10 cm. The string was circled round the base of the teat, and the circumference was recorded. Contact between teat and string was ascertained without pressure on teat or space between teat and string.

The color of the vulva as well as signs of mucous discharge and edema were assessed. Arbitrary scores were assigned to changes in each parameter, ranging from 1 (no change) to 5 (severe change). The sum of the scores for changes in the three parameters was recorded for each period of vulva examination.

The uterus was examined by rectal ultrasonography using the Aloka scanner, model SSD-210DXII (Aloka Co. Ltd., Tokyo, Japan) with a real-time B-mode 2-dimensional scanner. It is equipped with a linear array 5 MHz transducer which is suitable for rectal use in nonpregnant, small ruminants (14). Measurements of uterine diameter were made with integral electronic callipers used for measuring linear distances. Because of errors in defining the endometrial limits we decided to remeasure the distances and standardize them on the ultrasonographs. The measurements from the pictures were from perimetrium (outer wall of uterine horn) to perimetrium.

Hormone Assays. Selected samples from each ewe were assayed for progesterone and 17 β -estradiol to confirm the absence of ovarian activity.

Samples were assayed for progesterone by luminescence immunoassay (Amerlite; Kodak Clinical Diagnostics Ltd., Amersham, England) previously validated for canine plasma (15). Serial dilutions of ovine plasma containing high concentrations of progesterone produced curves parallel to the standard curve. The sensitivity of the assay system was <1 nmol/l. Intra-assay variation for progesterone was 13.5% and 6.0% for low and high assay controls, respectively. The corresponding interassay variation was 10.0% and 8.4%.

17 β -Estradiol was analyzed (16) using standards supplied with the Coat-A-Count radioimmunoassay kit (Diagnostic Products Cooperation, Los Angeles, CA). Serial dilutions of ovine plasma containing high concentrations of 17 β -estradiol produced displacement curves parallel to the standard curve. The detection limit of the assay was 5 pmol/l. The intra-assay variation calculated from the precision profiles of two assays was <17% for concentrations between 5.6 and 180 pmol/l. The interassay coefficient of variation for corresponding control samples was 7.6% (8.6 pmol/l) and 10.2% (198 pmol/l), respectively.

Statistical Analysis. Changes in teat length, teat circumference, and uterine diameter were expressed in percent of individual pretreatment values (i.e., 10 days before the beginning of red clover feeding).

Changes for all these parameters and vulva scores were computed and analyzed for treatment effects using the analysis of variance and the Duncan multiple range test from Statgraphics (STSC Inc., Rockville, MD). Probabilities <0.05 were considered statistically different.

Results

Feed Analysis. A homogeneous sample from the red clover silage, consisting of 24.6% dry matter, was analyzed to determine its nutritional quality and content of phytoestrogens. The red clover silage contained a total of 1.74 g estrogens (1.01 g formononetin, 0.03 g daidzein, 0.61 g biochanin-A, and 0.08 g genistein) per kg wet weight. Each ewe was fed 3.5 kg silage, corresponding to a daily intake of 6.1 g phytoestrogens (of which 3.5 g is formononetin). No estrogens were detected in samples of the hay or concentrates.

Clinical Findings. During the experiment in November, the teat length increased significantly on Day 4 in 100% of red clover fed ewes. The increase remained significant, and maximum length was reached on Day 15. Teat length was back to control levels on Day 39 (i.e., 25 days after withdrawal of the red clover) (Fig. 1). Teat circumference increased significantly on Day 1, peaked before withdrawal on Day 13 and dropped to control levels on Day 39 (i.e., 25 days after withdrawal of the red clover) (Fig. 2). Milky fluid secretion was observed in six of eight ewes. Two ewes began secretion on Day 5 and 10, respectively, and stopped on Day 25. The third showed secretion for only 9 days (from Day 19 to 28), and the fourth for 51 days (from Day 12 to 63). In the last two, starting on Day 7 and 13, respectively, milky fluid secretion lasted a few months. The amount of milky fluid varied from a few drops to a few milliliters per animal. In appear-

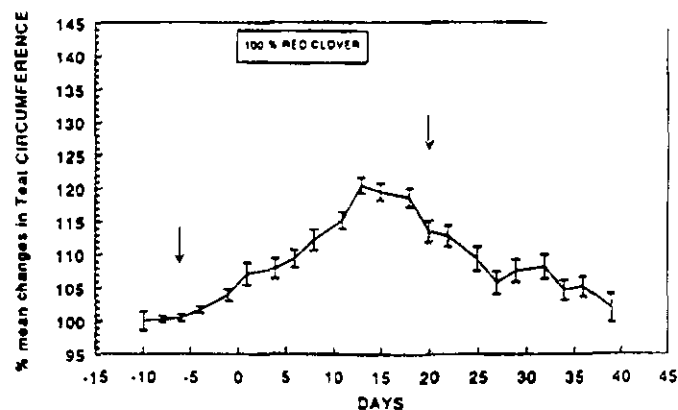


Figure 2. Mean (\pm SEM) changes (in percentage of initial values) in teat circumference ($100\% \pm 3.9\%$ is equal to 41.3 ± 1.6 mm) in ovariectomized ewes exposed to phytoestrogens in November. The timing of feeding with only red clover silage is shown by the box entitled "100% RED CLOVER." Arrows point to first and last day of feeding with red clover silage in addition to hay.

ance, the secretion was initially clear, serous, and colored, but later resembled the milk. Mammary glands were noticeably more voluminous, and palpation revealed the presence of irregular, solid lumps of various sizes towards the end of the feeding period and long afterwards.

The vulva gradually changed from pale to pink, and then to red with increasing edema. Vulva mucous discharges were negligible and brief in two ewes. On Day 4 in the ovariectomized ewes eating red clover diet, the vulva scores were significantly different from that seen before the initial treatment. Maximum changes were observed between Day 15 and 18, and the scores had dropped to control level on Day 36 (i.e., 22 days after withdrawal) (Fig. 3). The preliminary measurements made directly from the ultrasound images indicated that uterine diameter increased with exposure to phytoestrogens, and the standardized measurements from the photographs confirmed this trend

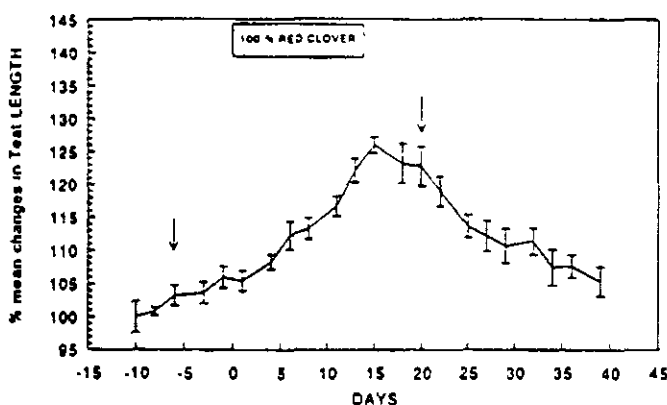


Figure 1. Mean (\pm SEM) changes (in percentage of initial values) in teat length ($100\% \pm 6.8\%$ is equal to 22.0 ± 1.5 mm) in ovariectomized ewes exposed to phytoestrogens in November. The timing of feeding with only red clover silage is shown by the box entitled "100% RED CLOVER." Arrows point to first and last day of feeding with red clover silage in addition to hay.

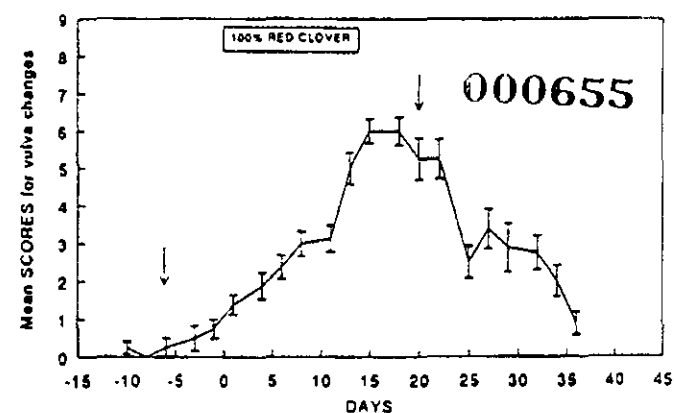


Figure 3. Mean (\pm SEM) vulva scores in ovariectomized ewes exposed to phytoestrogens in November. The timing of feeding with only red clover silage is shown by the box entitled "100% RED CLOVER." Arrows point to first and last day of feeding with red clover silage in addition to hay.

(data not shown). Uterine diameters tended to increase, with the highest value observed on Day 29 (i.e., 15 days after withdrawal of silage ($124 \pm 6\%$)) and subsequently decreased.

Plasma concentrations (mean \pm SEM) of 17β -estradiol during the hay and red clover periods were 7.1 ± 0.7 pmol/l and 8.8 ± 0.9 pmol/l, respectively, and were not significantly different. Plasma concentrations for progesterone were below the detection limits (1 nmol/l) of the assay.

Abrupt changes occurred in all parameters of the reproductive organs observed in ewes treated with 17β -estradiol. There were already significant changes in teat length on Day 4 after insertion of one implant (Fig. 4). Two 17β -estradiol implants resulted in an increase of teat length on Day 12. The teats decreased in size to pretreatment values by Day 26 and 19 in ewes with one and two implants, respectively. In regards to teat circumference, the effects of 17β -estradiol were seen until Day 30–31 (i.e., 16–17 days after withdrawal of either one or two implants) (Fig. 5). Two 17β -estradiol implants resulted in a more abrupt and higher increase in teat circumference (Fig. 5) than that during exposure to phytoestrogens (Fig. 2). A milky secretion occurred in five of six ewes. Four ewes began to lactate on Day 9, and the fifth began on Day 14. The secretion lasted between 36 and 74 days.

In less than 24 hr, the vulva color changed abruptly from pale pink to red, with obvious edema. Only one ewe showed a mucoid vulva discharge, which lasted throughout the period with implants. The sum of the vulva scores was already significantly increased on Day 1 in ewes with one implant (Fig. 6). Thereafter, the scores remained significantly higher than those obtained from Day 0 until Day 20 (one implant) and Day 25 (two implants). Uterine diameters measured by means of ultrasonography tended to increase after insertion of the implants and tended to fluctuate around the same level as was seen during red clover feeding.

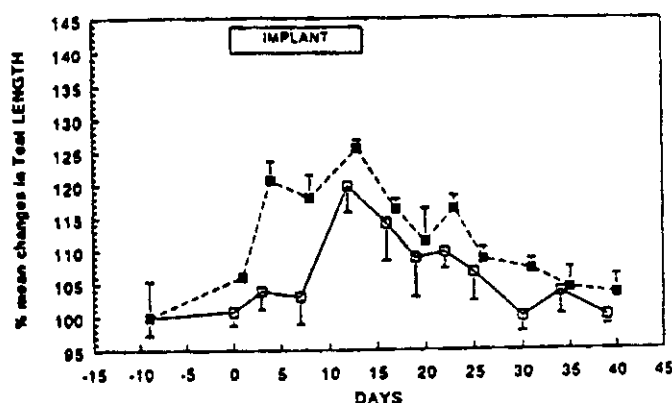


Figure 4. Mean (\pm SEM) changes (in percentage of initial values) in teat length in ovariectomized ewes treated with 17β -estradiol. Duration of treatment with one (—) or two (---) implants is shown by the box entitled "IMPLANT."

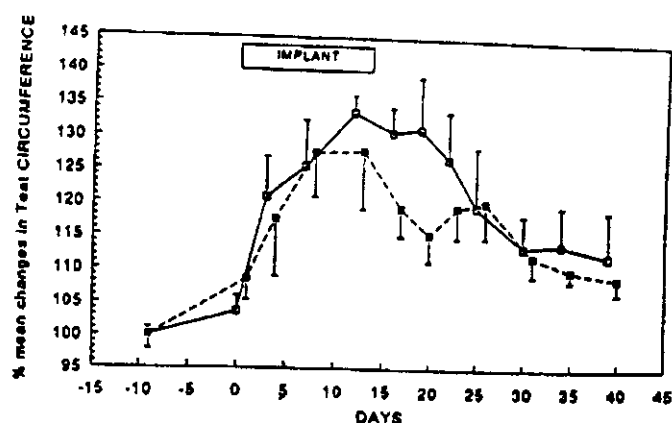


Figure 5. Mean (\pm SEM) changes (in percentage of initial values) in teat circumference in ovariectomized ewes treated with 17β -estradiol. Duration of treatment with one (—) or two (---) implants is shown by the box entitled "IMPLANT."

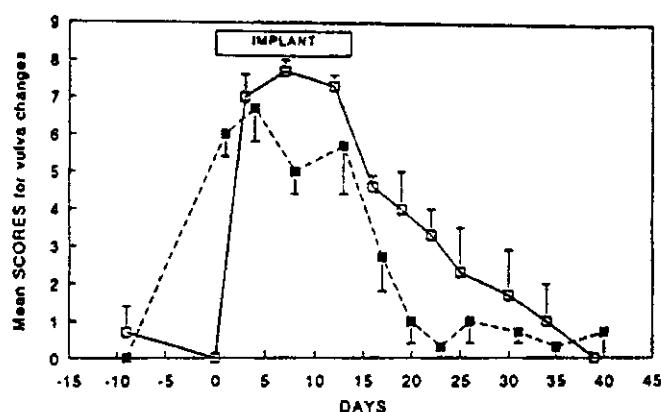


Figure 6. Mean (\pm SEM) vulva scores in ovariectomized ewes treated with 17β -estradiol. Duration of treatment with one (—) or two (---) implants is shown by the box entitled "IMPLANT."

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The plasma concentrations (means \pm SEM in pmol/l) of 17β -estradiol on Day 5–6, 7–8, and 12–13 after insertion of one or two implants were 43 ± 6 , 36 ± 5 , 32 ± 3 or 126 ± 40 , 97 ± 38 , 75 ± 23 , respectively.

During the experiment in May, the percentage increase in teat length and circumference on Day 11 was significantly higher in ewes exposed to red clover than that in control animals ($114\% \pm 4\%$ vs $102\% \pm 2\%$ and $113\% \pm 3\%$ vs $101\% \pm 1\%$, respectively). On the same day, the sum of vulva scores significantly differed between experimental and control animals (7.0 ± 1.2 vs 0.3 ± 0.1). In general, there was no difference in responses to red clover exposure in November and May except for the sum of vulva scores. The changes in vulva were more dramatic in May, and on Day 1 and 11 the scores were 3.8 ± 0.6 and 7.0 ± 1.3 , respectively, the values in both cases being significantly higher than those in November, as depicted in Figure 3. After slaughter in May, on Day 14, a significant difference was observed in the uterine weights between ewes fed silage and hay (65.3 ± 9.7 g vs 40.4 ± 2.8 g). Regarding the udder, only two of the four ex-

perimental animals showed an increase in weight (i.e., approximately 40% heavier udder than that in control ewes.

Discussion

This paper reports on the influence of red clover silage on the clinical status of reproductive organs in ovariectomized Swedish Finewool Landrace ewes.

The changes in teat length are in accordance with those previously reported in both ovariectomized ewes (17) and castrated rams (18) fed estrogenic clover. It was reported (19) that there was reasonable correlation between the mean increase in teat length at Day 14-17 and the mean uterine weight. The teat length method is simple and relatively sensitive, but the range of stilbestrol doses for which a linear log dose-response relationship was found to be narrow. The relationship between uterine weight responses and temporary infertility appears to be slightly better than that between teat length and infertility (20). Teat length responsiveness in wethers seemed to have been renewed by 35 days after their removal from estrogenically active clover pasture. In the present study, both teat length and circumference reached the pretreatment levels after 25 days from withdrawal of the silage. Mammary gland development and milky fluid secretion, which occurred in our ewes, have been reported in previous studies on sheep exposed to phytoestrogens (18, 21). However, the lobule-alveolar epithelial tissue pattern that normally develops prior to physiological lactation (22) is not established in lactating mammary glands from ewes grazing estrogenic pasture (18). In this experiment, the irregular solid lumps of tissue found during clinical examinations looked like developing tumors. Genistein, but not formononetin (23), and the estrogenic metabolite of formononetin called equol (24), can stimulate the growth of estrogen-dependent breast cancer cells in vitro. The estrogenic activity of biochanin-A and genistein in ruminants is limited to the first few days of exposure when the unadapted rumen microbes (2) cannot convert them to their nonestrogenic metabolites p-ethylphenol and phenolic acid (25). On the other hand, formononetin and daidzein in the rumen are mainly metabolized to equol (25), which is known to be estrogenic and relatively stable (8). The red clover used in this experiment contained relatively high concentrations of formononetin, which is thus considered to be the cause of the overall clinical changes observed in this experiment.

It was interesting to note the more dramatic increase of vulva scores in May than in November. However, the significance of that finding should be elucidated by using intact ewes. Swelling of the vulva was also reported earlier for ewes fed red clover (26), and it is a typical symptom of estrus in the ewe (27).

The increased blood flow that accompanies hyperplastic and hypertrophic enlargement of the reproductive organs exposed to estrogens (2) is assumed to be responsible for the color changes observed in the vulva when ewes were fed red clover and treated with 17 β -estradiol. It was reported (28) that ovariectomized ewes injected with estradiol benzoate for 12 days displayed estrus. One 17 β -estradiol implant used in the present study created average concentrations similar to those previously reported (29, 30). These concentrations resemble the values of 17 β -estradiol in ewes during the follicular phase of the estrous cycle (31).

In the present study, we attempted to use rectal ultrasonography to monitor changes in the uterus. The uterus became enlarged in ewes fed red clover and in ewes treated with 17 β -estradiol, but decreased in size again once the red clover silage or estradiol implants were withdrawn. Unfortunately, we could not assess these uterine changes statistically owing to some uncertainties in the measurements. However, we assume that the validation of ultrasonography on several more animals may permit the studying of estrogenic effects in ewes by this noninvasive technique. The increase of uterus weight during feeding with red clover silage found here is in agreement with the previous studies (18, 19, 20). Additionally, it was reported (32) that 17 β -estradiol treatment in ovariectomized ewes significantly increased uterine fresh and dry weights.

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Hormone Studies on Ewes Grazing an Oestrogenic (Yarloop Clover) Pasture During the Reproductive Cycle

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J. M. Obst^a and R. F. Seamark

Department of Animal Physiology and Department of Obstetrics and Gynaecology,
University of Adelaide, Adelaide, S.A. 5001.

^a Present address: Department of Agriculture, Straus Research Centre, Naracoorte, S.A. 5271.

Abstract

The endocrine function of Merino and Corriedale ewes grazing an oestrogenic (Yarloop clover) pasture has been studied during the oestrous cycle, pregnancy and parturition, and the results compared with those from a study of similar ewes grazing a neighbouring grass pasture. Plasma progesterone, oestrogen and corticoids were measured using competitive protein binding assay procedures.

During the oestrous cycle clearly anomalous patterns in hormone content were evident in ewes grazing Yarloop, and this related to their significantly poorer ($P < 0.001$) fertility.

The first mating, when ewes were 1½ years of age, was particularly affected. Successful conception took place in only 27% of ewes mated on Yarloop, compared with 95% on grass. Evidence of disturbance in the normal patterns of both plasma oestrogen and progesterone was found in infertile ewes, including a shortened period of luteal function.

Disturbance of endocrine function caused by Yarloop clover ingestion was also found in pregnant ewes, with the mean plasma progesterone concentrations during the latter half of pregnancy reduced ($P < 0.05$) and the plasma oestrogen and corticoid levels tending to be higher in these animals.

In detailed hormone studies in the periparturient period, both groups showed a similar fall in plasma progesterone and rise in plasma oestrogen prior to parturition. Where excessive time was taken for parturition (more than 30 min) this was reflected in higher plasma corticoid levels ($P < 0.05$) within 8 h of birth.

Introduction

Reproductive difficulties in sheep grazing subterranean clover pastures were first described by Bennetts (1944) and Bennetts *et al.* (1946). Characteristic signs were ewe infertility, dystocia and uterine prolapse resulting in high ewe and lamb mortality. This syndrome of aberrant reproductive functions on clover pastures is now commonly referred to as 'clover disease' and its occurrence has been widely recognized in association with many different clover species throughout the world (Moule 1961; Moule *et al.* 1963; Bickoff 1968).

The results of extensive studies have revealed that the aetiology relates to oestrogenic isoflavones present in the plants (Batterham *et al.* 1965). However, the exact way in which these substances exert their adverse effects on reproductive function is as yet unresolved. In the present study, an investigation has been carried out to examine the possibility that the clover acts by causing disturbances in the endocrinology of reproduction.

Preliminary investigations (Obst *et al.* 1971a) suggested that good indices of reproductive endocrine function for grazing flock sheep can be obtained by using relatively simple radio-ligand assays for plasma hormones. Comparison of these indices throughout the reproductive cycle was, therefore, made between a group of

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sheep grazing an oestrogenic pasture (*Trifolium subterranean* cv. Yarloop) and a non-oestrogenic grass pasture.

Preliminary reports on this study have been published previously (Obst and Seamark 1970, 1972; Obst *et al.* 1971b; Obst 1972).

Materials and Methods

Merino ewes and wethers (Koonoona strain) were obtained from flocks of the Agricultural Research Station at Wanbi, S.A., and were born and reared on non-clover pastures. Corriedale ewes and wethers were obtained from flocks at the Kangaroo Island Research Centre (KIRC), maintained on mixed 50/50 pastures of perennial rye grass and clover (cv. Mt. Barker, Yarloop and Woogenellup). The Merino rams (Bungaree strain) and Corriedale rams were also from KIRC flocks.

The Yarloop clover pasture of 17.2 ha (area A) was cleared and sown with Yarloop in 1959. Visual estimations of species composition of the pasture during the period of study (1969-1971) indicated that more than 70% of available feedstuff was Yarloop, with annual grasses and weeds constituting the remainder of the pasture. The grass pasture of 35 ha (area B) had a history similar to the Yarloop pasture until 1967 when a renovation program successfully established a pasture dominant in perennial rye grass. Contamination with Yarloop regrowth after renovation was less than 5% of the total pasture in 1969, but this increased to about 15 and 20% in 1970 and 1971 respectively.

Procedure

The investigation was initiated in February 1969 when ewes were 3½ years of age. Two groups of animals were formed each of 50 Merino and 50 Corriedale ewes and 40 wethers. One group was then maintained on the Yarloop clover pasture and the other on grass pasture throughout the period of the experiment. Ewes and wethers were weighed monthly. To maintain approximately equivalent liveweights in the two groups during and between years the stocking rates were adjusted with additional KIRC wethers. From January to April 1970, when dry Yarloop residues were limited, the Yarloop group was supplemented with Yarloop clover hay.

Study I. Two fertile rams were introduced into the flock grazing Yarloop in February 1969 and the first six Merino and six Corriedale ewes mated were used for blood sampling and hormone measurement. During this period the pasture consisted mainly of green germinating Yarloop clover.

The ewes on the grass pasture were first joined with a vasectomized ram and the first six ewes mated were kept separately with the teaser ram for blood sampling and hormone determination. The remaining ewes in each group were mated to four fertile rams. Venous blood samples of 10 ml were collected into heparinized containers every second day commencing on the day of mating (day 0) or the first day after mating (day 1). Blood sampling continued for a period of 42 days.

Study II. Four vasectomized rams fitted with Sireline harnesses and crayons were introduced on 2 October 1969 to the ewes which had grazed the Yarloop pasture since its germination in February 1969. The identity of the animals mated was recorded daily. Of the ewes which did not produce a lamb in study I, the first 20 to mate (13 Merinos and 7 Corriedales) were confined to a smaller area (C) of green Yarloop clover within the same area (A) to aid the collection of blood samples.

Blood samples of 20 ml for hormone determination were collected daily at 0900 h from the 20 ewes commencing on 20 October until 21 November 1969. Plasma progesterone determinations were made on all samples collected, but plasma oestrogen concentrations were determined only on samples selected from ewes which exhibited specific patterns in the function of their corpora lutea. Corpus luteum function was classified on the basis of peripheral plasma progesterone concentrations as follows.

- (1) Shortened—progesterone levels declined from day 12 to values less than 1.0 ng/ml on day 14 and remained below 1.0 ng/ml until oestrus.
- (2) Normal—progesterone levels were maintained above 1.0 ng/ml from day 12 to day 14 inclusive and then declined to below 1.0 ng/ml by day 19.
- (3) Prolonged—progesterone levels were maintained above 1.0 ng/ml from day 12 to beyond day 19.

Study III. Merino and Corriedale ewes on each pasture were mated separately for 6 weeks from mid-February to the end of March 1970 with two rams of the same breed per group. Ewes were

then 2½ years old. Twenty ewes (5 Merino and 5 Corriedale from each pasture group) which did not return to service during the 6-week mating period were selected for hormone measurement.

To facilitate blood collection, the selected ewes were confined to smaller areas on the same pastures and jugular vein blood samples of 20 ml were taken daily at 0800 h from day 40 of gestation to 2 days post-partum. Additional blood samples were taken at 1600 and 2400 h from day 137 of gestation onwards to follow periparturient changes in more detail.

Lambing Observations

Lambing ewes (10) on the Yarloop pasture whose blood was being sampled were continuously observed with the aid of spotlights and binoculars to allow the recording of time of birth, duration of parturition and signs of dystocia. Duration of parturition (in minutes) was defined as the time from first appearance of the lamb's front feet at the vulva to complete delivery of the lamb. The 10 lambing ewes on grass pasture were observed every 30 min, and time of birth, incidence of dystocia and approximate length of parturition were recorded.

General lambing observations on all other ewes were performed daily at approximately 0800 h when each lamb born was identified with its mother, ear-tagged and weighed. The incidence of dystocia was recorded and a post-mortem examination made on ewes which died near term, or were unable to lamb even with manual assistance, to ascertain the number of dead lambs *in utero*.

Hormone Assay Procedures

(i) *Progesterone assay.* The competitive protein binding (CPB) procedure used was essentially the same as that described by Thorburn *et al.* (1969), employing light petroleum as the plasma extractant and dog or hen plasma as a source of corticosteroid binding globulin with [³H]₂cortisol as the competing tracer. Free and bound steroids were separated on small Sephadex G25 (fine) columns (Bassett and Hinks 1969).

Progesterone standards containing 0, 0.2, 0.4, 1.0, 2.0 and 4.0 ng added to 0.2 or 0.5 ml of oophorectomized ewe plasma were run with each assay and results are expressed as progesterone equivalents.

(ii) *Oestrogen assay.* An index of the level of plasma oestrogen was determined using a CPB method based on that described by Korenman (1968, 1969). Uteri of 6-day-pregnant rabbits were used as a source of binding protein and the cytosol was prepared as described by Korenman (1968).

For assay, plasma (2-4 ml) was extracted with 2 volumes of ethyl acetate and the residue, after removal of the solvent, was reacted with the uterine cytosol. The competing tracer was [³H]₂oestra-3,17β-diol. Competition for binding sites was allowed to occur for at least 6 h, usually overnight, at 4°C and the free and bound fractions separated by small Sephadex columns using 4 ml of 0.01M tris buffer (pH 8) as eluant.

Oestrogen standards containing 0, 20, 50, 100, 200 and 400 pg of oestra-3,17β-diol (E₂-17β) were run with each assay. As a check on procedure, samples of oophorectomized ewe plasma and water (2 or 4 ml) were also included as blanks.

(iii) *Cortisol assay.* Plasma corticoid concentrations were determined by the method of Bassett and Hinks (1969). Results expressed as cortisol equivalents (ng/ml) were corrected to allow for interference by progesterone present in the ethanol extract.

Assessment of Oestrogenicity of the Pastures

(i) *Isoflavone content.* Samples from Yarloop pasture taken in July-August 1970 were kindly analysed for the isoflavones, genistein, formononetin and biochanin A by Dr R. C. Roslter of the Division of Land Resources Management, CSIRO, Wembley, Perth, W. A.

(ii) *Bioassay.* At various times during 1969 and 1970, the size of the bulbo-urethral gland of wethers grazing the two pastures was assessed by digital palpation through the rectum, and the size and state of the glands were scored as follows to assess any accumulative effects of the oestrogenic activity of the pasture:

Score	Diameter (mm)	Description
1	5	Normal, soft palpable
2	5-15	Slightly enlarged, hard
3	15-25	Enlarged, hard
4	25-35	Very enlarged, hard
5	35	Grossly enlarged with formation of a <i>cul de sac</i>

(iii) *Analysis of results.* Comparison of reproductive performance between pastures were made using the χ^2 test (Snedecor and Cochran 1968) and the significance of differences in the mean plasma hormone concentrations assessed using Student's *t*-test (Snedecor and Cochran 1968). Attempts were also made to relate individual ewe hormone levels with lambing observations.

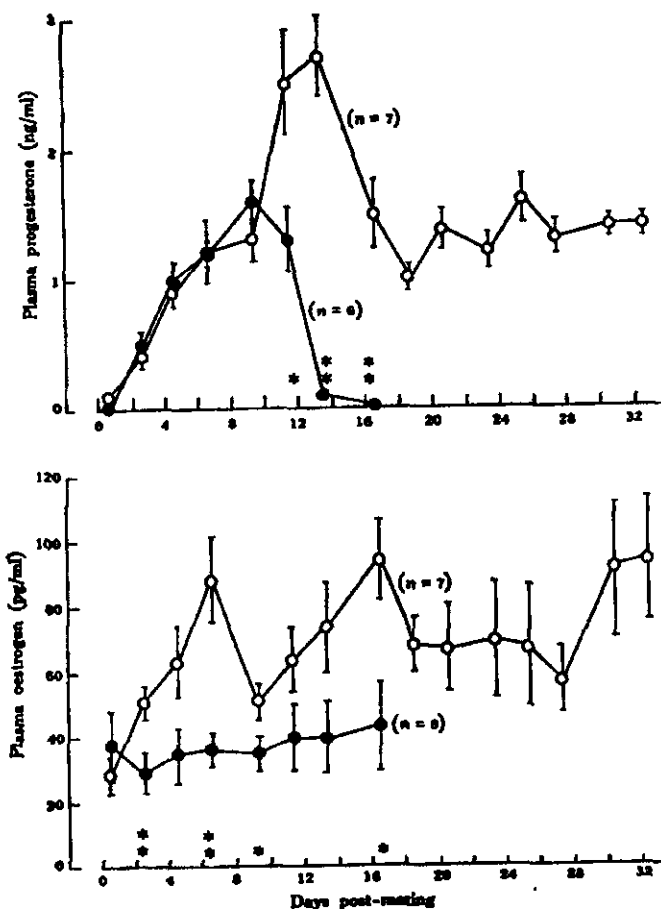


Fig. 1. (a) Plasma progesterone and (b) oestrogen following mating in ewes grazing green Yarloop clover (study I). Vertical bars represent \pm s.e.m. for fertile (○) and infertile (●) ewes. The numbers of ewes in each group are shown in brackets. Statistically significant differences are denoted thus: * $P < 0.05$, ** $P < 0.01$.

Plasma Oestrogenicity

The mean content of the isoflavones formononetin, genistein and biochanin A in Yarloop clover during July and August 1970 was 1.2, 2.5, and 0.54%, respectively. The mean dry leaf weight

The bulbo-urethral glands of the wethers were palpated at intervals of approximately 2 months from February 1969 to July 1970, and during this time 34 out of 40 wethers grazing Yarloop pasture and 13 out of 40 wethers grazing grass showed evidence of gland enlargement. On Yarloop, 24 of the 34 were given a score of 3 or more for at least two of the six palpations, whereas only 5 of the 13 on grass pastures received a score of 3 or more.

Fertility and Reproductive Performance

The overall fertility of ewes grazing Yarloop pastures (Table 1) was severely reduced compared with ewes on grass ($P < 0.001$). The largest difference in fertility between groups of animals on the different pastures occurred at the first mating in 1969. Following this mating only 14% of the Corriedales and 40% of the Merino ewes produced lambs while grazing green Yarloop pasture compared with over 90% of Corriedales and Merinos grazing the grass pasture. The percentages of Merino and Corriedale ewes which lambed 0, 1, 2 or 3 times during the 3 years whilst grazing on Yarloop were 22.2, 37.4, 28.3 and 12.1%, respectively, whereas the comparable values for ewes on the grass pastures were 3.1, 9.5, 34.7, and 52.6%.

Lamb survival was lower ($P < 0.01$) on the Yarloop pasture (55%) than on the grass pasture (71%). The differences between pastures in numbers of lambs born and in lamb and ewe mortality were most marked in the Corriedales. Uterine prolapse following parturition was observed only on the Yarloop pasture.

Endocrine Investigations: Assay Assessment

For the progesterone assay, estimates of the coefficient of variation at different points on the standard curve ranged from 2.6 to 6.5%. The mean recovery \pm s.e.m. of added amounts of progesterone (0.2–8.0 ng) was $90 \pm 2\%$ ($n = 12$) with no plasma and $86 \pm 2.5\%$ ($n = 12$) with oophorectomized ewe plasma.

For the oestrogen assay the coefficient of variation of determinations of various amounts of $E_2-17\beta$ over the useful range (20–400 pg) of the standard curve varied from 2.9 to 6.1%. Recovery estimates of non-labelled $E_2-17\beta$ (0–200 pg) added to ewe plasma were 93–96%.

Of the isoflavones tested, genistein in 10-ng amounts assayed as equivalent to 35 pg of $E_2-17\beta$. However, 200 ng or more of diadzein, biochanin A or formononetin was required to be present in samples before significant interference was recorded from these compounds. No equol was available during these investigations but the results of Shutt and Cox (1972) indicate that equol may react similarly to genistein under the conditions of assay employed.

Study I. All of the ewes on Yarloop selected for hormone assessment remated at least once during the 42-day period of study (17 matings total) and five lambs resulted, two to the second mating. The mean \pm s.e.m. plasma progesterone and oestrogen concentrations following these infertile and fertile matings of animals on Yarloop are presented in Fig. 1. Plasma progesterone concentrations were similar up to days 9–10 in both groups. At days 11–12, however, progesterone concentrations of the infertile group began to fall to reach oestrous levels at days 13–14, indicating a shortened period of corpus luteum (CL) function. Infertile matings were also characterized by lower plasma oestrogen levels than were found in fertile matings (Fig. 1).

Study II. Following 9 months of grazing on green Yarloop pastures, nearly all ewes which were infertile at their first mating and showed evidence of shortened periods of CL function had normal luteal phases. Of the 32 luteal phases studied, 24 were of normal length and were followed by oestrus, three had normal levels of plasma progesterone but there was no subsequent oestrus in the period of study, two luteal phases were shortened and the remaining three were prolonged. Patterns of plasma progesterone and oestrogen that are representative of each type of CL function are presented in Figs 2 and 3. Plasma progesterone concentrations (Fig. 4) on days 6-14 post-oestrus were significantly ($P < 0.05$) higher in ewes grazing Yarloop in October 1969 (study II), than in those grazing Yarloop or grass in February-March 1969 (study I).

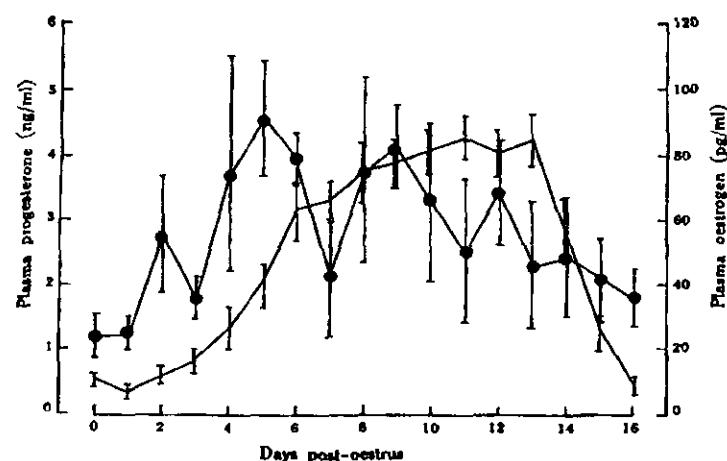


Fig. 2. Plasma progesterone (—) and oestrogen (●) in five ewes exhibiting normal CL function while grazing Yarloop clover pastures (study II). Vertical bars represent \pm S.E.M.

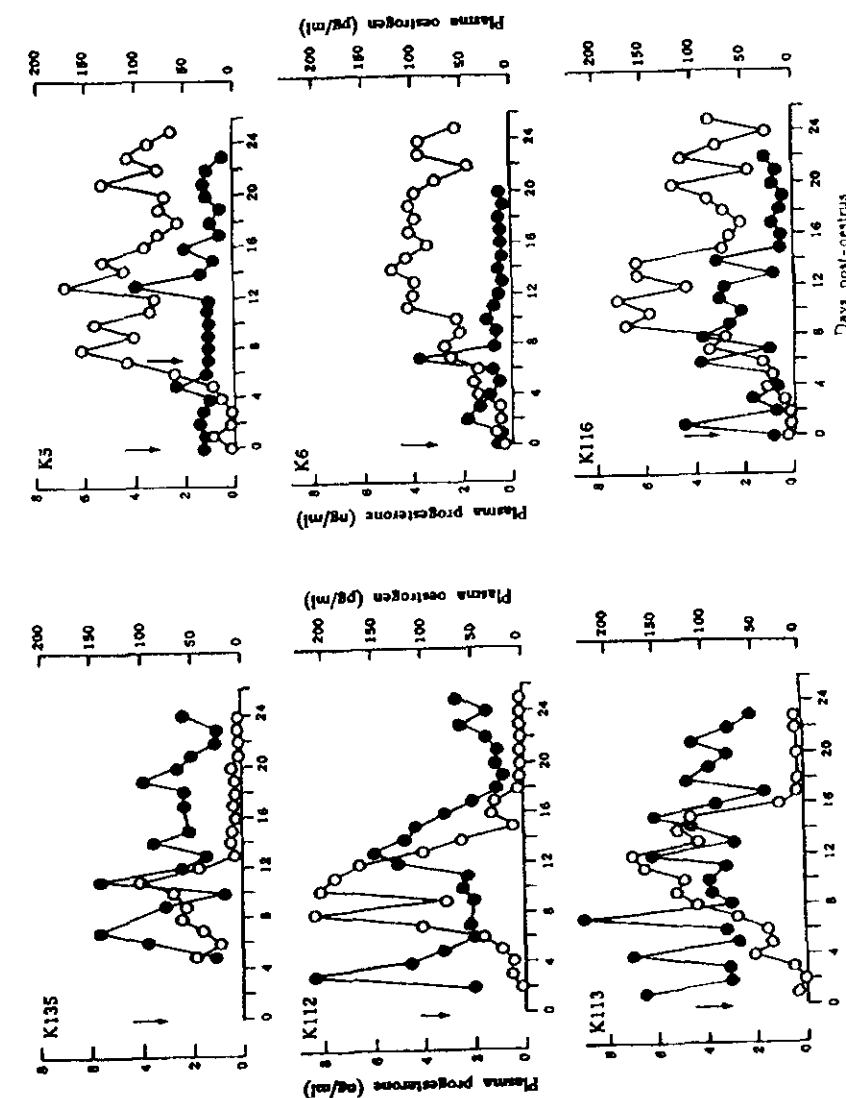
The higher percentage (84%) of normal luteal phases among the selected ewes in study I compared with study II is reflected in the improved fertility of the experimental flock during the mating in February and March 1970 (Table 1).

Study III. The mean plasma progesterone concentration of pregnant ewes grazing Yarloop was significantly ($P < 0.05$) lower than in those grazing grass pasture from 90 days gestation to term (Fig. 5a). Plasma corticoid concentrations for ewes on both pastures increased significantly ($P < 0.001$) from 40 days gestation to 100-120 days gestation (Fig. 5a). Thereafter, concentrations declined towards term but the mean values for ewes on Yarloop remained higher ($P < 0.05$) from 120 to 140 days gestation than the mean values for those on grass pasture.

Significantly ($P < 0.05$) higher concentrations of plasma oestrogen were observed from 110 to 120 days and at 140 and 145 days gestation (Fig. 5a) in the ewes grazing Yarloop pasture compared with the ewes grazing grass pasture throughout pregnancy. No differences, however, were apparent between the two groups in the characteristic rise in plasma oestrogen occurring within 24 h of birth (Fig. 5b).

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Fig. 3. Plasma progesterone (○) and oestrogen (●) in individual ewes exhibiting irregular oestrus or CL function or both while grazing Yarloop clover (study II). Arrows indicate the occurrence of oestrus



The mean (\pm S.E.M.) gestation lengths and birth weights of lambs were similar between the groups selected from Yarloop (151.4 ± 1.70 days, 4.15 ± 0.25 kg) and grass (150 ± 1.06 days, 4.05 ± 0.15 kg) pastures. However, four ewes on the Yarloop pasture experienced lambing difficulties compared with only one on grass. In these ewes the mean plasma corticoid concentration within 8 h of birth was higher ($P < 0.05$) than in the other ewes where parturition took less than 30 min. No relationship was apparent, however, between plasma progesterone or oestrogen concentration and duration of parturition.

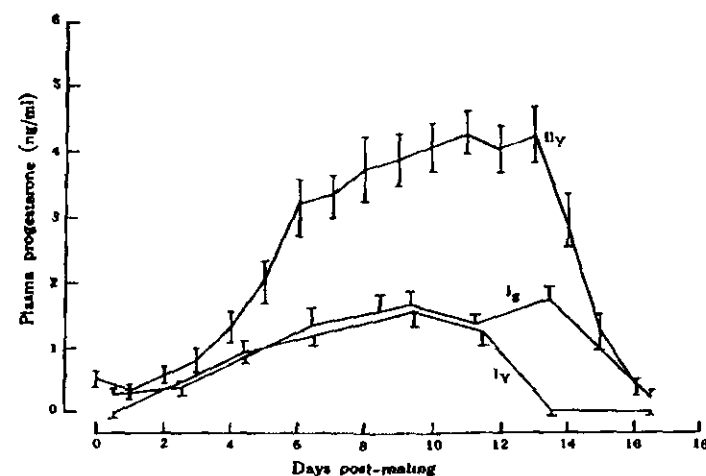


Fig. 4. Plasma progesterone in ewes grazing Yarloop clover in study I (I_Y) and study II (II_Y) compared with ewes grazing grass pasture in study I (I_G). Vertical bars represent \pm S.E.M. $n = 5$. Progesterone concentrations in study II were significantly higher ($P < 0.05$) from day 6 to day 14 post-mating.

Discussion

The study reveals that the poor reproductive performance of ewes grazing Yarloop is associated with marked disturbances in reproductive endocrine function. That hormonal disturbances may occur in ewes grazing oestrogenic pasture was suggested by the anomalies in both the incidence of oestrous and inter-oestrous intervals observed by previous workers (Underwood and Shier 1952; Turnbull *et al.* 1966; Fels and Neil 1968).

The cause of the particularly low fertility of ewes grazing Yarloop in 1969 remains unresolved. It appears to have been a temporary effect, as the majority of the ewes had improved fertility in study II despite continued grazing of the Yarloop in the intervening 9-month period. The improvement in fertility was accompanied by a higher incidence of normal periods of luteal function and, interestingly, higher plasma progesterone levels than found in study I. These changes probably relate to age of the ewe and seasonal factors, but it is possible, as suggested by Morely *et al.* (1969), that some ewes adapted to adverse effects of ingesting the large amounts of

oestrogenic isoflavones present in the pasture. Certainly, the pastures remained oestrogenic as indicated by the effects on the bulbo-urethral gland of the wethers.

Table 1. Reproductive performance of Merino and Corriedale ewes grazing Yarloop or grass pasture during the years 1969, 1970 and 1971

	Merinos			Total or mean	Corriedales			Total or mean	Merinos and Corriedales 1969-1971
	1969	1970	1971		1969	1970	1971		
Yarloop pasture									
Ewes mated	50	43	44	137	50	44	45	139	276
% Ewes fertile	40	67	59	55	14	50	44	38	46
Lambs born									
Singles	20	24	21	65	7	17	20	44	109
Multiples	0	10	10	20	0	10	8	18	38
Total (% of total ewes)	40	79	70	62	14	61	62	45	53
Lamb deaths									
Singles	11	7	7	25	3	11	9	23	48
Multiples	0	5	3	8	0	7	3	10	18
Total (% of lambs born)	55	35	32	39	43	67	43	53	45
Ewe deaths	0	3	5 ^a	8	0	4	6 ^b	10	18
Lamb deaths in utero	0	4	4	8	0	6	5	11	19
Lambs marked (% of ewes mated)	18	51	48	38	8	20	36	21	29
Grass pasture									
Ewes mated	52	46	47	145	48	38	43	129	274
% Ewes fertile	90	74	74	80	100	87	74	78	84
Lambs born									
Singles	44	24	21	89	47	25	24	96	185
Multiples	6	20	28	54	2	16	17	35	89
Total (% of total ewes)	96	96	104	99	102	108	95	102	100
Lamb deaths									
Single	12	4	2	18	11	5	4	20	38
Multiples	4	13	17	34	2	2	3	7	41
Total (% of lambs born)	32	39	39	36	27	17	17	21	29
Ewe deaths	0	2	2	4	0	0	0	0	4
Lamb deaths in utero	0	4	3	7	0	0	0	0	7
Lambs marked (% of ewes mated)	65	59	64	63	75	89	79	81	71

^a Includes two ewes with prolapsed uterus

^b Includes one ewe with prolapsed uterus

The basis for the endocrine disturbances caused by Yarloop ingestion during pregnancy is also not understood. Whether the different amounts of plasma hormones in ewes grazing Yarloop reflect changes in the rates of hormone synthesis or metabolic clearance or both is unknown.

Recent studies on the origin of plasma progesterone in sheep have shown that up to 110 days gestation the CL is a major contributor, but between 130 days and birth

the ovarian contribution is small (Edgar and Ronaldson 1958; Mattner and Thorburn 1971). Albeit, bilateral oophorectomy at 110-124 days gestation resulted in a 50% reduction of peripheral plasma progesterone concentrations in late pregnancy (Fylling 1970), a difference similar to that seen between the ewes on Yarloop and those on grass pasture.

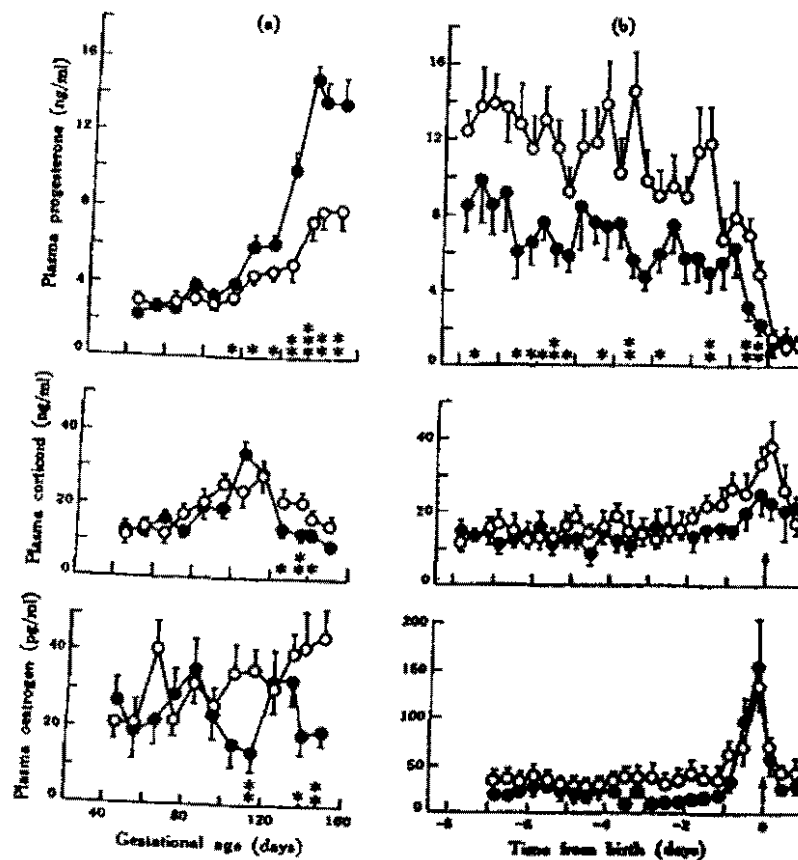


Fig. 3. Plasma progesterone, corticoid and oestrogen (a) during pregnancy and (b) about parturition in nine ewes grazing grass (●) and nine ewes grazing Yarloop clover (○). Vertical bars represent \pm SEM. Statistically significant differences are denoted thus: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The pattern of plasma corticoid found in ewes on grass pastures is very similar to that reported by Basson *et al.* (1969). The reduction in plasma corticoid concentration occurring after 120 days gestation has been attributed to an expansion in plasma volume or, alternatively, to increase in the metabolic clearance rate of cortisol (Paterson and Harrison 1968). Thus the higher than normal concentration of maternal

corticoid in the Yarloop group may reflect disturbances in the factors regulating these functions.

Undernourishment combined with cold stress can also cause higher than normal maternal plasma corticoid concentrations in the latter part of pregnancy (Lindner 1959, Saba 1965). However, because both the ewes on Yarloop and those on grass produced lambs with similar birth weights, and because both groups were exposed to the same climate and handling regimen, it is unlikely that these factors would explain the higher plasma corticoid levels in ewes grazing Yarloop pasture.

The apparent association between duration of parturition and plasma corticoid concentrations in the 8 h prior to birth may simply relate to the fact that the ewes with dystocia were stressed. It is known, however, that a functioning foetal pituitary-adrenal axis is necessary for successful parturition (Liggins *et al.* 1967; Drost and Holm 1968), and it is feasible that on account of high maternal corticoid concentrations some corticoid could cross the placenta (Dixon *et al.* 1970) and affect the development of this axis resulting in dystocia and the poor post-partum survival.

The pattern of plasma oestrogen concentration during pregnancy was similar to that reported by Challis (1971). No evidence was obtained in the present study to suggest that the characteristic pre-partum oestrogen rise was related to the length of parturition. Oestrogen peaks were observed in ewes which showed signs of dystocia as well as in ewes which were able to deliver their lambs unaided, both on Yarloop and on grass pasture. However, it is possible that in ewes exhibiting dystocia, the normal response by tissues was mollified by competition or previous effects of the high levels of circulating isoflavones in the plasma. Alternatively, the lambing difficulties may have been due to disturbances in the oestrogen/progesterone ratio seen in the ewes grazing Yarloop. Such an imbalance may have prevented not only normal myometrial activity, but also the normal preparation or relaxation of the uterus, cervix and vulva (Hindson *et al.* 1967; Csapo 1969).

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Effects of plant oestrogens on animal reproduction

Donald A. Shutt

In some pasture plants substances are present that can produce a temporary or permanent infertility in livestock eating the plants. Such oestrogenic compounds can occur in high concentration in some legume species. The author reviews the progress in understanding of this plant-animal interaction and the chemical nature of the substances involved. In particular, the oestrogenic substances implicated in the infertility syndrome in sheep in Australia known as 'clover disease' are discussed and possible control measures, including immunization of the animals, are considered.

Some plants that are commonly grazed nevertheless contain substances that are harmful to the animals ingesting them, and one such group of compounds can cause reproductive disorders in the female [1]. Plants containing these compounds include some of the economically important pasture or forage plants of the Leguminosae family, and the biologically active compounds have been found to be oestrogenic substances that mimic the activity of the animal oestrogens [2, 3]. These plant oestrogens present in high concentration in some pasture or forage legumes have been implicated in reproductive disturbances in sheep, cattle, and rabbits and may be a factor controlling rodent populations [4-6]. They may also account for the biological activity of some plants used by native communities for contraceptive purposes [7].

An oestrogen can be defined as a compound that acts on the central nervous system and induces oestrous (mating) behaviour in female mammals. Oestrogens also specifically stimulate the growth of the reproductive tract and breasts of mammals. In female mammals and in the human female, oestradiol, the primary oestrogen, is secreted by the ovaries and passes via the blood stream to specific target tissues identified by the presence of oestrogen receptor proteins. Oestrogens such as oestradiol and ethinyloestradiol, a synthetic oestrogen widely used in oral contraceptive pills, belong to the group of compounds known as steroids. This steroidal structure, however, is not exclusive to substances having oestrogenic activity. In 1923 E. Allen and E. A. Doisy [8] established a vaginal smear test in mice for oestrogens, based on the cornification of the vaginal epithelium that occurs in mice in response to oestrogen stimulation. By this method it was shown that oestrogenic substances occur in some species of plants, and some of these plant oestrogens were subsequently found to have a non-steroidal structure.

The potential deleterious and widespread influence of plant oestrogens on animal reproduction was highlighted in a report by H. W. Bennetts, E. J. Underwood, and F. L. Shier [9] of the Department of Agriculture in Western Australia in 1946. The affected animals were ewes (female sheep) grazing on the Dwalganup strain of subterranean clover (*Trifolium subterraneum* var. Dwalganup). The fertility of the sheep was found to decline progressively over the period 1941 to 1944 in the affected areas of Western Australia and the number of lambs born fell to about 30%. The infertility was expressed as a failure to conceive and was accompanied by a cystic condition of the reproductive tract. Lactation in non-pregnant ewes and in wethers (castrated male sheep) grazing on the subterranean clover suggested that a plant oestrogen was

implicated in the infertility in the breeding ewes. This type of infertility syndrome in sheep eventually became known as 'clover disease'.

Plant oestrogens in pasture legumes

By 1954 R. B. Bradbury and D. E. White [2] were able to report the isolation of two potentially oestrogenic compounds in subterranean clover named genistein and formononetin (figure 1). These compounds are isoflavones and are chemically related to the flavonoid plant pigments [10]. They occur in the plant mainly in the form of water-soluble glycoside conjugates [11]. Formononetin showed no oestrogenic activity when tested in mice but genistein was active at 1 mg; thus genistein was thought to be the oestrogen responsible for 'clover disease' in the sheep [12]. Two other isoflavonoid compounds were subsequently isolated from pasture legumes and were named coumestrol (figure 1) and biochanin A (see figure 5 for structure of other plant oestrogens). Biochanin A was found to have weak oestrogenicity similar to genistein in mice, probably because it is demethylated to genistein in the liver, whilst coumestrol was found to have greater oestrogenic activity than genistein or the other isoflavones [13].

The isoflavones genistein, biochanin A, and formononetin occur in variable concentrations in the leaves of many clover species. They are usually present in low concentrations in white clover (*Trifolium repens*) (figure 2) but can occur in especially high concentrations in certain cultivars of subterranean clover (*Trifolium subterraneum*) (figure 3). The isoflavones can account for as much as 5 per cent of the dry matter content of the leaves from healthy clover plants. Coumestrol can be found in low concentration in clover and in legumes such as (*Medicago sativa*) alfalfa (U.S.A.) or lucerne (Australia) (figure 4). However, the concentration of coumestrol in either clover or alfalfa can increase in response to fungal disease and may be associated with the defence mechanisms of plants exposed to foliar pathogens [6]. Otherwise coumestrol is

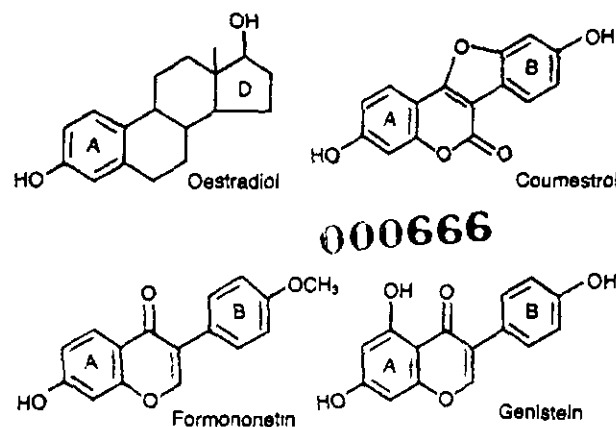


Figure 1 Comparative structure of the ovarian oestrogen oestradiol with the plant oestrogens coumestrol, genistein and formononetin

Donald A. Shutt, M.Sc., Ph.D.

Was born in England in 1931 and educated at Burnley Grammar School. After emigrating to Australia he received the degree of M.Sc. from Adelaide University and his Ph.D. from Macquarie University in Sydney. After several years' research on plant oestrogens with the CSIRO he joined Sydney University in 1972 as a Senior Research Fellow in Reproductive Endocrinology with the Department of Obstetrics and Gynaecology, to continue studies on natural and synthetic compounds controlling fertility in animals and in women.



Figure 2 White clover (*Trifolium repens*) usually contains a low concentration of isoflavones and is generally found to have negligible oestrogenic activity



Figure 3 Subterranean clover (*Trifolium subterraneum*) may contain up to 5 per cent of the dry matter content of the leaves in the form of the isoflavones formononetin, biochanin A, and genistein, and some strains have relatively high oestrogenic activity when tested green



Figure 4 Lucerne (*Medicago sativa*) contains variable amounts of coumestrol and related compounds, and the oestrogenicity and coumestan content of these plants is greater when the plants are infected with pathogenic fungi.

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not usually present in sufficient concentration in pasture or forage plants to be harmful to grazing animals

Comparative activity and metabolism of plant oestrogens in animals

Evidence against genistein being responsible for 'clover disease' in sheep was presented in 1964 by A. J. Millington, C. M. Francis, and N. R. McKeown [14]. They found the oestrogenic activity of nine different strains of clover pasture, as assessed by the criterion of increase of test length in wethers grazing the test pastures, was correlated with the amount of formononetin in the pasture and not with the genistein content of the pasture. Other workers [15-17] confirmed and extended this significant finding.

This led to a closer look at the digestion, absorption, and blood levels of the plant oestrogens in the sheep and other animals [18-21], and the reason why formononetin is oestrogenically more active than genistein in the sheep was found to lie in the digestion of plant constituents in the modified digestive system of the sheep. In common with other ruminant species the digestive tract preceding the stomach in the sheep has been enlarged to form an additional compartment, the rumen. This contains a large population of micro-organisms which aid in the digestion of cellulose, and these micro-organisms are capable of carrying out considerable degradation of other plant constituents. In the sheep it was found that biochanin A and genistein are degraded in the rumen to *p*-ethylphenol and a phenolic acid, both oestrogenically inactive; by contrast, formononetin is

first demethylated to daidzein and then metabolized by reductive step to a weakly oestrogenic compound named equol [22] (figure 5). It has been shown that the degradation of biochanin A and genistein to inactive phenol in the rumen of the sheep becomes increasingly more efficient over a period of about five days. On the other hand the conversion of formononetin to equol, once established, remains fairly constant, and further degradation of equol does not occur [23]. When present, coumestrol is rendered oestrogenically less active in the sheep over an adaptive period of one to two weeks, probably by the formation of less active metabolites, so far unidentified [24]. Other metabolic pathways for the degradation of clover isoflavones have been found in individual sheep [25] but the most common metabolic pathway leads to the formation of *p*-ethylphenol from genistein and equol from formononetin.

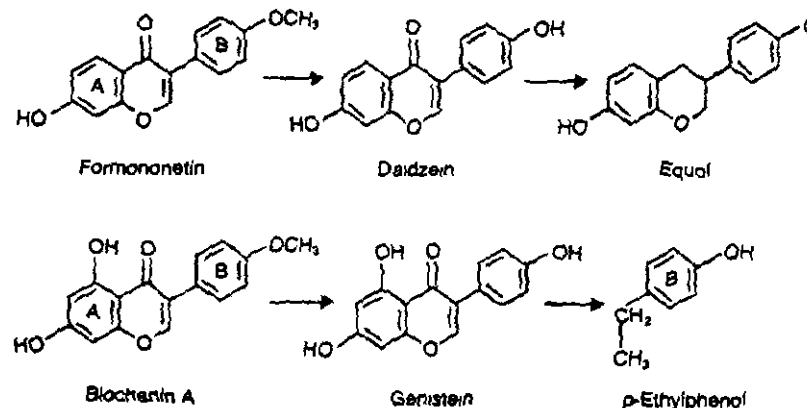


Figure 5 Metabolism of clover isoflavones in the sheep occurs primarily by the action of micro-organisms in the rumen of the sheep. Formononetin is first demethylated to daidzein, the latter then being reduced to an isoflavan, equol. Biochanin A is demethylated to genistein which then undergoes ring fission to form *p*-ethylphenol from the B-ring and a phenolic acid from the A-ring.

The metabolism of clover isoflavones in cattle [26] and guinea pigs [22] has also been examined. When the animals fed on red clover containing a high concentration of biochanin A and formononetin it was found that demethylation of the compounds occurred in both species. In the cow, biochanin A and genistein were observed to disappear rapidly from the blood plasma, and equol was found to be the major metabolite in the plasma after 6 days of feeding on red clover. This suggests that, in the cow, degradation of clover isoflavones is comparable to that found in the sheep. However, there appears to be a shorter adaptive period in the cow and the detoxication and excretion of the absorbed isoflavones and metabolites are more efficient. This probably accounts for the cow being less susceptible to the oestrogenic effects of clover isoflavones. In contrast, in the guinea pig preliminary observations suggest that biochanin A and genistein concentrations are maintained at a higher level in the plasma than in either the sheep or the cow, and support bioassay data indicating that the guinea pig responds mainly to the biochanin A and genistein content of the clover [16]. Equol was found in the guinea pig plasma, indicating that formononetin is converted to equol in this species.

The significance of equol

Historically equol is an interesting compound. It was first isolated from pregnant mares' urine and named equol by G. F. Marrian and G. A. D. Haslewood [27] in 1932. The plant origin of the precursor of equol was unknown then and compared with the highly oestrogenically active oestrogens of ovarian origin it was considered to be inactive when tested in mice. It was subsequently isolated from the urine of goats, sheep, cattle, and fowl and identified in the plasma of sheep, cattle, and guinea pigs ingesting clover isoflavones. In the sheep more than 80 per cent of the formononetin ingested in clover was found to be absorbed from the rumen in the form of equol [28] and most of the absorbed equol was conjugated, probably in the liver, to form equol glucosiduronate before being excreted in the urine. Equol can reach levels of 300-500 µg/100 ml in blood plasma of which small amounts occur as sulphaconjugates [29] and about 1-2 per cent can be present in a 'free' or unconjugated form. It is now thought that the maintenance of this high level of equol in the blood plasma is responsible for the infertility in sheep grazing legume pastures that contain a high concentration of formononetin; the sulphaconjugates and 'free' fractions probably represent the biologi-

cally active forms of the compound. On retesting equol it was found to have weak oestrogenicity in mice of the same order as that of genistein [22].

Mechanism of action

The problems caused by the oestrogens of plant origin result from their high concentration in the blood plasma of the animals ingesting the plants relative to the endogenous oestrogens. In high concentration a weak plant oestrogen can exert a significant oestrogenic effect in the animal and can produce a hormonal imbalance [30-31] even though their activity is only 10^{-3} to 10^{-5} times the oestrogenic activity of oestradiol. Plant oestrogens can also act as anti-oestrogens by competing for receptor proteins in oestrogen-sensitive tissues with the more active endogenous oestrogens which occur in much lower concentration in blood plasma [32-33]. Though weak plant oestrogens have less affinity than oestradiol for the oestrogen receptor proteins from tissues such as the uterus (figure 6), when a high blood concentration is maintained, they can exert a maximal oestrogenic effect even when the rate of breakdown of the oestrogen-protein complex is probably rapid and the compounds have a short biological half-life [34].

Physiologically, clover-induced infertility is brought about by a combination of factors, including interference with spermatozoan transport through the genital tract of the ewe, abnormal transport of ova, and interference with implantation. There is also evidence that the neuro-endocrine centres in the brain controlling the reproductive cycle of the ewe are suppressed. In long-term grazing on oestrogenic pastures a cystic condition of the cervix and uterus and an increased fluidity of the cervical mucus in affected sheep reduces the number of eggs that are fertilized. Shorter-term grazing of oestrogenic clover during mating can produce a temporary infertility in the ewe, possibly due to a disturbance in ovum transport. Neither short- nor long-term grazing of oestrogenic pastures however, appears to affect the fertility and sperm production in the ram (see [25]).

Control measures

To control the deleterious effects of clover oestrogens in sheep a number of approaches have been tried [35]. These include agronomic measures aimed at replacing existing oestrogenically active clover in the pasture with other types of plants. This is difficult in some areas as some of the more oestrogenic strains of subterranean clover are well adapted to their environment and have built up large reserves of seed in the soil. Where possible, animal husbandry procedures have been adopted to reduce the number of breeding ewes grazing on the oestrogenic pastures. However, even with modern methods, and the knowledge that is now available, it was recently

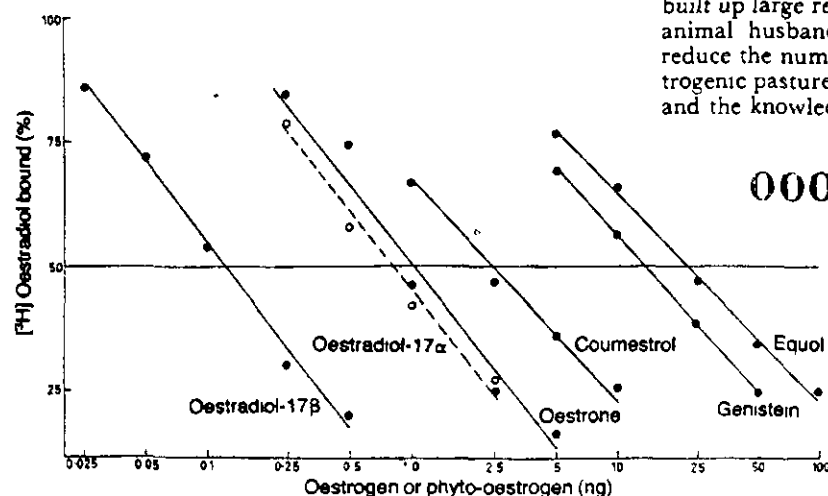


Figure 6 Competitive binding curves for some mammalian steroid oestrogens and some plant oestrogens. Test substances plus [3 H] labelled oestradiol were incubated with oestrogen receptor protein in sheep uterine cytosol

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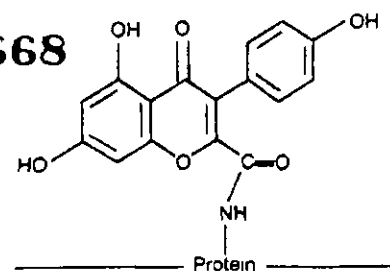


Figure 7 Covalent linkage of the 2-carboxylic acid of the plant isoflavone genistein to protein. Injection of this antigen into animals results in the production of antibodies to the protein and to the attached isoflavone

estimated that each year one million ewes of the Australian flock fail to lamb due to 'clover disease'. A further approach to the problem is an attempt to develop an immunization procedure that would offer some protection to breeding sheep in areas where changes in animal or pasture management are not economic. In a pilot study on rabbits H. R. Lindner and colleagues [36], of the Weizmann Institute in Israel, demonstrated the production of antibodies against the plant oestrogen genistein when genistein-2-carboxylic acid was coupled to a synthetic polypeptide (figure 7). Conjugation of a plant oestrogen with a polypeptide in this way allows formation of antibodies both to the carrier and to the oestrogen, as unattached oestrogens are non-antigenic. It was further shown that these antibodies would not cross-react with endogenous steroid oestrogens and would not therefore disrupt normal oestrogenic activity. Because of the availability of genistein and formononetin these compounds were then used as model compounds for preliminary trials with sheep [25]. Sheep immunized with a genistein-protein antigen showed that some protection was afforded against the oestrogenic activity of injected genistein (although genistein is rendered oestrogenically inactive in sheep when ingested in pasture plants, it is oestrogenically active when given by parenteral injection). Further work showed that specific antibodies could also be raised in sheep to formononetin when coupled as the 2-carboxylic derivative to bovine serum albumen.

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Antibodies against formononetin and genistein were found to persist in sheep for about a year, after which a booster injection resulted in a marked increase in antibody formation. Further work is now in progress in Australia to raise antibodies in sheep against the more important compounds equol and coumestrol. With equol it will be necessary to see whether the antibodies against equol in the sheep will afford protection to animals ingesting gram amounts of formononetin in clover daily, and also whether fertility can be improved economically by this procedure.

Because of the magnitude of the effects of plant oestrogens on the reproduction of grazing sheep there has been a greater research effort directed towards alleviating the problem in the sheep. However, the techniques of identification and quantitative assessment of oestrogens in plants should allow a quicker screening for possible oestrogenic compounds when they are suspected of reducing fertility in other animals, or having a contraceptive action in women. The possibility of immunizing animals against individual plant oestrogens is also at an advanced stage of development, and points the way to methods of reducing other disorders in grazing animals due to plant constituents. From the wider viewpoint of evolution it is interesting that compounds have evolved in plants that not only give the plant some protection from foliar pathogens, but also reduces the fertility of animals ingesting the plant.

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Organizational and Activational Effects of Phytoestrogens on the Reproductive Tract of the Ewe (43837)

NORMAN R. ADAMS¹

CSIRO Division of Animal Production, PO, Wembley WA 6014, Australia

Abstract Ewes exposed to phytoestrogens may display two forms of infertility, categorized as temporary or permanent. Temporary infertility results from actions of estrogen that are similar to the activational effects of estrogen in most species of mammals. The permanent infertility results from changes to the cervix which are analogous to the organizational effects of estrogen reported in other species treated during organogenesis. However, in the ewe these effects may be produced after organogenesis by prolonged treatment during adult life. It has recently become apparent that the level of nutrition and metabolic hormones influence the degree of uterus-like histological change in the cervix produced by prolonged treatment with estrogen. It is hypothesized that, under some nutritional conditions, the hormonal milieu in adult ewes may simulate hormonal patterns that are normally experienced by fetal lambs in utero, thereby allowing the cervix of the adult ewe to give an organizational response to estrogen.

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Infertility in female sheep grazed on estrogenic subterranean clover (*Trifolium subterraneum* L.) was described almost 50 years ago (1), as part of the condition known as "clover disease". The fertility of male sheep was rarely affected. It soon became apparent that there were two forms of infertility, a temporary form that resolved when sheep were returned to nonestrogenic pasture and a permanent form that became progressively worse with continued exposure. The biological basis for these two different forms of infertility has been determined over the past 15 years. Adult female sheep can show either activational or organizational responses to estrogen, and these have different effects on fertility. This review summarizes knowledge of these responses, and describes briefly the epidemiology of infertility.

Both coumestans and isoflavone compounds can be estrogenic in sheep. Coumestans such as coumestrol occur in alfalfa or annual medics, particularly in plants suffering from a foliar disease. Isoflavone compounds,

including genistein, biochanin A and formononetin, occur in subterranean clover or red clover (*Trifolium pratense*). Genistein and biochanin A are usually broken down by microbial activity in the rumen of sheep, while formononetin is converted to the isoflavan equol, which is rapidly absorbed and is responsible for most of the estrogenic activity in ruminants. Subterranean clover is an annual plant that contains estrogenic isoflavones only while it is green and growing. The concentrations of isoflavones depend primarily on the genotype, and are relatively independent of disease status of the plant.

Activational Effects of Phytoestrogens

Functional Change. Fertility is reduced in sheep that are mated on estrogenic pasture, although these effects normally resolve within 3 to 5 weeks after return to nonestrogenic pasture. Most of the decrease in fertility arises from decreased twinning rate (2), probably caused by direct effects of phytoestrogen on the ovarian follicles. Follicular function may be compromised to the extent that some ewes fail to ovulate at all. Embryo mortality may be slightly increased due to abnormalities of ovum transport and uterine function (3). Effects on the central nervous system are relatively slight. There are no substantiated reports of estrus behaviour produced by phytoestrogens, and neither isoflavones nor coumestans have much effect on

¹ To whom requests for reprints should be addressed at CSIRO Division of Animal Production, PO, Wembley WA 6014, Australia

* Typical of Japan; referred to in Theo Colborn's book "Chemically Induced Alterations in Sexual Development" by Prof Pat Whitten.

Dietary Intervention Study to Assess Estrogenicity of Dietary Soy among Postmenopausal Women

DONNA DAY BAIRD, DAVID M. UMBACH, LYLE LANSDELL,
CLAUDE L. HUGHES*, KENNETH D. R. SETCHELL, CLARICE R. WEINBERG,
ARTHUR F. HANEY, ALLEN J. WILCOX, and JOHN A. MCLACHLAN

National Institute of Environmental Health Sciences (D.D.B., D.M.U., C.R.W., A.J.W., J.A.M.) and
Survey Research Associates (L.L.), Research Triangle Park, North Carolina; Duke University Medical
Center (C.L.H., A.F.H.), Duke University, Durham, North Carolina, and Children's Hospital
(K.D.R.S.), University of Cincinnati, Cincinnati, Ohio

ABSTRACT

We tested the hypothesis that postmenopausal women on a soy-supplemented diet show estrogenic responses. Ninety-seven postmenopausal women were randomized to either a group that was provided with soy foods for 4 weeks or a control group that was instructed to eat as usual. Changes in urinary isoflavone concentrations served as a measure of compliance and phytoestrogen dose. Changes in serum FSH, LH, sex hormone binding globulin, and vaginal cytology were measured to assess estrogenic response. The percentage of vaginal superficial cells (indicative of estrogenicity) increased for 19% of those eating the diet compared with 8% of controls ($P = 0.06$ when tested by ordinal logistic regression). FSH and LH did

not decrease significantly with dietary supplementation as hypothesized, nor did sex hormone binding globulin increase. Little change occurred in endogenous estradiol concentration or body weight during the diet. Women with large increases in urinary isoflavone concentrations were not more likely to show estrogenic responses than were women with more modest increases. On the basis of published estimates of phytoestrogen potency, a 4-week soy-supplemented diet was expected to have estrogenic effects on the liver and pituitary in postmenopausal women, but estrogenic effects were not seen. At most, there was a small estrogenic effect on vaginal cytology. (*J. Clin. Endocrinol. Metab.* 80: 1685-1690, 1995)

PHYTOESTROGENS ARE nonsteroidal plant compounds of diverse structure that produce estrogenic responses (1-5), they are found in many fruits, vegetables, and grains. Most are relatively weak estrogens, but they can have potent biological effects when ingested in large quantities. The most striking example occurred in the 1940s when an epidemic of infertility decimated the sheep breeding industry in southwest Australia. Red clover forage that contained large quantities of estrogenic isoflavones caused the outbreak [reviewed by Moule *et al.* (6)]. More recently, infertility and liver disease in captive cheetahs was explained by the presence of estrogenic isoflavones in soy meal contained in a commercial feline diet (7).

Hypothesized effects of dietary estrogens in humans include developmental changes (8-10), reduced fertility (11, 12), reduced severity of menopausal symptoms (13), cardiovascular reactions (3), and increased or decreased risk of hormonally related cancers, especially breast and endometrial cancer (3-5, 8, 14-16). However, few studies have been undertaken to measure biological effects of dietary phytoestrogen intake in humans. Two studies of premenopausal women reported alterations in menstrual cycle characteristics (17-18), and a study of postmenopausal women in Australia suggested estrogenic effects on vaginal epithelium (19).

Soybeans warrant particular interest because they are a widely used food source for humans and domestic animals. Concentrations of estrogenic isoflavones in most soy protein products reach levels of 0.1-0.2% (20), the major substances being daidzein and genistein. After ingestion of soy protein by humans, intestinal flora can convert the soy isoflavones to equol, a more potent estrogenic isoflavone that is absorbed along with the unconverted genistein and daidzein. The urinary excretion of equol in humans eating soy-supplemented diets can greatly exceed the concentration of urinary endogenous estrogens. Such high concentrations enhance the plausibility of human health effects (8).

We designed a dietary intervention study in postmenopausal women to assess estrogenic effects of a soybean-supplemented diet. Conjugated estrogens produce rapid biological changes in postmenopausal women: documented effects include reductions in LH and FSH, increases in sex hormone binding globulin (SHBG), and increased maturation of vaginal epithelium as reflected by vaginal cytology (21-22), with SHBG being the most sensitive (21). We hypothesized that these same biological changes, especially the increase in SHBG, would be detectable in postmenopausal women eating large quantities of soy protein.

Materials and Methods

Study design

Ninety-seven women were randomly assigned (in approximately a 3:1 ratio) to a soy diet group or a control group after a 2-week period when baseline measurements were taken. During the 4 weeks after

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Address all correspondence and requests for reprints to: Donna Day Baird, Epidemiology Branch, A3-05, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709.
*Current address: Bowman Gray School of Medicine, Winston-Salem, North Carolina.

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randomization, the soy diet group ate daily portions of soy foods (provided by the study) as a substitute for approximately one third of their caloric intake. Members of the control group were instructed to eat as usual during the dietary intervention period. The following markers of estrogenicity were measured at baseline and again at the end of the dietary intervention: serum LH, serum FSH, serum SHBG, and cytology of the vaginal epithelium, as reflected by the maturation index or percentage of superficial cells in vaginal smears. The concentrations of serum estradiol and urinary soy estrogens (urinary daidzein, genistein, and equol) were also measured at baseline and at the end of the dietary intervention period. All laboratory analyses were conducted without knowledge of treatment status. The study began with a pilot phase ($n = 8$ women) and was then completed in two separate sessions, one in the fall ($n = 40$ women) and one in the spring ($n = 49$).

Study participants

Study participants were volunteers recruited through newspapers, fliers, and radio announcements in the three-county area of Research Triangle Park, North Carolina. Criteria for entry were age 65 yr or younger, at least 2 yr past last menses, no use of antibiotics or estrogen replacement therapy in the preceding 6 months, no use of prescription drugs known to affect outcome measures, e.g., corticosteroids. Women received \$50 per week compensation for time and travel expenses. This study was approved by the Human Subjects Review Committee at the National Institute of Environmental Health Sciences, and informed consent was obtained from all participants.

Questionnaire data

Before randomization, the women completed an extensive self-administered questionnaire which included an adaptation of the *Health Habits and History Questionnaire* (23) that collects information about dietary habits during the previous year. In addition, the women completed a short daily questionnaire that included body weight and a record of soy food intake for those in the soy diet group.

Soy foods

The major daily soy food was a main dish made from whole soybeans or textured vegetable protein (dried defatted soybean flour). The soybeans were a single variety, organically grown, and purchased in a single batch. Soy splits (dried soybeans) were provided as a daily snack. The soy foods were analyzed for daidzein and genistein by high performance liquid chromatography mass spectrometry, as described previously (7). The daily intake of soy consisted of 38 g of dry textured vegetable protein (2.1 mg/g daidzein, 0.6 mg/g genistein) or 114 g of dry whole soybeans (0.7 mg/g daidzein, 0.2 mg/g genistein). In addition, women ate 25 g of soy splits daily (1.8 mg/g daidzein, 0.7 mg/g genistein). Thus, daily intake of isoflavones was 165 mg/day. This is approximately equivalent on a molar basis to 0.3 mg/day of conjugated steroidal estrogen, assuming that the estrogenic activity of the phytoestrogens is about 0.1% that of conjugated estrogen.

Blood, urine, and vaginal smear collection

Participants visited 1 of 4 medical clinics 4 times during the study: twice in the prediet period, midway through the diet period, and at the end of the diet period. All appointments were scheduled between 0800 and 1000 h, and women were instructed to fast from 2400 h the previous night until after their appointment. At each clinic appointment, the women were weighed, and blood was drawn 4 times at 20-min intervals via venipuncture. Blood was centrifuged, and equal aliquots of serum from each sample were pooled and stored at -20°C . The pooled serum sample was used for assays in order to reduce the variability caused by the pulsatile release of LH and FSH from the pituitary. First morning urine specimens were collected and frozen daily after the first week of the study, and a 24-hour urine specimen was collected on the same day as each clinic appointment. At the second prediet and final clinic visits, samples of vaginal epithelial cells were taken from the left and right midlateral vaginal walls by making 5 to 10 scraping strokes with vaginal spatulae. A separate slide was prepared and fixed for each wall.

Measurement of urinary phytoestrogens

Urinary concentrations of soy isoflavones were measured to demonstrate compliance with the diet and to provide a crude measure of phytoestrogen dose for each participant. To minimize the effect of day-to-day variations in urinary isoflavone levels, we pooled first morning urine samples from before the diet (6-mL aliquots from each of the 7 days before randomization) and during the diet period (2-mL aliquots from each day of the last 3 weeks of the diet) and measured the phytoestrogens in the pooled sample. Concentrations were expressed relative to the creatinine concentration in the pooled sample. A pilot study of 20 paired specimens had been conducted to measure phytoestrogen concentrations in 24-h and first morning urine specimens from the same 24-h period to verify that first morning urine specimens (corrected for creatinine) were valid indicators of total urinary excretion.

Daidzein, genistein, and equol were extracted from urine by solid-phase extraction after addition of an internal standard 5 α -androstane-3 α ,17 α -diol (5 μg). Conjugates were hydrolyzed with β -glucuronidase and sulfatase enzymes. Unconjugated estrogens were extracted by liquid-solid extraction, and phenolic compounds were separated from neutral steroid hormone metabolites using an anion exchange gel, triethylaminoxypropyl Sephadex LH-20. Trimethylsilyl ethers were prepared, separated by gas chromatography on a DB-1 capillary column, and quantified by mass spectrometry using selected ion monitoring (8).

Measurement of serum LH, FSH, SHBG, and estradiol

LH, FSH, SHBG, and estradiol concentrations in sera were measured with commercial kits. Time-resolved fluoroimmunoassays for LH, FSH, and SHBG were performed with the appropriate LKB-Wallac DELFIA kits (Electronuclear, Inc., Columbia, MD). Estradiol was measured by RIA (Leeco Diagnostics, Inc., Southfield, MI). All samples from an individual woman were assayed together. For all analytes, the intraassay coefficient of variation was less than 5%, and the interassay coefficient of variation was less than 10% on the basis of quality control standards.

Vaginal cytology

Specimens from each vaginal wall were read separately by a single trained technician who was unaware of which slides were paired. From each slide, 200 cells were examined to determine the percentage of parabasal, intermediate, and superficial cells (24). The values from the 2 walls were averaged. Of the 364 slides, 51 (14%) had too few cells to count and were not included in the calculations. This resulted in 4 women with no vaginal smear data and 34 women with vaginal smear data based on only 1 wall for at least 1 of the time periods. A maturation index was calculated as the percentage of superficial cells plus half the percentage of intermediate cells.

Statistical analyses

For each of the four dependent variables (change in FSH, LH, SHBG, and maturation index), we tested for the effect of dietary intervention by including treatment as a term in a basic linear regression model that also included season of study and the clinic that the woman attended. Thus, the null hypothesis was that the mean change for the soy diet group was not different from the mean change in the control group. FSH, LH, and SHBG concentrations were logarithmically transformed before calculating change variables. Baseline concentrations were estimated as the geometric mean of two prediet values. Change in serum estradiol level (difference in natural logarithms of end-of-diet and baseline concentrations), change in weight, and age (by chance, controls were younger on average than women in the soy diet group, although not significantly so) were also added one at a time to the basic model to adjust for possible effects of these factors. Change in percentage of superficial cells, the cells considered most indicative of estrogen stimulation (25), was examined separately with ordinal logistic regression. Because only 27 women exhibited a change in superficial cells during the study (most remained at 0%), we defined three levels of the dependent variable: decrease, no change, and increase. Adjusting for other variables was done as described above.

In further analyses to explore a possible dose response, we replaced

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treatment as a term in the models with each of three different measures of urinary soy estrogens: equol concentrations alone, an unweighted sum, or a weighted sum of concentrations with weights of 4 for daidzein, 8 for genistein, and 100 for equol, on the basis of laboratory data on their relative estrogenicity (26-27).

To evaluate possible interaction effects, i.e. that subsets of the population responded differently to the dietary intervention, we added terms that represented interactions with treatment for age, length of time since menopause, smoking status, weight, Quetelet's index, and estradiol level at baseline to the models for FSH, LH, SHBG, and maturation index.

Results

Ninety-seven women began the study. Of these, 3 were found to be ineligible (1 was still premenopausal, 1 was taking corticosteroids, and 1 was taking medication for diabetes). Three others dropped out during the study (2 because of emergencies in their families, and 1 because she could not tolerate the soy foods). The remaining 91 women (66 in the soy diet group and 25 in the control group) completed all aspects of the study. First morning urine samples were more than 98% complete.

The participants were well educated women, ages 45-65 yr, about half of whom were employed outside the home. Demographic, lifestyle, and reproductive characteristics for the 91 participants are shown in Table 1. Dietary habits were similar for the soy diet and control groups as were baseline estradiol levels (mean \pm SD for treatment and control groups were 41.85 ± 12.48 pmol/L and 44.79 ± 14.32 pmol/L, respectively).

Compliance with the soy diet appeared to be good. Most (73%) women reported that they ate all of their assigned foods. Eighteen women reported that they ate only part of their soy foods on at least one day, but only four women missed days completely (3 missed one day, 1 missed two days), and these occurred at the time of an illness. Consistent with these reports, urinary soy estrogens increased markedly for most women in the soy diet group (average of a 105-fold increase in the unweighted sum) but increased little for those in the control group (average of a 2-fold increase, which was not statistically significant). The distribution of changes in soy estrogens for the soy diet group and for the control group is shown in Table 2. As expected, there was little overlap between the control and the soy diet group, but the variation among the women in the soy diet group was broad, with some women showing extremely large increases, others showing more modest changes, and a few showing little change.

Women maintained fairly stable body weights through the diet intervention period. The average weight change was a gain of 0.5 pounds (0.4 for the diet intervention group and 0.9 for the control group). No one gained or lost more than 5 pounds, and most (82%) varied by no more than 2 pounds. Endogenous estrogen levels, as reflected by serum estradiol concentrations, decreased slightly during the study for both the diet intervention and the control group. The decline was slightly larger for the diet intervention group than for the control group (5.14 pmol/L vs. 3.30 pmol/L) but not significantly so. Weight change and change in estradiol level were both considered potential covariates in all analyses.

TABLE 1. Characteristics of the sample of 91 postmenopausal women participating in the soy study

	Total (n = 91)		Soy diet (n = 66)		Control (n = 25)	
	Number	%	Number	%	Number	%
Race						
White	88	97	63	95	25	100
Nonwhite	3	3	3	5	0	0
Age (yr)						
≤ 55	30	33	20	30	10	40
56-60	28	31	18	27	10	40
61-65	33	36	28	42	5	20
Years since menopause						
≤ 5	28	31	21	32	7	28
6-10	27	30	20	30	7	28
> 10	36	40	25	38	11	44
Education (yr)						
≤ 12	16	18	11	17	5	20
> 12 and < 16	53	58	38	58	15	60
≥ 16	22	24	17	26	5	20
Smoking						
Yes	15	16	10	15	5	20
No	76	84	56	85	20	80
Alcohol						
None	23	25	20	30	3	12
Drink/wk (< 1)	19	21	13	20	6	24
Drinks/wk (1-6)	29	32	20	30	9	36
Drinks/wk (≥ 7)	20	22	13	20	7	28
Quetelet's index						
< 23	39	43	25	38	14	56
23-25.99	27	30	21	32	6	24
≥ 26	25	27	20	30	5	20
Recreational activity						
0 min/day	14	15	12	18	2	8
1-30 min/day	30	33	22	33	8	32
31-60 min/day	19	21	14	21	5	20
> 60 min/day	20	22	18	27	10	40
Vegetarian						
Yes	4	4	2	3	2	8
No	87	96	64	97	23	92
Number of pregnancies						
None	8	9	6	9	2	8
1-2	34	37	22	33	12	48
> 2	49	54	38	58	11	44
Prior use of replacement estrogen						
Yes	33	36	25	38	8	32
No	58	64	41	62	17	68

TABLE 2. Change in urinary soy estrogen concentration (ng/mg creatinine) during the diet intervention period of the soy study

Change	Control		Soy diet	
	Number	%	Number	%
Decreases				
≥ 1000	1	4	0	0
0-999	10	40	1	2
Increases				
0-999	13	52	3	5
1000-4999	1	4	11	17
5000-9999	0	0	10	15
10,000-14,999	0	0	14	21
15,000-19,999	0	0	11	17
20,000-24,999	0	0	11	17
$\geq 25,000$	0	0	5	8

Baseline and end-of-diet measurements for LH, FSH, SHBG, and maturation index are shown in Table 3. Changes in these outcomes during the 4-week diet period are shown

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TABLE 3. Baseline and end-of-diet measurements of FSH, LH, SHBG, and maturation index for the controls and soy diet group of postmenopausal women

Outcome	Number	Observed mean* (SD)	
		Baseline	End-of-diet
FSH (U/L)			
Controls	25	63.4 (24.5)	61.6 (25.8)
Soy diet	66	61.1 (22.1)	58.4 (21.1)
LH (U/L)			
Controls	25	30.3 (14.4)	29.7 (14.0)
Soy diet	66	32.6 (14.0)	31.8 (14.0)
SHBG (nmol/L)			
Controls	25	57.9 (35.2)	54.6 (25.4)
Soy diet	66	51.5 (32.3)	48.0 (26.8)
Maturation index			
Controls	24	16.1 (18.8)	14.2 (18.4)
Soy diet	63	14.6 (18.7)	17.4 (22.2)

* Geometric mean is shown for FSH, LH, and SHBG, arithmetic mean for maturation index. Standard deviation (SD) gives variation about the arithmetic mean for all outcome measures.

TABLE 4. Changes in outcome measures during 4-week diet period for the control group and soy diet group of postmenopausal women

Outcome	Measure of change*		Effect of Soy Diet	
	Crude	Adjusted*	Test statistic ^c	P value
FSH (U/L)				
Controls	-0.028	-0.028	-0.017	
Soy diet	-0.045	-0.045	(-0.052, 0.018)	0.33
LH (U/L)				
Controls	-0.020	-0.025	0.004	
Soy diet	-0.024	-0.021	(-0.058, 0.067)	0.89
SHBG (nmol/L)				
Controls	-0.058	-0.073	0.007	
Soy diet	-0.071	-0.066	(-0.086, 0.010)	0.89
Maturation index				
Controls	-1.9	-0.9	3.3	
Soy diet	+2.8	+2.4	(-4.5, 11.1)	0.40

* For FSH, LH, and SHBG, change is measured as the natural logarithm of the end-of-diet geometric mean minus the natural logarithm of the baseline geometric mean. Exponentiating this difference gives the end-of-diet value as a proportion of the baseline value. For maturation index, change is measured as end-of-diet arithmetic mean minus baseline arithmetic mean.

* All outcomes are adjusted for season and clinic; additionally, SHBG is adjusted for estradiol change and FSH for both estradiol change and weight change.

* The test statistic is the adjusted change for the soy diet group minus the adjusted change for the controls. If the two groups were identical, the test statistic would be zero. Values in parentheses are 95% confidence limits.

in Table 4. Adjustment for season, clinic, age, change in body weight, or change in estradiol concentration had little effect on these relationships. LH and FSH were predicted to decrease with soy intervention, and both did tend to decrease slightly (FSH more so than LH). However, controls also showed small average decreases, and the soy diet and control groups did not differ from each other ($P = 0.33$ for FSH, $P = 0.89$ for LH). SHBG was predicted to increase with soy intervention, but SHBG tended to decrease for both the soy diet and the control group to a similar degree ($P = 0.89$). Maturation index was predicted to increase with soy intervention. Although there was a slight average increase in the

soy group compared with a slight decline in the control group, the groups did not differ significantly ($P = 0.40$).

When we examined vaginal epithelium data further by focusing on superficial cells (the cells most indicative of estrogen stimulation), the changes were again in the predicted direction (increased superficial cells with dietary intervention). In unadjusted data, 43 (68%) women in the soy diet group showed no change in percentage of superficial cells during the diet, 9 (13%) showed a decrease, and 12 (19%) showed an increase compared with 17 (71%), 5 (21%), and 2 (8%) in the respective categories of the controls ($\chi^2_{2df} = 2.03$, $P = 0.36$). We found the same trend after adjustment for other factors with ordinal logistic regression. In comparison with women in the control group, women in the soy diet group tended to have no change or to have their proportion of superficial cells increase rather than decrease during the diet period [odds ratio = 2.5 (0.8, 7.8)], but the difference was consistent with chance ($P = 0.06$).

Concentration of urinary soy estrogens was not a better predictor of the outcome measures than was treatment alone, whether the change in soy estrogens was modelled as a linear term or as four separate categories corresponding to quartiles of change. Adjustment for other variables did not affect this result.

We also examined potential interactions to determine whether subsets of the study participants may have responded to the diet as predicted even though the soy diet group as a whole showed no clear estrogenic responses. We systematically looked for effects that might depend on age, length of time since menopause, smoking status, weight, Quetelet's index, and estradiol level at baseline. Of the 24 tests conducted, 2 showed significant interaction effects ($P < 0.05$), but neither were consistent with biological predictions. The first suggested that the decrease in FSH was similar in magnitude at all ages among dieters, but among controls the decrease tended to be larger in older women. The other suggested that SHBG decreased slightly for both smoking and nonsmoking dieters, whereas among controls, SHBG decreased slightly for nonsmokers but increased slightly for smokers. Considering the multiple tests conducted, the significant interactions probably resulted from chance.

Discussion

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A diet high in soy resulted in significantly increased urinary isoflavone excretion in postmenopausal women, indicating that large quantities of soy estrogens were being ingested and absorbed. However, after 4 weeks of soy-supplemented diet estimated to have estrogenicity similar to a 0.3 mg/day dose of Premarin (Wyeth-Ayerst brand of conjugated estrogens), there was little evidence of estrogenic effects from the plant estrogens. Geola et al. (21) reported that Premarin at 0.15 mg/day resulted in significant increases in SHBG ($n = 21$ postmenopausal women). In their study, SHBG was a more sensitive indicator of estrogenic response than FSH (which decreased significantly at 0.3 mg/day), LH (which showed no significant decrease below a dose of 0.625 mg/day), or the percentage of superficial cells in vaginal epithelium samples (which showed no significant increase

below a dose of 1.25 mg/day). In our study of soy estrogen, the order of sensitivity seems to be reversed if the increase in superficial cells of the vaginal epithelium is real. The overall maturation index did not differ between women in the soy diet and the control group, but the percentage of superficial cells tended to increase with dietary intervention ($P = 0.06$). We reported similar preliminary analyses (based on examination of cells from only one vaginal wall by a commercial laboratory) at a soy workshop sponsored by the National Cancer Institute (28) and then had slides of both vaginal walls read by a research laboratory. The more complete data are reported here. FSH and LH decreased during the intervention period, but the changes were small and occurred in both the soy diet and the control group.

Contrary to expectation, SHBG tended to decrease in both the control and the soy diet group. Adlercreutz *et al.* (16, 29-30) reported an association between urinary phytoestrogen concentrations and increased SHBG in observational studies, and genistein was reported to stimulate SHBG production in cultured human liver cancer cells (31). However, we saw no evidence of such effects *in vivo* nor did Cassidy *et al.* (17) in premenopausal women. The association in observational studies may not have been causal, or the differences in results could be a result of differences in length and type of dietary intake. Premarin may be effective when phytoestrogens are not because Premarin increases levels of estradiol (32), a much stronger estrogen, whereas the phytoestrogens remain weak estrogens, albeit in high concentrations.

Urinary concentrations of soy isoflavones were measured in order to document intake and to provide a possible measure of dose. Equol, the most potent soy-related estrogen, is produced by bacterial conversion in the gut, and the conversion rates are extremely variable (8). Thus, we hypothesized that the urinary equol level could be a more precise measure of soy estrogen exposure than treatment status could. However, when we looked for dose-response effects in relation to urinary equol level or other measures of soy estrogen dose, no stronger relationships emerged between the soy diet and outcomes. In addition, we looked for differential effects in different women (e.g., stronger effects in women with low estradiol levels or in women with low body mass index), but no such interactions emerged.

One other study looked for estrogenic effects of phytoestrogens in postmenopausal women and reported significant increases in vaginal cell proliferation during dietary intervention, as measured by the maturation index (19). In this Australian study, baseline measures were compared with measures taken during a 6-week diet period when 25 women ate soy flour, red clover sprouts, or linseed, each for a 2-week period. The Australian study included no control group so that effects of diet could not be separated from other changes over time, but vaginal smears taken 8 weeks after return to a normal diet showed maturation index values similar to those at baseline. The other difference between this study and ours is that they made no mention of laboratory personnel performing analyses in a blinded fashion, which may be important for somewhat subjective measures such as cytology. The daily amount of soy estrogen eaten by the North Carolina women should have been more than that

ingested by the Australian women (isoflavone content of various soy flours is reported to be from 178-306 mg per 100 g) (20); therefore, the 45 g/day ingested by the Australian women would have included from 80-138 mg/day of isoflavones compared with the 165 mg/day for the North Carolina women. Possibly, the linseed and clover sprouts may have been more highly estrogenic than soy, and the effects on the Australian women that appeared to be soy-related may have resulted from residual effects of the other foods.

The Australian and North Carolina studies did not seem to differ appreciably in enrollment criteria, but despite the similarity in the ages of participants, their baseline maturation index was much higher than ours (31 vs. 16), suggesting that they had fewer women with completely atrophic smears. Atrophic vaginal epithelia may be less likely to respond to the dietary estrogen. However, when we limited analysis to the 52 women whose vaginal epithelia were not atrophic at baseline, there was still limited evidence for increased proliferation.

The absence of any clear estrogenic effects in our study was surprising, but several factors may have played a role. Phytoestrogens do not bind well to SHBG (33), and the vast majority of molecules may be conjugated before they reach target organs. Competitive binding at the estrogen receptor (27) could reduce estrogenicity of the more potent endogenous estrogens, thus counteracting predicted estrogenic effects. Direct inhibition of aromatase has been reported (34-35), which would lower endogenous estrogen production. We saw little direct evidence of antiestrogenic responses, but such effects would be more easily observed in premenopausal women with high estrogen levels. Finally, effects of weak, nonsteroidal estrogens may also be biphasic, as observed in some laboratory studies of phytoestrogens (36-38). Biphasic responses would have been difficult to evaluate in this protocol.

In summary, this study showed no clear estrogenic effects in postmenopausal women eating a soy-supplemented diet for 1 month, despite evidence of absorption of high quantities of estrogenic isoflavones. Reproductive tract epithelial cell proliferation may have increased with the diet, but the effect was weak. A longer dietary exposure may be required for estrogenic effects. This was the first intervention study of postmenopausal women to measure urinary levels of phytoestrogens. The lack of clearly detectable effects of the diet suggests that more information about the metabolism of these compounds is needed, as well as more sensitive, site-specific markers of estrogenic and antiestrogenic effects.

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in mycological cultures of the meals. Neither zearalenone nor any other mycotoxin was detected.

The mouse uterine weight bioassay data are summarized in Table 1. Samples 2 and 3 were oestrogenic when fed as whole meal but their extracts were not tested. Nine other samples were active when fed as whole meal and also when fed in the form of ethyl-acetate extracts, while five further samples were active only when fed as ethyl-acetate extracts. Thus all sixteen samples showed biological activity. No oestrogenic activity was found in extracts in 70% aqueous ethanol, which is routinely used to extract substances with oestrogenic activity from red and white clover (Bickoff, Loper, Hanson, Graham, Witt & Spencer, 1967; Saba *et al.* 1974). Neither was it found in extracts in acetonitrile, which has previously been used to isolate an active fraction 6b11 from oestrogenic rat cake (Drane *et al.* 1975). However, all of the ethyl-acetate extracts were oestrogenically active, although when appropriate comparisons were made, it was found that the recovery of the source of the activity present in the original samples of soya meal was poor. Hydrolysed ethyl-acetate extracts examined by TLC were found to contain genistein and daidzein

and preliminary experiments suggested that the former isoflavone contributed most of the oestrogenic activity. No other reference oestrogen was detected.

Various reproductive disturbances in animals have been traced to the ingestion of oestrogenic feeds. Cattle became infertile whilst grazing lucerne containing high concentrations of coumestrol (Adler & Trainin, 1967), hyperoestrogenism was reported in pigs fed diets containing 0.1-6.8 µg zearalenone/g (Mirocha, Pathre & Christensen, 1977), the conception rate was lowered in sheep fed 8-16 µg DES/day and conception was prevented altogether in sheep given 32 µg DES/day (Morley, Bennett & Axelsen, 1963). The present results suggest that comparable levels of oestrogenic activity might be provided by diets containing soya products, in those whole soya meals in which quantifiable amounts of oestrogenic activity were present, levels equivalent to 8-37 ng DES/g soya were detected. On the basis of our own estimate that the potency of the mycotoxin zearalenone in the mouse bioassay is 8.5×10^{-4} that of DES, the observed oestrogenic activity of these soya meals was equivalent to 9.4-43.3 µg zearalenone/g soya.

There is little published information on the oestro-

genic activity of foodstuffs for human consumption (Schoental, 1977) and this report helps to remedy the situation. Since soya meal is an important source of protein for animal feeds and is now increasingly used in human food, we feel that this apparently constant source of oestrogenic activity should not be overlooked, even though it is at a low level. However, species differ greatly in their susceptibility to the effects of oestrogens and caution must therefore be exercised when attempting to extrapolate data from species to species or from the biological effects of one oestrogenic substance to another.

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Table 1. Oestrogenic activity of whole soya meal and of ethyl-acetate extracts of whole soya meal in the mouse uterine weight assay

Sample no.	Results for mice fed whole soya meal				Results for mice fed ethyl-acetate extracts of soya meal			
	Maximum total dose† (g whole soya meal/mouse)	Uterine wt (geometric mean, mg)	DES equivalent (ng/g soya)	Calculated zearalenone equivalent‡ (µg/g soya)	Maximum total dose† (g soya meal extracted/mouse)	Uterine wt (geometric mean, mg)	DES equivalent (ng/g)	Calculated zearalenone equivalent‡ (µg/g soya)
1	6	10.4*	<10		NT			
	6	16.5**		9.4	15	19.4***	5	5.9
2	6	11.7	<10		NT			
	6	13.9*		19.9	NT			
3	6	16.0***	10-20		NT			
	6	17.4***	17	19.9	NT			
	6	19.0***	10	11.7	NT			
4	6	11.3***	<20		NT			
	6	13.6***	10	11.7	20	18.6***	3.5	4.1
5	3	12.2**	<10		15	16.6***	4	4.7
6	3	8.5	0.0		15	19.0***	5	5.9
7	3	11.0*	<10		14	33.5***	8	9.4
8	2.5	6.3	0.0		12	29.1***	7	8.2
	5	10.5	<10		NT			
9	2.5	7.7	0.0		14	21.3***	4	4.7
	5	9.0	<10		NT			
10	2.5	10.1**	<10		12	24.0***	5	5.9
11	3	7.8	0.0		12	15.5***	3	3.5
12	2.5	9.9	<10		12	22.8***	5	5.9
13	3	31.5***	37	43.3	15	56.5***	12	14.0
14	3	13.6***	12	14.0	15	32.7***	7	8.2
15	5	34.0***	24	28.1	15	36.3***	8.7	10.2
16	4	15.5***	16	18.7	15	25.3***	7	8.2

NT = Not tested

†The mice ate poorly, and therefore the dose is only approximate.

‡By the mouse uterine weight assay the zearalenone equivalent per unit wt of DES = 1170 (850-1600).

§Oestrogenic activity present at levels equivalent to <10 ng DES/g soya could not be quantified.

The values marked with asterisks differ significantly from those of the controls that were given soya-free semi-synthetic feed (*P < 0.05, **P < 0.01, ***P < 0.001). Sample 1 was a soya-bean meal used in the manufacture of rat food. Samples 1-14 were soya-bean meals used for the manufacture of farm-animal feeds. Samples 15 and 16 were soya-bean products intended for human consumption.

Calcium at calving

Letters to Editor

Sir, — Further to the article in *Dairy Exporter* (March, page 32) I continue the discussion on the crucial role of calcium around calving.

Scientists at the national animal disease centre USA (1969) found that when calcium levels of dairy cows declined, hydrocortisone hormone is an antagonist of the action of vitamin D, parathyroid hormone and insulin: a metabolic disaster.

It is now known that plasma zinc levels decline (in hours) as plasma corticosteroid levels rise, which indicates that calcium and zinc levels decline together.

In 1976 a professor in Greece wrote to me that in their situation they needed to double the dietary zinc intake of ruminants for optimal performance. Fodder zinc levels in Greece and NZ are similar (about 35ppm). Greek researchers considered a plasma zinc level kept at about 100ppm was normal and a level of 50ppm was deficient.

In August 1974 Massey University studied plasma zinc status of dairy cows in the periparturient period. A marked fall in blood plasma zinc values was recorded before and at the onset of calving in mature dairy cows. Plasma zinc levels in 14 cows were below 100 ppm (6 ranged from 20ppm to 60ppm) and in 3 cows above 100 ppm in the 17 cows monitored. The reason was not identified; I suggested it was hypocalcaemia (calcium not measured).

A US study of the zinc-deficient rat concluded an outside source of zinc was required at parturition because zinc body stores (like cal-

cium and magnesium) could not be mobilised fast enough during the high demand of stress. Animals with low plasma zinc status consume little food and nibble all day. The zinc-deficient rat ate an average 2.5g of food on the day of delivering the young, the zinc-supplemented rat ate 12.5g of food. The following day average food consumption was 10g in the deficient rat, 15g in the supplemented rat. Within a week the zinc-supplemented rats were eating about twice as much as the deficient rats, with appropriate weight differences.

My detractors are reported as stating zinc-deficient NZ livestock are unknown. They have forgotten the Canterbury episode in the late 1970s when 70 pigs were slaughtered in a foot and mouth scare; the pigs were found to be zinc-deficient.

Gladys Reid,
Te Aroha

Zinc deficiency

Sir, — I recently described the crucial role of zinc in uterine fluid in nutrition of the embryo (February, page 94). Another crucial nutrient in the developing mammal is the thyroid hormone. Both of these nutrients are crucial to the development of epithelial cells (skin cells and neural epithelial cells of the embryo and cells lining the lumen of the thyroid gland).

Research into parakeratosis (the thickened horny folded layers of flaking skin) in the pig in 1969 by the goitre inducer thiouracil demonstrated that the epithelial cells lining the lumen of the thyroid gland failed to secrete

the compound (colloid) which absorbs iodine in the thyroid gland, and goitre developed.

Thiouracil is a goitre inducer produced in brassica plants and in some species of *Escherichia coli* (e. coli) bacteria found in animal and human excreta. The classic experiments of McCarrison in 1906 demonstrated the incidence of goitre increased in villages as they went downstream from 12% to 45%. Poor sanitation increased bacterial pollution of river water from each village. A high stocking rate on NZ farms, particularly in winter, increases e. coli levels on pasture and in puddles from animal dung.

In a German experiment (1969), 2 groups of pigs (39kg weight) were either treated with 1g methyl thiouracil daily or left untreated. After the 11th week of the test every animal in both groups was given 1g zinc sulphate daily. For the first 18 days no difference was noted in food consumption of poisoned and control pigs, after which the poisoned pigs ate less, gained less weight and huddled together for warmth. In pigs butchered at 10 weeks of the experiment, the thyroid gland and skin of thiouracil-treated pigs was typical of parakeratosis of zinc deficiency. The thyroid epithelium was thickened and no longer secreted the colloid which absorbs iodine.

All animals left were given zinc sulphate from 11 to 16 weeks and butchered at 16 weeks. The thyroid poisoned pigs showed clear improvement of skin and thyroid gland. The epithelial cells lining the thyroid lumen became normal and again secreted colloid. It was stated the addition of thiouracil to the animal diet, in spite of sufficient zinc and normal calcium, led to parakeratosis in the pig. It was considered the zinc dosage was too low to counter the effect of this poison in the thyroid gland, yet improvement was noted.

In 1980 research at Michigan State University described goitre in dairy cows, poisoned with excess iodine. Illustrations of this syndrome in cows are identical to zinc-responsive cows in Greece and thyroid poisoned pigs in Germany — scaly skin, no hair around the eyes, tears and runny nose. The goitrous cows in USA had severe reproductive problems, a greater frequency of infections and a high calf mortality.

In 1976 a professor in Greece

wrote that for optimal performance the zinc content of the ration needed to be 80 to 100ppm. Ruminant rations were seldom above 35ppm. An NZ survey (Oct-Nov 1970) described pasture zinc levels mostly about 30ppm and plasma zinc in ewes ranging from 38 to 70ppm with 1 sample reaching 104ppm. The report stated the results were unexpected.

The disaster for NZ farming is that for a generation agricultural authorities have failed to take into account the requirement of zinc above dietary needs, except for facial eczema protection.

It appears to me that zinc protection of the thyroid gland may have a profound effect on herd fertility. The estrogenic mycotoxin zearalenone may be a thyroid gland poison. Estrogens are involved in thyroid function, which suggests a toxic estrogen is likely to be a thyroid poison. It is time to find out.

Goitre has long been known to induce serious reproductive failure and failure of foetal development. Iodine supplementation for goitre, induced by thiouracil blocking iodine uptake by the thyroid, is useless. Research in the pig demonstrated that zinc reversed the toxic effect of thiouracil.

Supplementation of the thyroid hormone (thyroxine, T4) or with selenium in the presence of thiouracil is also likely to be futile, as the active form of T4 is triiodothyronine (T3). Conversion of T4 to T3 requires a selenium-converting enzyme, the function of which is blocked by thiouracil. To the best of my knowledge there has been no research into the effect of zinc in reversing thiouracil blocking of the function of this selenium-converting enzyme in T4 to T3 conversion.

To apply this research to the NZ situation means that this role of selenium is blocked when pastures are highly contaminated with animal excreta.

It is an indictment on farm leaders that they have never questioned the statements of spokesmen, unqualified to voice an opinion on this field of research, debunking the role of zinc above dietary needs to counteract the effect of certain poisons.

A professor of pathology pointed out to me that the hair loss and skin lesions of zinc deficiency were typical of thyroid insufficiency, which confirms the German research on thiouracil in the pig.

Gladys Reid,
Te Aroha

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Cows need clover in later lactation

Clover is important in cows' diet in the second half of lactation, says Dr Sharon Harris, Dairying Research Corporation feed production group, Hamilton.

Loss of white clover to clover root weevil from a typical Waikato or Bay of Plenty dairyfarm would cost \$14,300 in extra nitrogen fertiliser to compensate for N not fixed by clover, and \$13,200 in lost production from December 1 to the end of the season.

The average farm would be of 76ha milking 210 cows over 232 days with normally 16% clover content in the pasture.

"Can you afford to lose around \$360/ha/year from loss of white clover," she asked dairyfarmers at a meeting near Morrinsville on clover root weevil.

Nitrogen fixation

Clover fixes on average 210kg N/ha/year. This rate is affected by a number of factors, but mainly the level of clover in a pasture. If all clover disappeared, the soil N levels would gradually run down, increasing the cost of N fertiliser application.

Boosting N fertiliser use may have environmental costs.

Nutritive value

White clover has the highest nutritive value of any grass or legume commonly found in dairy pastures.

Milk yields increase with increased levels of white clover in pastures because of increased drymatter intake, increased nutritive value of feed. There is no change in milk composition (fat and protein %).

Summer / autumn

The biggest benefit from white clover lies in increased summer/autumn milk yields rather than in spring because clover growth (at maximum in summer / autumn) complements ryegrass growth (at maximum in spring), clover maintains its nutritive value during summer while ryegrass quality decreases after flowering.

Greg Mills, Livestock Improvement Advisory consulting officer, Hamilton, told the Tātuanui farmers meeting that information from Australia suggests dairyfarmers should aim for greater milk solids before December 31. Production

TABLE 1: A selection of characteristics of perennial ryegrass, tall fescue, white clover, red clover and chicory (scale, 1 = poor, 5 = excellent).

	Perennial ryegrass	Tall fescue	White clover	Red clover	Chicory
Drought tolerance	2	4	2	4	4
Mixture compatibility	3	3	4	4	3 to 4
Ease of establishment	5	2	3 to 4	4	4 to 5
Ease of management	4 to 5	4	4	4	4
Animal acceptance	3	3 to 4	4	5	5
Summer growth	1	5	3 (5)	5	5
Autumn growth	3	5	5	5	5
Winter growth	3 (5)*	3	3 (5)	1 (3)	0
Spring growth	5	3	5	3	5

* The bracketed score reflects the modern cultivars of the species.

before this date is a key target for profitability.

Northern Waikato TAPP group averages 525kg MS/ha but the top 5% of farmers reach 700kg MS/ha before December 31.

If clover root weevil makes inroads into clover production, options include -

- 1) Replacing lost clover N with fertiliser N;
- 2) Making every attempt to maintain white clover in the sward;
- 3) Accepting that clover root weevil will affect white clover and adjusting farm management accordingly.

Option 1 provides a benchmark cost of about \$360/ha. Any other option must cost less than that.

"White clover is only part of the pasture growing system - don't forget the rest of it."

Dr Han Eerens, AgResearch agronomist, Ruakura, reminded farmers of the potential of red clover for milk solids production. Red clover is more tolerant of clover root weevil and gives more summer and autumn growth than white clover, but less in winter/spring. It is sensitive to stock treading, breaking its taproot would kill the plant.

Researchers are screening large numbers of white clovers to find a resistant line. "The chances are

not good." It would take 10 years before a resistant line could be bred in quantities to tackle the clover root weevil problem.

Red clover was 1 of 3 alternative species to white clover discussed by Dr Eerens.

Red clover does not appear to be as sensitive to clover root weevil attack. It is deeper rooting than white clover which provides it with better drought tolerance.

Red clover plants develop a

tolerance, grass grub tolerance and higher summer quality. Farmers have used tall fescue successfully in Waikato and, according to their observations, the key to success is ensuring that it is never allowed to become rank (quality and palatability will decline rapidly).

Summer decline in milk solids production is far less on tall fescue than on ryegrass, resulting in a higher daily production over the summer and an extended season.

Nutritive value of summer ryegrass and clover:-	Perennial ryegrass	White clover
Protein (g/100g DM)	16.9g	23.3g
Digestibility (%)	67.8%	74.0%
Metabolisable energy (MJ/kg DM)	10.5	12.0

single seeding taproot, damage (generally stock treading in moist conditions) to this taproot needs to be avoided. Phyto-oestrogens in the new cultivars are 65% lower than in the older cultivars, greatly reducing the risk of problems with animal production.

Friendlier

Tall fescue is a more 'clover friendly' grass than perennial ryegrass, with up to 60% more clover measured in tall fescue/white clover pastures than ryegrass / white clover pastures, Dr Eerens said.

Added to that is better drought

Chicory is a high-quality, high-productive forage herb. Its deep root confers drought tolerance. Animal production levels on chicory pastures are on a par with white clover, and the bulk of its drymatter production takes place in summer. As with red clover, the plant forms a single taproot and damage to this root needs to be avoided.

Animal acceptance of this herb is high, but judicious management is required to maintain this.

Early impact 000679

AgResearch Ruakura scientist Dr Stewart Ledgard told the meeting that in its early years of establishment, red clover is higher producing and fixes more N than white clover.

Dr John Hay, assistant general manager AgResearch Grasslands in Palmerston North, confirmed that red clover is more tolerant of clover root weevil than white clover. But it is not resistant to the pest.

Milk yields of cow grazing pasture with different clover contents (Dec 1997):-

Clover content (% total DM)	Milk yield (litres/cow/day)	ME of pasture (MJ/kg DM)	Intake (kg DM /cow/day)
1%	12.9	10.8	13.5
22%	15.6	11.3	14.1
47%	17.6	11.9	15.4
70%	17.8	12.1	15.5

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- 1** ■ New to evaluate
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Recommendation	Cultivated	Spray Drill
Bronsyn perennial ryegrass	18-22	14-18
Aran white clover	2	1
Sustain white clover	2	1
Total kg/ha	22-26	16-20

- 2** ■ Proven performance
 ■ Most robust mix

Recommendation	Cultivated	Spray Drill
Yatsyn 1 perennial ryegrass	8-10	7-9
Vedette perennial ryegrass	8-10	6-8
Kara cocksfoot	2	1
Sustain white clover	4	2
Total kg/ha	22-26	16-20

- 3** ■ High yields
 ■ Summer & winter growing varieties
 ■ High rust resistance

Recommendation	Cultivated	Spray Drill
Vedette perennial ryegrass	9-11	7-9
Dobson perennial ryegrass	9-11	7-9
Aran white clover	2	1
Sustain white clover	2	1
Total kg/ha	22-26	16-20

- 4** ■ Spread of flowering for easier management
 ■ Impact (late flowering) plus Vedette (early growth)

Recommendation	Cultivated	Spray Drill
Impact long rotation ryegrass	9-11	7-9
Vedette perennial ryegrass	9-11	7-9
Aran white clover	2	1
Sustain white clover	2	1
Total kg/ha	22-26	16-20

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Line	Year 4 1990/91	Year 5 1991/92	Year 6 1992/93	Mean
Prop	799 bc	1682 b	1668 bc	1383 c
Tahora	1060 b	2598 a	1792 b	1822 b
Demand	587 c	1530 b	1332 c	1180 c
Hula	669 c	1279 b	1510 c	1182 c
Kopu	605 c	1166 b	1308 c	1026 c

Total annual white clover Herbage accumulation (kg DM/ha/vr) from 6 white clovers in years 4 to 6 at Ballantrae Research Station

Source: Chapman and Caradus 1996
Ag Research Ballantrae Results



GRASSLANDS COLENZO RED CLOVER.

KEEPS ON KEEPING ON

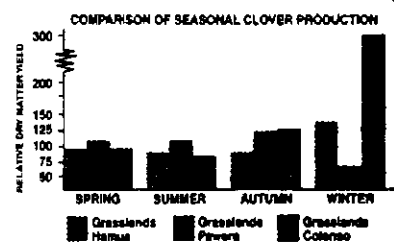
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Effects of plant oestrogens in ruminants

M.F. McDONALD

Department of Animal Science, Massey University,
Palmerston North

ABSTRACT

Research on phytoestrogens in herbage and metabolites in the ruminant has arisen owing to their deleterious effects on reproduction, particularly in sheep and to a limited extent in cattle. Plants containing these compounds include some of the economically important pasture or forage plants of the Leguminosae family and it is a paradox that while they play a valuable role in the improvement of soil fertility, and animal growth and production, they also can reduce animal fertility and prolificacy. The female reproductive cycle is markedly affected in contrast to sperm production in the male. Oestrogenic activity in forage plants also can have positive effects on mammary gland growth, lactation, and animal growth.

With breeding ewes grazed on oestrogenic clover prior to, and during mating they suffer from a temporary infertility from which they recover within a few weeks after removal to non-oestrogenic pasture. Prolonged grazing on oestrogenic clover for several years, may result in a permanent and progressive infertility in ewes. Formononetin, the main isoflavone present in subterranean clover and red clover is implicated in these reproductive problems; it is not oestrogenic itself but is metabolized mainly to equol in the rumen, and equol is oestrogenic. Coumestans and other oestrogenic substances associated with fungal growth on plants will also reduce reproductive performance particularly ovarian function and the early events in development of the embryo.

Protection against the deleterious actions of phytoestrogens, such as immunisation against equol or coumestrol in sheep grazing clovers or lucerne is feasible but not developed as a control technique. Forage plants with low levels of phytoestrogens is the long term goal. Fortunately management plans can be devised to avoid feeding oestrogenic herbage at crucial times to breeding livestock.

INTRODUCTION

The realisation that some pasture plants can cause serious infertility in sheep was first observed in Western Australia and it has been the stimulus for much of the work on phytoestrogens and their actions in the grazing animal. Dramatic reductions in lambing percentages have occurred when breeding ewes have grazed pastures containing a high content of subterranean clover (*Trifolium subterranean*) and the infertility resulting has frequently been termed "clover disease". Affected sheep grazing subterranean clover will have ingested phytoestrogenic compounds of variable potency over several months and even years, causing some animals in extreme cases to become sterile or show reduced fertility and flock lambing percentage to progressively decline.

Other varieties of clover also contain phytoestrogens but usually in reduced amounts compared to that in subterranean clover. An exception is red clover, (*T pratense*) which is widely used in pastures in many countries especially in temperate regions and reproductive problems have frequently been noted (Moule *et al.*, 1963). Fortunately white clover (*T. repens*) which is the main legume in most New Zealand pastures has low levels of

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phytoestrogens, although if subjected to fungal attack it too may show increased oestrogenic levels.

The main role that clovers have in mixed grass - clover swards through their N-fixing ability is to increase soil fertility and thus higher total herbage production. As well, clovers are highly digestible in the ruminant and will cause increased liveweight gain compared to other herbages. Oestrogenicity in the pasture may also give an added contribution to the growth rate in animals such as with lambs being fattened for slaughter (Oldfield *et al.*, 1960; Trenkle, 1969).

Other herbages like lucerne (*Medicago sativa*) which are intermittently grazed by sheep and cattle or which are grown for hay crops and used in concentrate feeds, may also contain oestrogenic compounds. Lucerne will have high levels of oestrogenic substances especially when it has suffered from aphid attack and this will be associated with climatic conditions (Kain and Biggs, 1980). With newly grown lucerne, and particularly when grazed at a young age, the plants should be free of these oestrogens. This will not be the case with mature stands of herbage and particularly when infected with leaf spot or some foliar disease. Spraying the herbage with fungicide may be adopted but utilisation of the lucerne before any build-up of oestrogen should be the preferred option for avoiding potential problems in breeding animals.

Mycotoxins that ruminants acquire while grazing may also induce oestrogenic effects. Thus zearalenone, an oestrogenic mycotoxin produced by *Fusarium* spp. is a common contaminant of pastures in New Zealand and ingestion of zearalenone is associated with poor fertility in ewes on some farms. This is illustrated by the results from the farm surveys conducted by Towers *et al.*, (1992) shown in Figure 1.

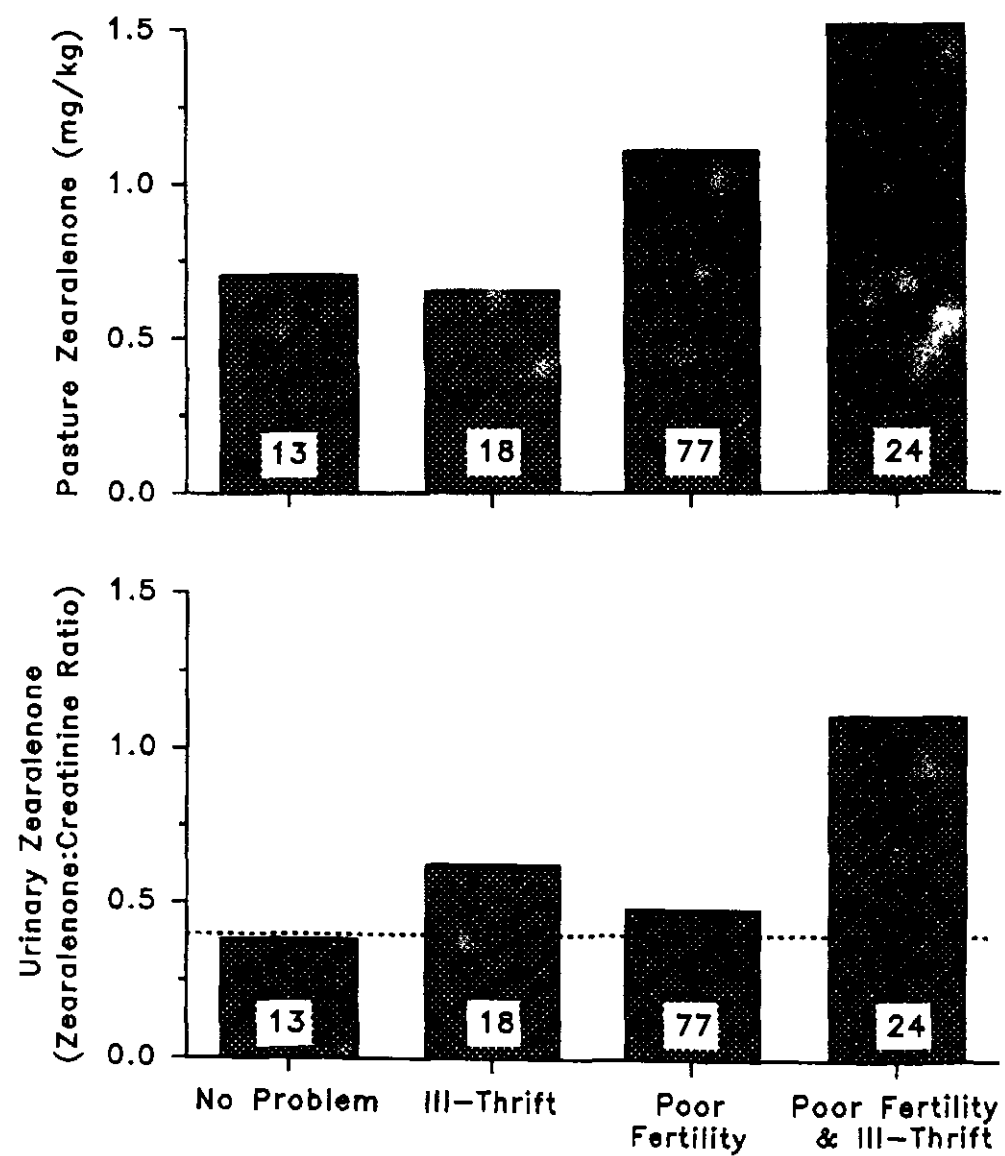
Phytoestrogens and Metabolic Products

The compounds in plants which cause oestrogenic effects in the animal are from two major groupings: Isoflavones - mainly formononetin, genistein, biochanin A and daidzein, Coumestans - principally coumestrol, and methyl coumestrol. The isoflavones are mainly found in the clovers and coumestrol is important in lucerne and other medics but the groupings are not specific to particular species of plant. Some of the isoflavones are found in high concentration with combined levels of up to 5% of leaf dry matter (Beck, 1964). Although only weakly oestrogenic (about 10^{-5} times as active as oestradiol) they have a significant biological effect in animals because of the large quantities ingested (Collins and Cox, 1985). Isoflavones are present in the plant usually as glycosides and are hydrolysed by glycosidases during digestion.

Figure 2 shows the metabolic pathways for the isoflavones. These compounds when biologically tested in mice show different oestrogenic effects than when tested in the sheep. Genistein and biochanin A are metabolized in the sheep rumen to non-oestrogenic phenols. In contrast formononetin is metabolized to equol with small amounts of methyl equol, daidzein, and desmethyl angolensin, all of which are oestrogenic in sheep (Shutt *et al.*, 1970). Rumen micro-organisms will have a major effect on the oestrogenicity that develops for particular phytoestrogens.

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Figure 1: Numbers of farms surveyed for zearalenone levels and production problems in sheep (Towers et al., 1992).



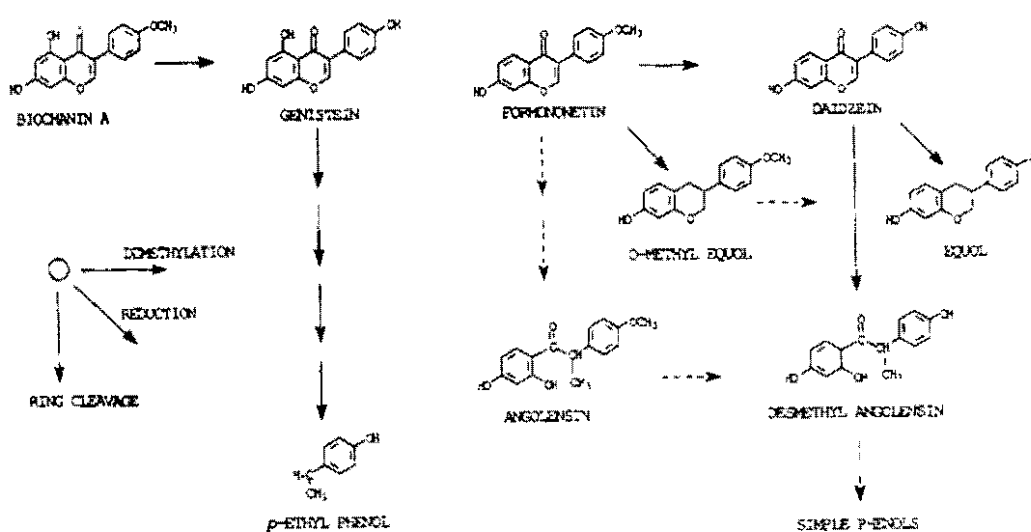
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Figure 2. Metabolic conversions of isoflavones in the sheep. Broken arrows indicate probable changes. The reactions in the metabolic lattice are shown in the insert (Cox et al., 1984).

5-HYDROXY ISOFLAVONES

5-DEOXY ISOFLAVONES

(occurring as glycosides in legumes)



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Very little formononetin will pass in the digesta from the rumen. Equol production is almost entirely in the rumen and it is readily absorbed at this site (mean residence time of about 2h). The isoflavones and equol are present in blood plasma mainly conjugated as glucuronides or small amounts (5-15%) as sulphotoconjugates (Cox and Braden, 1974). Equol can reach levels of 300-500 µg/100ml plasma with about 1% present in the biologically active unconjugated free form. Excretion of phytoestrogens and their metabolites is mainly in the urine. Most of the equol (80%) produced is excreted in 48 hours. Coumestrol and methyl coumestrol and other coumestans in small amounts have been isolated from lucerne and medic. These substances are about 10^{-3} times as active as oestradiol but their levels in plants are usually low. In the absence of fungal infestation controlled by spraying with a fungicide, no coumestans were found in lucerne. In contrast coumestan concentrations will reach appreciable levels in lucerne and even in white clover in response to fungal diseases (Smith *et al.*, 1979; Wong *et al.*, 1971). Other environmental stresses imposed on the plant by aphid damage, water deficit or lax grazing management will increase coumestrol levels to high values (>1000 ppm) whereas normal levels should be only a few ppm and not produce oestrogenic effects. Coumestrol is absorbed from the rumen and high proportions (20 to 50%) circulate as unconjugated or sulphotoconjugated products.

The effects of oestrogenic pastures and forages

The effects of ingesting oestrogenic compounds in the ruminant should be anticipated with a variety of animal responses. These include anabolic and lactation responses as well as effects on reproduction. A spurt in mammary gland growth and even secretion is often noticed in immature sheep and breeding ewes grazed on some clovers and lucerne. There is a biological assay for plant oestrogens in sheep based on the increase in teat length of wethers (castrate males).

Serious reproductive problems occur mainly in sheep. There are relatively few major problems reported for other ruminants but there are sufficient isolated instances of reproductive disturbance in pasture-fed cattle to suggest the possibility of phytoestrogens being a factor.

Sheep - Oestrogenic subterranean clover and red clover can cause infertility by two distinct mechanisms which results in either temporary or permanent infertility. The two conditions may occur concurrently, but they differ in both mechanism and epidemiology (Adams, 1987). Exposure to green, oestrogenic subterranean clover for 6 to 8 months annually may result in permanent, cumulative infertility after two years which persists after removal from oestrogenic pasture (Adams, 1987). The syndrome of clover disease included uterine prolapse, dystocia, and permanent infertility with very low lambing rates. Permanent infertility has been induced experimentally in ewes after prolonged grazing on red clover (Barrett *et al.*, 1965; Shackell *et al.*, 1993) or on subterranean clover (Davenport, 1967). There is a lack of ovum fertilisation due to impaired transport of semen through the cervix. Not all ewes in the group will be completely barren (Lightfoot *et al.*, 1967) and may show resistance to the effect of ingesting oestrogen. Adams (1987) considers that under some conditions ewes may graze oestrogenic clover with impunity. Selection of ewes for resistance to clover infertility has given promising results (Croker *et al.*, 1989).

Temporary infertility is recorded in ewes grazing oestrogenic clover around mating time and is characterised by a lower ovulation rate and an increase in returns to service (Lightfoot and Wroth, 1974); fertility recovers by 5 weeks after removal from non-oestrogenic pasture. Reproductive disorders in ewes after ingestion of coumestans include

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inhibition of oestrus (Kelly *et al.*, 1979) and a reduction in ovulation rate (Smith *et al.*, 1979). There have been no reports of coumestans causing permanent infertility. Rams which are grazing with the ewes appear to be unaffected and have normal levels of fertility (Marshall, 1973).

Cattle - The extent to which phytoestrogens may cause deleterious effects in cattle is not clear. Although there are many instances in the literature of reproductive disturbances in cattle consuming oestrogenic forages, a permanent infertility has not been noted. Anoestrus, and difficulty in the detection of oestrus, and a high incidence of service returns can occur in cows grazing subterranean clover (Thain, 1966). Lactation in maiden heifers, cystic degeneration in the ovaries, irregular heats, indeterminate discharges, anovulation and abortions have also been reported. However, in Western Australia, where cows are frequently exposed to oestrogenic subterranean clover and where problems in sheep are so common, there are no authenticated reports of associated infertility in cows (Adams, 1987).

Deer - There does not appear to be any reports of reproductive problems in breeding hinds or stags associated with grazing clovers which might contain phytoestrogens. At Massey University over 3 years breeding hinds have been offered red clover pasture during the year except for one month prior to and during the period of rut; fertility was similar to that in hinds grazing ryegrass - white clover (control) pasture (P.N. Wilson and T.N. Barry, unpubl.). Also the developmental changes in the fawns on red clover were similar to control fawns. Trials have yet to be conducted that record the ingestion of phytoestrogen during the mating period.

Mechanism of temporary infertility

As oestrogens act at a range of body sites it is not surprising that several critical events in the reproductive process may be modified when breeding ewes consume phytoestrogens. Oestrus - oestrogenic pasture may cause a decrease in the incidence of oestrus (Lightfoot and Wroth, 1974), induce oestrus without ovulation (Chang, 1958) or the incidence be unaffected. Ovulation rate - a depression in ovulation rate is frequently noted after oestrogenic pasture has been consumed. Disturbances in the development of follicles leading to ovulation are implicated (Adams, 1987). The deleterious effect of phytoestrogen on ovulation rate was not related to the failure of oestrogenic pasture to sustain body weight/condition, as the sheep grazing such pastures either had liveweights similar to the controls (Smith *et al.*, 1979) or gained more weight (Kelly *et al.*, 1980). Ovum fertilisation and gamete transport - A reduction in fertilisation rate of ova, and fewer sperm attached to the egg suggest some impairment of the movement of sperm to the site of fertilisation in sheep ingesting phytoestrogen (Holst and Braden, 1972). Sperm transport will be affected by disturbances in cervical mucus production (Adams, 1987). Embryo mortality - Grazing ewes on oestrogenic clover may cause oviductal hyperplasia and uterine oedema (Adams, 1987) with a likely effect on the developing embryo. However, the evidence for additional embryonic mortality induced by phytoestrogens is inconclusive (Anwar *et al.*, 1993). Returns to service and lambing performance - Many studies show that oestrogenic pasture causes reduced conception rates and a reduction in the number of multiple births. Feeding red clover for 8 days before and during the first cycle of mating resulted in higher returns to service than in control animals (Kelly *et al.*, 1980). The effects on conception persisted for at least 3 weeks after removal from red clover, but an earlier recovery of ovulation rate occurred.

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Finally the long term goal in avoiding some of the above problems is the need to develop strains of pasture plants with lowered levels of phytoestrogens consistent with good agronomic and nutritional features.

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Mechanism of permanent infertility

The prolonged exposure to phytoestrogen in the sheep causes permanent changes in the brain and in the genitalia but the most severe abnormality was in the structure and function of the cervix (Adams, 1990). The cervical folds fuse together, the lamina propria becomes thicker and more cellular and coiled tubular glands similar to uterine glands develop. The mucus produced is abnormal being thin and watery and no increase in secretion occurs at oestrus. Few sperm therefore traverse the cervix and reach the oviduct; failure of fertilisation commonly occurs.

Avoidance of reproductive disturbances

Recognition by livestock managers of the potential losses that might occur after ruminants consume oestrogenic compounds is important. In New Zealand the message to avoid feeding sheep and cattle with potential oestrogenic herbage is probably acted upon in most cases or at least the risks assessed. Isolated occurrences of reduced fertility due to the presence of phytoestrogens will be more an individual farmer problem than a national one.

In New Zealand subterranean clover is important in the drier parts of the country. The effect of sowing varieties of subterranean clover relatively low in phytoestrogen will have reduced potential problems. As well, when sheep are mated in the autumn subterranean clover will be a small component of the diet. This is in contrast to systems in Australia where mating both in the spring and autumn occurs.

The use of red clovers in the pasture mix can be managed according to the percentage of clover composition and the level of phytoestrogen can be "diluted" by other herbage. Where clover dominance occurs then the pasture should be used for other stock rather than breeding ewes prior to mating. Using the oestrogenicity in clover to promote growth in lambs or other young stock is an alternative option. Hay and silage made from herbage containing oestrogens (such as pastures predominantly of red clover or lucerne in the summer - autumn period) will retain most of the phytoestrogen after harvesting and storage. Breeding sheep and cattle are often fed these supplements but as it comprises only a small part of total intake or fed at non critical times for the animal's reproductive process, then practical problems are avoided. Immunisation of sheep against phytoestrogens offers a procedure for reducing some of the effects of these compounds. Antibodies specific to some isoflavones have been produced and include neutralization of biologically active equol. Further, some of the potential reduction in ovulation rate (and hence twinning rate) noted in ewes fed lucerne containing coumestans can be overcome when antibodies against oestrone or androstenedione previously had been developed in the ewes (Smith *et al.*, 1982). Knowledge that sheep had been treated with commercially available immunisation products might allow less risk in the feeding of some clovers and lucerne as flushing feeds for ewes.

Modification of rumen metabolism to reduce the formation of oestrogenic products has been suggested as a control technique; formononetin might be degraded in the rumen to form inactive non-oestrogenic products similar to that with genistein and biochanin A, or demethylation of formononetin might be impeded (Adams, 1990). In flocks studied for clover disease some animals will show strong resistance to the damaging effects of phytoestrogens. It seems quite likely that such animals may well have developed systems to more rapidly detoxify and reduce the high levels of oestrogenic compounds and avoid the anticipated depression in fertility. In New Zealand the dramatic effects of very high intakes of phytoestrogens are unlikely or can be avoided through management actions, however, as these compounds are widespread in herbage the grazing ruminant will perform against a background of at least low levels of phytoestrogen intake.

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REVIEW ARTICLE

Potential Value of Plants as Sources of New
Antifertility Agents I *

NORMAN R. FARNSWORTH*, AUDREY S. BINGEL, GEOFFREY A. CORDELL,
FRANK A. CRANE, and HARRY H. S. FONG

Keyphrases □ Antifertility agents—review of plant sources, classified by anatomical mechanism and folkloric route of administration □ Medicinal plants—sources of antifertility agents, classified by anatomical mechanism and folkloric route of administration, review □ Plant extracts—potential sources of antifertility agents, review □ Contraceptives—plants with active constituents, review □ Abortifacients—plants with active constituents, review □ Oxytocics—plants with active constituents, review □ Estrogenic plants—review of active principles

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*Editor's note: Part II of this article will appear in the May 1975 issue of the Journal of Pharmaceutical Sciences

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The world population explosion has pointed out the need for new and effective contraceptive agents and/or methods, having a minimum of side effects and giving a maximum protective effect. To date, the most effective and widely used contraceptives have been steroids, but these are not without side effects (1, 2).

Practically all major research to date involved with the search for new oral contraceptives has been of the synthetic type, particularly the preparation of steroid derivatives. Very little attention has been directed to the plant kingdom. There are those who continue to argue that plant products are of little importance as drugs. However, an analysis of some 1.05 billion new and refilled prescriptions dispensed from community pharmacies during 1967 showed that 25% contained one or more active principles derived from higher plants (3). Only three of the higher plant products (papaverine, ephedrine-pseudoephedrine, and caffeine) are produced commercially by synthesis, the remainder still being produced by extraction from

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their variables, abnormal mental status, would mandate roentgenograms because an adequate history could not be obtained. We were a bit surprised that in analyzing our data abnormal neurologic findings did not mandate roentgenograms. Dr Binns informed us that of the eight patients in their series who would have been missed if our criteria had been used, six had abnormal neurologic findings and two had direct neck trauma. Again, we assumed that if abnormal neurologic findings were present then cervical spine films would be indicated.

Our purpose in reporting our findings was to (1) direct attention to the overuse of cervical spine films in childhood trauma and (2) to encourage others to test the validity of our clinical assessment model. We feel both of these purposes have been fulfilled by our publication. We hope that clinical judgment will continue to be the mainstay in all decision-making processes, especially when using algorithms or assessment models, since models are only accurate when used in conjunction with sound clinical judgment.

BURRIS DUNCAN, MD
Department of Pediatrics
JOHN BJELLAND, MD
Department of Radiology
University Medical Center
1501 N Campbell
Tucson, AZ 85724

BARBARA SIBLEY
School of Medicine
W. THOMAS BOYCE, MD
Child Study Unit
University of California—
San Francisco
400 Parnassus Ave—Room A-203
San Francisco, CA 94143
INGRID RACHESKY, MD
316 W 11th St
Panama City, FL 32401

Firearms and Child Safety

Sir—Patterson and Smith¹ state "children are frequently victims of gunshot wounds." It puzzles me that in over 17 years of pediatric practice in a large city, not one of my patients has been wounded, let alone killed, by firearms.

The authors further state that "more than half of all deaths from firearms occur in homes where guns are frequently kept loaded and easily accessible." This is possibly true, but it does not say whether innocent bystanders or criminals are being shot.

The authors state "the often-quoted statistic that a gun in the home is six

times more likely to kill a friend or family member than an intruder." It is true that the statement is often quoted, but that does not make it true! In fact, the statement is false as documented by Federal Bureau of Investigation statistics.

Thousands of honest citizens successfully use a handgun to protect themselves and their loved ones every year. A gun in a child's environment is not as dangerous as an automobile, but like anything else to which a child is exposed, the parents must assume a protective role.

I think pediatric journals are an excellent forum for discussion of the firearm controversy, but I wish the editors were as critical of the authors' sources of "facts" as they are about scientific discussion.

GENE W. HOLLINGSWORTH, MD
Pediatric Associates, SC
5758 N California Ave
Chicago, IL 60659

¹ Patterson PJ, Smith LR. Firearms in the home and child safety. *AJDC* 1987;141:221-223.

In Reply.—The week I received Dr Hollingsworth's letter dismissing the threat of firearms, five children, aged 3 to 13 years, were injured by firearms in Houston (*Houston Chronicle*, June 22, 1987). Other areas of the country are also reporting unintentional deaths resulting from children shooting themselves or other children.¹

The majority of unintentional deaths by firearms occur in the home.² A recent study³ found that 53.6% of firearms-related deaths occurred in the home where the firearm was kept. Of these 398 deaths, only two cases (0.5%) involved an intruder being shot during an attempted entry.

I am not sure that Dr Hollingsworth can substantiate his statement that "a gun in a child's environment is not as dangerous as an automobile." Neither object should be used by children or any person who has not been properly trained to use it without endangering his or her own life or that of others.

Unintentional firearms deaths in children are preventable. The incidents are often precipitated by a gun being left loaded and accessible to a child.¹

Of course, parents must assume an active role in protecting their children. Pediatricians are also responsible for making every effort to ensure the safety and well-being of all children.

PATTI J PATTERSON, MD
Texas Department of Health
Personal Health Services Division
1100 W 49th St
Austin, TX 78756

¹ Wintemute GJ, Teret SP, Kraus JF, et al. When children shoot children: Eighty-eight unintended deaths in California. *JAMA* 1987;257:3107-3109.

² Rushforth NB, Hirsch CS, Ford AB, et al. Accidental firearm fatalities in a metropolitan county (1958-1973). *Am J Epidemiol* 1975;100:499-505.

³ Kellermann AL, Reay DT. Protection or peril? An analysis of firearm-related deaths in the home. *N Engl J Med* 1986;314:1557-1560.

Premature Thelarche in Puerto Rico

Sir—I read with interest the article about premature thelarche in Puerto Rico in the December 1986 issue of *AJDC*.¹ The authors found positive statistical associations between premature thelarche and consumption of various meat products and soy-based formula, and a maternal history of ovarian cysts. Although the retrospective nature of the study leads to the possibility of measurement bias due to inaccurate recall, the authors should be commended for the methods they used to minimize this bias via a standardized two-part interview administered by two different interviewers. The selection process utilized for obtaining case subjects and controls (matched pairs) add strength to the selection process. Other strengths of the study include the multivariate logistic regression analysis to compute odds ratio, with exact confidence intervals to determine significant statistical associations.

I agree with the authors' comments that the association found with consumption of fresh chicken may be due to selective recall because of media publicity about the condition and its possible association with chicken. I also agree with the authors' conclusions that the statistical associations observed are probably not sufficient to explain the increase reported for premature thelarche because of the number of cases in which there was no history of estrogen exposure. Although the authors suggest that the reported increase may be due to better diagnosing, or the presence of new unsuspected factors, they do not address the possibility of a dose-response relationship, which may be involved in some cases if there is history of exposure of an individual to several of the risk factors simultaneously. The article does not address this issue.

KARLA MONTAGUE-BROWN
Department of Community and
Occupational Health
Meharry Medical College
1005 D. B. Todd Bldg
Nashville, TN 37208

F. et al
J Pediatr
1987;110:252

B. et al
J Pediatr
1987;110:100

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1. Freni-Titulaer LW, Cordero JF, Haddock L, et al. Premature thelarche in Puerto Rico. *AJDC* 1986;140:1263-1267

In Reply.—The issue of a dose-response relationship, brought up by Ms Montague-Brown, is important. Finding a dose-response relationship is strong support that an association is not due to chance. Our data include information about the subjects' ages when various food items were introduced in the diet. We do not, however, have detailed information about the frequency of consumption and amounts of foods consumed over time. Even if such data were available, it would still not be appropriate to combine various exposures into one variable, as suggested by Ms Montague-Brown, because we would not know how much weight to assign to each factor.

LAMBERTINA W. FRENI-TITULAER, MD, MSPH

JOSE F. CORDERO, MD, MPH
Center for Environmental Health
Centers for Disease Control
Public Health Service
US Department of Health
and Human Services
Atlanta, GA 30333

Subperiosteal Bleeding With Acute Bone Infarcts in Children With Sickle Cell Disease

Sir—Infarction of the long bones in patients with sickle cell disease is associated with swelling, warmth, redness, pain, and tenderness over a part or the entire shaft of the affected bone. Bone destruction and periosteal reaction with new bone formation are usually evident approximately two weeks after the onset of symptoms.^{1,2} This clinical and radiologic picture is indistinguishable from that of osteomyelitis, and it is different from the "typical" painful vaso-occlusive crises that are probably caused by infarction of bone marrow.^{1,3} Periosteal elevation and new bone formation is believed to be due to an inflammatory reaction, edema, and exudate.^{2,4}

Report of Cases.—Needle aspiration was performed in 12 Saudi children with homozygous sickle cell disease who presented with fever and swelling, warmth, and tenderness of the long bones (five tibiae, three femora, and four humeri); 2 to 5 mL of bloody or serosanguineous fluid was obtained from each patient, which resulted in decreased tenderness within a short

time. The fluid contained few inflammatory cells; Gram's staining and aerobic and anaerobic cultures were negative. Patients were treated conservatively, with no antibiotic therapy, and all of them recovered.

Comment.—I believe that most and possibly all periosteal elevation and periosteal new bone formation associated with bone infarction in patients with sickle cell disease may be due to subperiosteal bleeding rather than an inflammatory reaction.

AHMAD A. MALLOUH, MD
PO Box 76
c/o Aramco
31311 Dhahran
Saudi Arabia

We believe that the above observation is unique but that it is possible that the "bleeding" may be secondary to inflammation.—Ed.

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Early Attainment of Sex and Race Differences in Skeletal Mass

Sir—The study by Specker et al¹ confirmed, through direct photon absorptiometry, what we previously learned about sex differences in the

growth of the skeletal mass by measurement on roentgenograms (i.e., diaphanometry).² Despite differences in biophysical measurements and the bone sites surveyed (distal forearm vs second metacarpal), the trends were much the same. Though little boys behind little girls in ossification time and skeletal development, they have larger bone mass, however it is measured. The two technical approaches are also parallel throughout the cycle.³

In our work with many thousands of children, sex differences in bone size, cortical thickness, and cortical area are a consistent finding both for boys and for girls in a longitudinal growth program³ and for those studied in national surveys.⁴ This is also demonstrable in somewhat abridged form for 12 white boys and girls who participated in the Ten-State Nutrition Survey (Table). From age 1 year onward, the boys are demonstrably larger than the girls in bone width and cortical thickness and, therefore, in cortical area. Among children in kindergarten at first grade, boys are some 4% to 5% larger in cortical area or bone mass (Table). Such early sex differences in skeletal mass have also been demonstrated in Costa Rica, Guatemala, Honduras, Nicaragua, Panama, and Salvador, despite differences in nutritional status.⁴

Besides the sex differences in bone area and bone mass that are demonstrable by radiogrammetry from an early age in children from the United States and six Central American countries,^{4,5} there are also consistent black-white differences. After the first year of life (when black infants are statistically smaller than their white age peers), black children, adolescents, and adults

Cortical Area of the Second Metacarpal at Midshaft in Boys and Girls*

		Race							
		White				Black			
		Boys		Girls		Boys		Girls	
Age, y	n	Mean Cortical Area, mm ²	n	Mean Cortical Area, mm ²	n	Mean Cortical Area, mm ²	n	Mean Cortical Area, mm ²	n
1	15	10.02	25	9.05	17	9.43	14	8.22	
2	45	12.06	35	11.00	20	12.43	30	11.60	
3	95	13.51	61	12.39	42	14.28	29	14.74	
4	123	15.30	124	14.57	55	16.93	64	16.81	
5	158	16.62	142	16.02	97	18.49	79	17.71	
6	169	18.47	171	17.60	105	20.83	123	19.90	

*Cortical area was calculated as $0.785(T^2 - M^2)$, as described by Garn,³ using data derived from the Ten-State Nutrition Survey.⁴

Examples of activities of phytochemicals

- 1) In the rodent, tobacco-specific nitrosamines induce lung tumours. When Phenethyl isothiocyanate is added tumour formation is inhibited (Chung *et al.*, 1992; Chung *et al.*, 1993). Phenethyl isothiocyanates is one of the isothiocyanate family found in water cress, green tea, and cruciferous vegetables.
- 2) Genestein, is a dietary phytoestrogen with a potency of action of 1/1000 that of oestradiol. Genistein has an inhibitory action on breast cancer cells while increasing normal apoptosis (Pagliacci *et al.*, 1994). Prostate cancer mortality is low in Japanese males who have a traditional diet high in genistein rich soy products. Genistein inhibits tyrosine-specific kinase which is necessary for angiogenesis needed for prostate cancer cell invasion
- 3) Flavonoids are widely distributed in vegetables and fruits. There are many individuals in this family and they are widely distributed in different foods at different concentrations (Herzog *et al.*, 1994). As anti-oxidants they individual specificity of site and activity of action

This symposium has been an opportunity to come up to speed with aspects of phytochemicals which increasingly have become imposed on the thinking and action of all nutritionists, medical professionals, health care workers and the food industry

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Phytoestrogens: Toxicology and regulatory recommendations

I.G.C. ROBERTSON

Department of Pharmacology and Clinical Pharmacology,
University of Auckland Medical School, Auckland

ABSTRACT

Humans are exposed to isoflavone phytoestrogens primarily through consumption of soy beans and other legumes. The main soy phytoestrogen, genistein, is well known as a biologically active agent. Most research to date has focused on the potential benefits of genistein and other phytoestrogens. However concern, supported by animal and *in vitro* data and by mechanistic considerations, has been expressed about possible adverse effects, particularly to the foetal-neonatal nervous and reproductive system. Adverse effects may occur by inhibition of the enzyme which converts the relatively impotent estrone to the much more potent oestradiol, and by occupying the estrogen receptor resulting in antagonism of the naturally produced oestradiol. Adequate oestradiol is necessary for the imprinting and development of many physical, physiological and behavioural characteristics during the neonatal period and infancy. Infants on soy-based formula have been identified as a high risk group because the formula is the main source of nutrient, and because of their small size and developmental phase. Infants absorb phytoestrogens and have a calculated daily dietary intake (per kg) 3-6 times that shown to have physiological effects in women, but no research has specifically investigated the likely effects of this exposure. In this country an estimated 800-1,600 infants per year use soy formula, ostensibly because of allergy or lactose intolerance. However, the incidence of allergy is much lower, intolerance is typically temporary, and alternatives are available. Although the risk is potential, it is avoidable. Also, soy contains a number of other potentially toxic agents that are not consistently, or at all, removed by processing. Advisories concerning trypsin inhibitors and aluminum have been placed on soy formula in the past. Possible additional regulatory approaches and recommendations for phytoestrogens in infant formula will be discussed.

INTRODUCTION

The aims of this paper are to discuss aspects of the toxicology of phytoestrogens and to make some comments on their regulation. This topic is addressed, not from the perspective of a nutritionist, an endocrinologist nor a cancer biologist, but from that of a mere toxicologist. However, it is the toxicologists who has the task of pulling together the available information from these and other disciplines and making recommendations on the use of a chemical.

Some general principles of toxicology are first discussed briefly. The human exposure to phytoestrogens and the concern expressed about such exposure are then examined (Seitchell *et al.*, 1984; Whitten *et al.*, 1995; Clarkson *et al.*, 1995; Sharpe and Skakkebaek, 1995), similar to that raised for synthetic environmental estrogens (Colborn *et al.*, 1993). The discussion concentrates on genistein, because our main exposure is to this soy bean phytoestrogen, although of course there are others (Murphy, 1982), and on infants on soy based infant formula as an example of a high risk group. Finally, some comments will be made on the regulatory recommendations.

A classical definition of toxicology is the study of the adverse effects of chemicals on living systems, or, to put it another way, the study of the chemical modification of physiological function (Conning, 1993). It must be emphasised that the circumstances may determine the perception of whether any modification is therapeutic, adverse or even neutral. This definition is very much pharmacologically based and, clearly the phytoestrogens are pharmacologically active compounds.

A search on Medline for 1991-1995 gave 696 citations for genistein. Genistein is described as a weak estrogen because of its relatively low affinity for the estrogen receptor (Shutt, 1976). Because of this its significance has been down played. But of course even a weak binder can have effects if the concentration is high enough. But there are other activities. Genistein is cited as a potent inhibitor of a number of enzymes, for instance, tyrosine kinase and topoisomerase II (see Barnes and Peterson, 1995). To this may be added the 17- β -hydroxysteroid oxidoreductase, Type 1 (Makela *et al.*, 1995) (see below). The point at this stage is that genistein is a bioactive compound with sufficient activity to be considered as a drug. There is no hesitation in regarding it as such and clearly not as a nutritional factor.

It must also be stressed that beneficial and adverse effects are not mutually exclusive. This is a very important point. If benefits are shown this does not eliminate the possibility that toxicity may also occur, possibly by the same mechanism. It is possible to have too high a dose, or different target tissues may respond in different ways, or the circumstances of use may be different and inappropriate.

The phytoestrogens may be considered as partial mixed agonists/antagonists. That is, they may have both estrogenic and anti-estrogenic activity (see Whitten *et al.*, 1995, Clarkson *et al.*, 1995). There may be benefits for post-menopausal women if phytoestrogens exhibit estrogenic activity but, on the other hand, there may be promotion of the growth of estrogen dependent tumours. Similarly, as antagonists they may inhibit the growth of tumour cells but at the same time reduce reproductive capability. Claims that phytoestrogen containing diets are beneficial are not reassuring in the least to a toxicologist. On the contrary, the greater the evidence that these compounds are active *in vivo* in humans, as opposed to in experimental studies, the greater the concern about adverse effects depending on the dose and the circumstances of use, or inappropriate use.

Why is this so? The fundamental principle of toxicology is that stated by Paracelsus that "All substances are poisons: there is none that is not. The right dose differentiates a poison and a remedy". Again, it should be stated that different targets may be affected, or that a drug may be used in an inappropriate situation giving rise to undesirable effects.

A classical definition of toxicology has been given above. There is also a modern definition (Conning, 1993). It is the study of adverse effects of chemicals, in order to predict chemical hazards to humans. There is a shift from a pharmacology base to a public health/regulatory base. At the same time there is a shift from a strict science to one involving something of an art, that of having to make predictions from a limited data base. There is often not enough information to make an unequivocal decision about the human toxicity of a chemical. If there is, then it is highly likely that the decision has been left too late. There has also been a shift from dramatic clinical effects to consideration of more subtle and delayed effects, as might be the situation here.

Exposure to phytoestrogens

In considering the possible adverse effects of phytoestrogens, a logical starting point is to ask - what are the high risk groups, who has the greatest exposure? There are other possible high risk groups with high soy intake, such as vegetarians or those on some weight reducing diets. However, the group of most interest currently is infants on soy-based infant formula. Here we have a group who receive such formula, initially at least, as their only source of food. The effective dose is magnified because of their size. Finally, this exposure is compounded because they are still at a stage where their development may be highly susceptible to hormonal based interference.

From measurements done in this country by Dr M G. Fitzpatrick (1994) on the total genistein and daidzein content of three different commercial soy formulae (it should be stressed that these measurements were made on the formulae themselves and not on the soy material before processing), together with the daily intake of formula recommended by the manufacturers, the likely daily dose of these two phytoestrogens can be calculated (Table 1). Exposures to total (conjugated and unconjugated) genistein and daidzein range from 7.4 mg/day in first few weeks to 26 mg/day after 6 months. (The variations in the groups is due to small variations in both the isoflavone content and the recommended intake between the three preparations.)

Table 1 Calculated phytoestrogen intake of infants consuming soy formula (Fitzpatrick, 1994)

Age (months)	Total isoflavones ¹ (mg/day)
< 1	7.4-9.1
1-2	11.0-15.5
2-4	13.2-19.3
4-6	14.1-25.7
> 6	14.1-25.7

¹ genistein 63%, daidzein 37%

Is this exposure significant? In a recent study (Cassidy *et al.*, 1994) women were fed 60 g of soy protein (containing 45 mg genistein and daidzein, an average daily dose of these isoflavones of 0.73 mg/kg) per day for a month. In these women the menstrual cycle was lengthened by 1.5 days. More importantly, their levels of mid-cycle luteinizing hormone and follicle stimulating hormone were decreased by 67 and 47% respectively. The effects persisted for up to 3 months and were considered to be due to the phytoestrogen exposure. These results were interpreted and discussed by the authors as being beneficial for breast cancer. However, these results would also appear to have adverse implications for fertility in young women.

Whatever the implications for fertility in women, it is clear that addition of quite modest amount of soy protein to a complex adult diet had definite physiological effects, which lasted for several months after the soy was removed from the diet. From Dr Fitzpatrick's data, the daily dose received by infants is calculated (Table 1) to be 2.3-4.8 mg/kg/day (assuming standard weights, at birth and at six months, of 3 and 6 kg, respectively) i.e. more than 3 to 6 times the dose having physiological effects in young women.

One has to be certain that these phytoestrogens are being absorbed. Cruz *et al.* (1994) measured the urinary excretion of phytoestrogens, at 4 months of age, in babies fed breast milk, cow's milk formula, or a cholesterol supplemented or normal soy formula. The data are presented as median and range (possibly to demonstrate the wide interindividual variation, but this may also reflect collection problems, which is also of concern). High amounts of genistein and daidzein were detected in the urine of babies on the two soy formulas. This indicates absorption and then excretion of these isoflavones by these infants. Also, of interest is the lesser, but detectable, amounts of genistein and daidzein in the urine of individuals fed cow's milk formula.

There has been frequent comment that Asian babies are exposed to phytoestrogens, but have never shown any adverse effects. However, there are no studies which have systematically looked for any such effects. It could also be said that there has been ample opportunity for selection and adaptation. However, at this stage what is of interest to concentrate on their exposure to phytoestrogens. Traditionally, Asian babies do not appear to be directly exposed to soy products. Although, of course, they would normally be breast fed. Therefore, an attempt has been made to estimate their exposure from breast milk.

There are no published data on concentrations of phytoestrogens in breast milk. Therefore, these have been estimated from plasma levels, assuming equal partitioning into the milk. Plasma levels of genistein are quoted to be 1-4 $\mu\text{mol/L}$ for individuals on a high soy diet (Barnes, 1995). However, there is very little published data on Asian individuals. There is only one report of plasma levels of total genistein (0.28 $\mu\text{mol/L}$ in Japanese men, Adlercreutz *et al.*, 1993). Assuming a daily intake of 500 ml of breast milk, this gives an estimate of 0.04 mg of genistein per day (based on extrapolation of the data from Japanese men) or 0.14-0.54 mg per day, based on the figures cited by Barnes (1995). These values again may be compared with those calculated for soy formula (i.e. between 4.7-5.7 and 8.9-16.2 mg of genistein per day, for babies less than 1 month and more than 4-6 months of age, respectively). The daily intake of genistein from soy formula is 10-400 times greater than that from breast milk. Similar values may be calculated for daidzein. Therefore, it would appear that the exposure of Asian babies to phytoestrogens is in fact significantly lower than that of infants on soy formula. They would also have the expected advantages of being on breast milk rather than an artificial substitute.

On the other hand there is the possibility of exposure *in utero*. This would depend, for instance, on how much passes across the placenta and how critical *in utero* exposure is. There is no information on these points. Similarly, there is no information on the pharmacokinetics and metabolism of the phytoestrogens in infants. The pH in the infant stomach and the gut flora, which is established shortly after birth, should enable breakdown of conjugated phytoestrogens. However, the bioavailability in infants may be greater than in adults if binding to fibre is significant.

Likely effects

The phytoestrogens have the ability to either mimic or interfere with the production and binding of endogenous estrogens. Again, any effects due to binding to the estrogen receptors are often dismissed because they are considered weak estrogens. However, there are also other estrogen related effects. For instance, recent data have shown genistein to be a potent inhibitor of the enzyme 17- β -hydroxysteroid oxidoreductase, type I (Makela *et al.*, 1995) which converts estrone into the much more potent estradiol, the main endogenous estrogen. Genistein may therefore both inhibit the production and the binding of estradiol. As with all these enzymes, these data are obtained from *in vitro* studies. Whether or not inhibition

occurs *in vivo* is not known. However, it is noteworthy that the inhibition of the enzyme activity is maintained in whole cells (T-47D breast cancer cell line). Genistein also inhibits the conversion of androstenedione to testosterone. Therefore there is the possibility of modulation of levels of both estradiol and testosterone. Finally, there are the possible anti-proliferative effects due to inhibition of topoisomerase II and tyrosine kinase (see Barnes and Peterson, 1995).

The main effects are those due to possible alteration of endocrine dependent regulation (Clarkson *et al.*, 1995). These might include carcinogenic, reproductive, and developmental outcomes, including effects on sexual maturation and behaviour, which would not necessarily be readily apparent either in time or in appearance. What appears to be critical here is imprinting of characteristics important in sexual differentiation and maturation, neurological and behavioural development. Adequate estradiol is necessary after birth for these processes to proceed normally (Faiman *et al.*, 1976; Dohler, 1987). There is also the surge in testosterone levels shortly after birth which, if altered, prevents normal development of male characteristics.

The experimental data with genistein are limited (see Clarkson *et al.*, 1995) but are supported by that from animal and some human effects observed with other estrogenic compounds such as diethylstilbestrol (DES) (see Colborn *et al.*, 1993), and other phytoestrogens (Whitten *et al.*, 1995). Obviously, there must be caution in making comparisons between compounds, species, period and extent of exposure. Equally there is no ground for complacency and the possible adverse effects of genistein and daidzein in humans must be examined thoroughly.

It is repeatedly said that soy formula has been used without any serious health effects. That raises the question of what is regarded as serious. The above effects may not be considered that severe on a population basis but would be to the individual. Certainly, there are other possible causes of such effects. However, it has to be asked - who has actually looked for a contribution from exposure of neonates to soy formula? The answer is no-one.

It is relevant to mention male reproductive tract disorders and possible endocrine causes (Sharpe, 1993; Sharpe and Skakkebaek, 1993). In the last 30-50 years there has been, amongst other things, a decline in sperm count, an increase in testicular cancer and an increase in cryptorchidism. The latter is only a minor abnormality (failure of a testicle to descend fully) but it is very frequent (2-3%) and a predisposing factor to testicular cancer. A hypothesis has been put forward by Dr Richard Sharpe of the MRC Reproductive Biology Unit in Edinburgh that these changes are due to exposure to environmental estrogens, for instance DES. The point here is that estrogen related adverse effects have been noted. Also the use of DES ended around 1970. It is entirely plausible that exposure to phytoestrogens in soy formula may be one factor contributing to these effects. Also of note is that, in comparisons between environmental estrogens and phytoestrogens, against a range of *in vitro* endpoints, the phytoestrogens have been shown to be more potent i.e. effective at lower concentrations (P. L. Whitten, unpublished).

All of the discussion so far on infant formula has concentrated on phytoestrogens. It is classic toxicology and pharmacology to consider chemicals in isolation. However, it is well known that the soy bean contains other compounds which may elicit toxic effects. For instance, phytic acid is well known to bind and reduce the bioavailability of divalent cations such as iron, calcium and zinc. Admittedly, the formula may be supplemented with these nutrients to compensate for the reduced bioavailability. However, it seems very much dependent on accurate measurement and good quality control. With zinc, over supplementation is also a potential problem. Trypsin inhibitors may also cause adverse effects

(Roebuck, 1987) They are removable by standard processing techniques. However, some data on soy formula (Peace *et al.*, 1992) show that the efficiency of removal varies significantly, perhaps reflecting the Murphy's Law of quality control. Saponins have detergent like effects but also appear to act synergistically with other soy toxins; and there are others.

Regulatory recommendations

There is no question that some infants are receiving significant quantities of phytoestrogens, and that this exposure is occurring at an inappropriate time. As noted above there is some experimental evidence with genistein and other estrogenic compounds. There are plausible mechanistic explanations. For instance, estrogenic compounds have been implicated in an increase in male reproductive disorders. However, there are no human clinical studies showing a link between any adverse effects and this exposure, but, on the other hand no one has actually looked. So, at this stage it can only be said that the risk is a potential one. What can be done?

A preliminary question is - how many infants are at risk? A rough calculation may be made based on 60,000 births per year, 10-20% of babies not being breast feed and soy formula comprising 13% of all formula sales. From this we can estimate 800 to 1600 infants per year, not an enormous number but still significant.

Also - is the risk avoidable? The main reasons for using soy formula are allergy and lactose intolerance. The actual incidence of sensitivity to cow's milk protein is quite low (0.3-0.7%) and around 35% of these individuals are allergic to soy protein anyway (see Witherley, 1990 and Erdman, 1990). Lactose intolerance in infants is typically temporary, following a gastrointestinal infection, for instance. Are there alternatives? Formulas based on goat's milk or hydrolysed casein are alternatives which may be tried. This means that the risk is largely avoidable. Most, if not all, of infants on soy formula are therefore being exposed to phytoestrogens unnecessarily.

There is a need for more information in a number of ways. The modern definition of toxicology incorporates prediction of hazards from a limited data base. Obviously, the more information the better. However, there are clear ethical problems with gaining more human data. How does one say to someone in a trial "we are not sure if this is harmful or not but we would like to test it on your child"? There is a need for the paediatricians and the endocrinologists, in particular, to get together and identify likely effects or biomarkers. Then retrospective epidemiology studies should be done. Plunket books and prescription numbers should be invaluable for identifying individuals and their past exposure. This study, hopefully, would allow the risk to be quantified. Meanwhile, parents and professionals have to be given the available information so that parents may make as informed a decision as possible.

There is no good reason why general sales of soy formula should continue, at least until the epidemiology study is done. Availability of soy formula by prescription could cater for those individuals with genuine needs. It should be noted that according to the Food Act 1981 the responsibility is clearly on the manufacturers and retailers to ensure that the food they offer for sale is safe. That does not seem to be known or enforced although all the onus need not be placed on the manufacturers. There may be no clinical evidence of adverse effects but again, who has looked? No-one.

Equally, there is no convincing evidence as to safety. The emphasis now has to be placed on demonstrating that the use of soy in infant formula is safe. There is no hesitation in taking a concerned cautionary approach regarding low level exposure to synthetic environmental estrogens. It is strange that a similar approach with phytoestrogens is dismissed, despite the effective exposure, particularly to infants, being much higher and the

potency of the phytoestrogens, greater, in some situations. Soy protein is a cheap soy protein. Soy phytoestrogens and other soy compounds are natural and may well be shown to be useful in the chemoprevention of cancer in adults. However, the question remains: use of soy appropriate in infant formula?

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000698

Effects of plant oestrogens in ruminants

M.F. McDONALD
Department of Animal Science, Massey University,
Palmerston North

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ABSTRACT

Research on phytoestrogens in herbage and metabolites in the ruminant has arisen owing to their deleterious effects on reproduction, particularly in sheep and to a limited extent in cattle. Plants containing these compounds include some of the economically important pasture or forage plants of the Leguminosae family and it is a paradox that while they play a valuable role in the improvement of soil fertility, and animal growth and production, they also can reduce animal fertility and prolificacy. The female reproductive cycle is markedly affected in contrast to sperm production in the male. Oestrogenic activity in forage plants also can have positive effects on mammary gland growth, lactation, and animal growth.

With breeding ewes grazed on oestrogenic clover prior to, and during mating they suffer from a temporary infertility from which they recover within a few weeks after removal to non-oestrogenic pasture. Prolonged grazing on oestrogenic clover for several years, may result in a permanent and progressive infertility in ewes. Formononetin, the main isoflavone present in subterranean clover and red clover is implicated in these reproductive problems, it is not oestrogenic itself but is metabolized mainly to equol in the rumen, and equol is oestrogenic. Coumestans and other oestrogenic substances associated with fungal growth on plants will also reduce reproductive performance particularly ovarian function and the early events in development of the embryo.

Protection against the deleterious actions of phytoestrogens, such as immunisation against equol or coumestrol in sheep grazing clovers or lucerne is feasible but not developed as a control technique. Forage plants with low levels of phytoestrogens is the long term goal. Fortunately management plans can be devised to avoid feeding oestrogenic herbage at critical times to breeding livestock.

INTRODUCTION

The realisation that some pasture plants can cause serious infertility in sheep was first observed in Western Australia and it has been the stimulus for much of the work on phytoestrogens and their actions in the grazing animal. Dramatic reductions in lambing percentages have occurred when breeding ewes have grazed pastures containing a high content of subterranean clover (*Trifolium subterranean*) and the infertility resulting has frequently been termed "clover disease". Affected sheep grazing subterranean clover will have ingested phytoestrogenic compounds of variable potency over several months and even years, causing some animals in extreme cases to become sterile or show reduced fertility and flock lambing percentage to progressively decline.

Other varieties of clover also contain phytoestrogens but usually in reduced amounts compared to that in subterranean clover. An exception is red clover, (*T. pratense*) which is widely used in pastures in many countries especially in temperate regions and reproductive problems have frequently been noted (Moule *et al.*, 1963). Fortunately white clover (*T. repens*) which is the main legume in most New Zealand pastures has low levels of

INTRODUCTION

to 1993, Second Internat. Conference on Phytoestrogens.

The Case for Expanded Phytoestrogen Research (43824B)

DANIEL M. SHEEHAN

Department of Health and Human Services, Food and Drug Administration, National Center for Toxicological Research, Division of Reproductive and Developmental Toxicology, Jefferson, Arkansas 72079-9502 and Department of Biochemistry, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

Phytoestrogens are estrogenic chemicals produced by plants. The estrogenic activity of clover was first described almost 50 years ago following the observation that sheep feeding on pastures that contained clover demonstrated hyperestrogenization and infertility (1). Progress in understanding the significance of plant estrogens has been slow, in part due to the scientific isolation of what was thought to be a somewhat idiosyncratic animal husbandry problem. Studies identifying the estrogenically active chemicals and the discoveries of additional plant sources for other estrogens were roughly contemporaneous with our developing understanding of the mechanism of action of animal estrogens. Phytoestrogens are now known to be diverse in their chemical structures as well as in their origins (2). The two major chemical classes, the coumestans and isoflavonoids, each have a number of representatives with different estrogenic potencies; they may have different patterns of biological activities as well (3, 4). Thus, while general statements regarding phytoestrogen effects can be made, additional properties may be associated with specific phytoestrogens.

It was predictable, therefore, that purified phytoestrogens would be examined in the experimental systems being used to elucidate the mechanism of action of estrogens. Observations that phytoestrogens competed with radiolabeled estradiol for binding to the estrogen receptor (ER) and elicited estrogenic responses in estrogen-responsive tissues and cells were crucial in demonstrating that phytoestrogens and traditional estrogens shared a common mechanism of action (2, 5). Even at this juncture, the major research emphasis was on estrogens with a high affinity for the ER, since these are generally the most biologically potent estrogens. In the pharmaceutical industry, this emphasis drove the discovery of estrogenic chemicals with high

affinity and biological potency. These studies resulted in major advances in both human health and population control, exemplified by the synthetic estrogen ethynylestradiol. However, the induction of benign abnormalities and malignancies in the reproductive tracts of the offspring of diethylstilbestrol (DES)-treated pregnant women (6) showed that estrogens can also adversely affect humans.

In the meantime, phytoestrogens continued to be of only marginal interest, in large part because they generally showed a low affinity for the ER and a low estrogenic potency in bioassays. However, as the number of plant species demonstrated to contain phytoestrogens expanded, interest increased in the potential for these chemicals to be effective estrogens in human and wildlife populations, and to be used for medicinal purposes. Specifically, the anticarcinogenic activity of some phytoestrogens suggested an antiestrogenic action (7). This hypothesis is consistent with the finding that Asian populations, which consume large amounts of phytoestrogens derived from a soy-based diet, have a lower incidence of breast and prostate malignancies than western populations, which consume a much lower quantity of phytoestrogens in their diet (7, 8). A sequence of findings in experimental animals demonstrated that phytoestrogens possess the same wide range of biological activities previously found with traditional estrogens. Nonetheless, the entire literature on phytoestrogens includes only some 900 references published since the mid 1940s (see preceding Announcement). Most of these studies are concentrated in four areas: effects on livestock, isolation and identification; metabolism; and estrogenic potency. While the literature is sufficiently large to demonstrate the importance of phytoestrogens, there are huge gaps in our knowledge.

At the Second International Phytoestrogen Con-

INTRODUCTION 3

* Since 1993, phytoestrogen references number in the 1,000's 000699

ference, formal presentations and individual and group discussions helped identify a number of significant deficiencies in our techniques and knowledge. These include the need to develop analytical methods for simple, rapid, accurate, and reliable identification of phytoestrogens. Currently, analytical methods are quite expensive and require substantial effort. Without improved techniques, important problems in various species of interest (such as the identification of sites of metabolism and metabolic products, pharmacokinetic studies, and the determination of phytoestrogen concentration in target tissues and human foods) cannot be pursued in the detail necessary.

Given the interest in the antiestrogenic activity of phytoestrogens, particularly with respect to prevention of breast cancer, there is a clear need to carry out detailed pharmacological studies to define relative agonist and antagonist activities in different tissues and species. The pharmaceutical industry has produced a variety of antiestrogens with a wide range of agonist/antagonist activities. Some of these antiestrogens, such as the mixed agonist/antagonist tamoxifen versus the pure antiestrogens ICI 164,384 and ICI 162,780 (9, 10), appear to act via different mechanisms. These findings suggest that the antiestrogenic actions of different phytoestrogens also could be mechanistically distinct. In addition to anticarcinogenesis, could such chemicals alter regulation of endocrine activity in both males and females? Are there new endocrine drugs awaiting discovery among the phytoestrogens? Only careful, detailed examinations can provide an answer to such questions. Another important issue is distinguishing between phytoestrogen agonist/antagonist effects elicited via the estrogen receptor- versus nonreceptor-mediated, chemical structure-specific outcomes (11). Given the large number of structurally different phytoestrogens, it would be naive to expect that all their effects are estrogen receptor-mediated.

As with any biologically active chemical, it is crucial to define adverse versus beneficial effects of the phytoestrogens. Some possible benefits have already been discussed. The major adverse activities of estrogens involve reproductive, carcinogenic, and developmental outcomes. Estrogen regulation of fertility is exemplified by oral contraceptives. Phytoestrogens are known to induce infertility in livestock (2) and probably in quail populations (12). It is clearly important to determine if there are additional effects on fertility in human, wildlife, and livestock populations. Estrogens are also involved in induction of malignancies in estrogen target tissues. In humans, chronic unopposed estrogen exposure is a major risk factor for the induction of endometrial adenocarcinomas (13), and tamoxifen also increases the risk for this outcome (14). Are some phytoestrogens also risk factors for malignancies? Finally, despite the compelling example of DES

developmental toxicity in humans, there are only a couple of dozen studies on the developmental effects of phytoestrogens in reproductive tract and brain of experimental animals. Even though they are few in number, these studies make it clear that phytoestrogens have some of the same capabilities to induce developmental toxicity as do other estrogens (3, 15-17). However, there are hints that they may differ from other estrogens in certain specific outcomes (3). Given the DES tragedy, it would be foolish to ignore the possibility that some phytoestrogens constitute a developmental hazard.

Even in the few areas highlighted in the foregoing discussion, it is apparent that an extensive biomedical research agenda is necessary. But where are the resources to come from to drive this emerging area? The National Institutes of Health (NIH) could accept a major responsibility for funding appropriate research projects on phytoestrogens. Absent such an initiative from the NIH, progress will be delayed. The food industry could devote more attention to the potential beneficial and adverse effects of phytoestrogens found in human foods such as soy-based products. The interests of the pharmaceutical industry would be well served by supporting basic research as well as drug discovery and development involving phytoestrogens. Regulatory agencies such as the Environmental Protection Agency, responsible for assuring a safe environment, and the FDA, which regulates the safety of drugs and foods, are aware of the regulatory issues involving phytoestrogens. The FDA is actively engaged in phytoestrogen research. Since phytoestrogen research has such clear relevance to so many private and public organizations, communication and coordination of resource allocation among them will be important in increasing our understanding of the impact of phytoestrogens on wildlife, livestock, and humans.

This volume constitutes the first published compendium focussed on phytoestrogen research and is intended to highlight the varied approaches and findings of current investigations. We are hopeful that in the fall of 1995 another phytoestrogen conference can be held that will not only attract even wider attendance but will demonstrate significant progress in our understanding of phytoestrogens.

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000701

OU



Dr Linda Kahl
Office of Premarket Approval
U.S. FDA

(202) 418 3131

Dear Dr Kahl

G. R. N # 000001

A scientist who may make a submission to you has been trying to ascertain whether isoflavones, or genistein and daidzein, have already been assessed for Defect Action Levels; he says he is interested in this because the National Academy of Sciences Food Protection Committee denounced them as a Toxicant in 1973.

He has not been able to get a compilation from the Center for Food Safety and Applied Nutrition, and wonders if you could fax me a copy as a matter of urgency, so that he can prepare his submission. My fax is 64 9 4340567

This is a new angle altogether, and I presume it was A.D.M.'s own reference to Good Manufacturing Practice for the removal of isoflavones which set him off on this thought train.

Below is the page which he has sent me and the reference is the last paragraph

Best Regards

~~(Signature)~~

000467

BEST ORIGINAL COPY

Food and Drug Administration, HHS

§ 110.110

employment of one or more of the following practices:

(i) Monitoring the a_w of food.
(ii) Controlling the soluble solids-water ratio in finished food.

(iii) Protecting finished food from moisture pickup, by use of a moisture barrier or by other means, so that the a_w of the food does not increase to an unsafe level.

(iv) Food such as, but not limited to, acid and acidified food, that relies principally on the control of pH for preventing the growth of undesirable microorganisms shall be monitored and maintained at a pH of 4.6 or below. Compliance with this requirement may be accomplished by any effective means, including employment of one or more of the following practices:

(i) Monitoring the pH of raw materials, food in process, and finished food.
(ii) Controlling the amount of acid or acidified food added to low-acid food.

(iii) When ice is used in contact with food, it shall be made from water that is safe and of adequate sanitary quality, and shall be used only if it has been manufactured in accordance with current good manufacturing practice as outlined in this part.

(iv) Food-manufacturing areas and equipment used for manufacturing human food should not be used to manufacture nonhuman food grade animal feed or inedible products, unless there is no reasonable possibility for the contamination of the human food.

§ 110.93 Warehousing and distribution.

Storage and transportation of finished food shall be under conditions that will protect food against physical, chemical, and microbial contamination as well as against deterioration of the food and the container.

Subpart F [Reserved]

Subpart G—Defect Action Levels

§ 110.110 Natural or unavoidable defects in food for human use that present no health hazard.

(a) Some foods, even when produced under current good manufacturing

practice, contain natural or unavoidable defects that at low levels are not hazardous to health. The Food and Drug Administration establishes maximum levels for these defects in foods produced under current good manufacturing practice and uses these levels in deciding whether to recommend regulatory action.

(b) Defect action levels are established for foods whenever it is necessary and feasible to do so. These levels are subject to change upon the development of new technology or the availability of new information.

(c) Compliance with defect action levels does not excuse violation of the requirement in section 301(a)(3) of the act that food not be prepared, packed, or held under unsanitary conditions or the requirements in this part that food manufacturers, distributors, and holders shall observe current good manufacturing practice. Evidence indicating that such a violation exists causes the food to be adulterated within the meaning of the act, even though the amounts of natural or unavoidable defects are lower than the currently established defect action levels. The manufacturer, distributor, and holder of food shall at all times utilize quality control operations that reduce natural or unavoidable defects to the lowest level currently feasible.

(d) The mixing of a food containing defects above the current defect action level with another lot of food is not permitted and renders the final food adulterated within the meaning of the act, regardless of the defect level of the final food.

(e) A compilation of the current defect action levels for natural or unavoidable defects in food for human use that present no health hazard may be obtained upon request from the Center for Food Safety and Applied Nutrition (HFS 565), Food and Drug Administration, 200 C St., SW., Washington, DC 20204.

(51 FR 23475, June 19, 1986, as amended at 51 FR 15489, Apr. 2, 1986)

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Prof. James M. Gentile
Biology Department
Hope College
HOLLAND
MI 49423 - 3698 (U.S.A.)

Dr. Suzanne M. Morris
Division of Genetic/Reproductive Tox.
HFT-120/DGRT/NCTR
3900 NCTR Road
Jefferson, AR 72079

Holland, Michigan,

5/22 98

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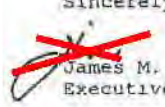
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**P53, MUTATIONS, AND APOPTOSIS IN GENISTEIN-EXPOSED
HUMAN LYMPHOBLASTOID CELLS**

**Suzanne M. Morris¹, James J. Chen², Olen E. Domon¹, Lynda J. McGarrity¹,
Michelle E. Bishop¹, Mugimane G. Manjanatha¹ and Daniel A. Casciano¹**

¹Division of Genetic and Reproductive Toxicology

²Division of Biometry and Risk Assessment

National Center for Toxicological Research

Food and Drug Administration

Department of Health and Human Services

3900 NCTR Road

Jefferson, Arkansas 72079

Corresponding author:

Suzanne M. Morris, Ph.D.

Division of Genetic and Reproductive Toxicology

HFT-120/DGRT/NCTR

3900 NCTR Road

Jefferson, AR 72079

(870)543-7580

(870)543-7393 (Fax)

E-mail: smorris@nctr.fda.gov

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KEYWORDS

genistein, human lymphoblastoid cells, thymidine kinase mutations, hypoxanthine phosphoribosyl transferase mutations, apoptosis, p53 functional status.

ABBREVIATIONS

tk, thymidine kinase; *hprt*, hypoxanthine phosphoribosyl transferase; LOH, loss-of-heterozygosity; TFT, trifluorothymidine; 6-TG, 6-thioguanine; FDA, fluorescein diacetate; PI, propidium iodide; FSC, forward scatter; EMS, ethyl methanesulfonate, DMBA, dimethylbenz(a)anthracene; DMN, dimethylnitrosoamine; BrdUrd, bromodeoxyuridine; anti-BrdUrd, monoclonal antibody against bromodeoxyuridine; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FCM, flow cytometry.

ABSTRACT

The phytoestrogen, genistein, is a naturally occurring isoflavone found in soy products. On a biochemical basis, genistein is a competitive inhibitor of tyrosine kinases and the DNA synthesis-related enzyme, topoisomerase-II. Exposure of mammalian cells to genistein results in DNA damage that is similar to that induced by the topo-II inhibitor and chromosomal mutagen, *m*-amsa. In order to determine the potential genotoxicity of genistein, human lymphoblastoid cells which differ in the functional status of the tumor suppressor gene, *p53*, were exposed to genistein and the induction of micronuclei quantified by microscopic analysis. In addition, the mutant fraction at the thymidine kinase (*tk*) locus (both the normal-growth and slow-growth phenotypes) was determined by resistance to trifluorothymidine (TFT) and at the hypoxanthine phosphoribosyl transferase (*hprt*) locus by resistance to 6-thioguanine (6-TG). Flow cytometric analysis of the percentage of viable, apoptotic and degenerating cells was utilized to determine the rate and kinetics of cell death after genistein exposure. The detection of micronuclei in both cell lines indicated that genistein-induced damage had occurred in both AHH-1 *tk*^{+/−} and L3. Linear regression analysis detected a significant increase in the number of 6-TG-resistant clones in both AHH-1 *tk*^{+/−} (*p53*^{+/−}) and L3 (*p53*^{+/−}). A comparison of slopes revealed no difference between the lines. In contrast, a significant, concentration-dependent increase in the number of TFT-resistant clones with the slow-growth phenotype was detected in AHH-1 *tk*^{+/−} (mutant *p53*), but not in L3 (wild-type *p53*). Cell death occurred primarily by apoptosis in both cell lines; however, a concentration-dependent decrease in the percentage of viable cells was detected immediately after exposure in L3, but not until 32 hours after exposure in AHH-1 *tk*^{+/−}. A comparison of the slopes of the concentration-response curves for the percentage of viable cells revealed no difference between the cell lines in the effect of genistein on cell viability. Our results may be interpreted that genistein is a chromosomal mutagen and that *p53* functional status affects the recovery of chromosomal mutants, possibly by signaling cells into the apoptosis pathways.

BACKGROUND:

The tumor suppressor gene, *p53*, plays an important role in signaling cells with DNA damage to undergo apoptosis through its ability to transcriptionally activate or repress the synthesis of apoptosis regulatory genes [1, 2]. For example, an increased rate of apoptosis is observed in ionizing radiation - exposed thymocytes obtained from mice with wild-type *p53* compared to mice with mutant *p53* [3]. A specific role of *p53* is thought to be the immediate down-regulation of the synthesis of the apoptosis repressor protein, *bcl-2* [4] and the up-regulation of *bcl-2* gene family member, *bax* [5, 6, 7], a death effector protein [4].

In cells with "loss-of-function" mutations in the *p53* gene, the G1 checkpoint is compromised, death by apoptosis may be delayed, the rate of apoptosis may be decreased, and cells with DNA damage (or the effects of that damage) may remain viable [8, 9]. This is clearly evident in studies with the TK6 family of human B-lymphoblastoid cell lines, TK6 (*p53*^{+/+}), WI-L2-NS (*p53*^{+/+}) and WTK1 (*p53*^{+/+}). A mutation in exon 7 of the *p53* gene has been described in the WI-L2-NS and WTK1 lines [10, 11]. Exposure of these lines to ionizing radiation consistently results in an increase in the frequency of chromosome aberrations or mutations at the thymidine kinase (*tk*) locus and a decrease in the frequency of apoptosis in WI-L2-NS or WTK1 compared to TK6 [12, 13, 14, 10, 15, 16, 17, 18, 19]. Further, differences in the mutational spectra between the two lines were found at the *tk* locus [20, 21], and in the mutation frequency, but not the spectrum, at the hypoxanthine phosphoribosyl transferase (*hprt*) locus [15, 16]. Different classes of chromosome aberrations have been recovered in these cell lines after exposure to ionizing radiation [22] and may reflect, at least partially, the difference in the mutational spectrum observed at the *tk* locus.

Similarly, members of the AHH-1 family of human B-cell-derived lymphoblast lines, AHH-1 *tk*^{+/+} and MCL-5, differ in the functional status of the *p53* gene [23]; a C → T mutation at codon 282 of exon 8 of the *p53* gene was detected in AHH-1 *tk*^{+/+} and the wild-type sequence in MCL-5. A 5-fold difference in the spontaneous mutation frequency at the *tk* locus was due to the recovery of large-scale chromosomal events (loss-of-heterozygosity, LOH) on the long arm of chromosome 17 in AHH-1 *tk*^{+/+} [24]. The MCL-5 line, transfected with the genes for human P-450's 1A1, 1A2, 2E1, 3A4 and epoxide hydrolase [25], was derived from an AHH-1 subclone, L3, that is also heterozygous at the *tk* locus (Dr. Bruce Penman, Gentest, personal communication).

The phytoestrogen, genistein, is a naturally occurring isoflavone that is found in soybean products, e.g., soy milk, soya meal and tofu (reviewed in [26]). Much of the interest in this compound comes from the results of epidemiological studies which indicate that the incidence of breast cancer and prostate cancer is reduced in populations which consume a "high soy" diet (reviewed in [27]). For example, in a study of breast cancer incidence in Singapore women, the consumption of soya protein was highly correlated with a decreased

risk of breast cancer [28]. Further, the incidence of prostate cancer was significantly lower in a cohort of Japanese men in Hawaii maintained on a diet high in rice and tofu [29]. Detectable levels (nmol/l) of genistein have been found in the plasma [30] and urine [31] of individuals consuming a "high soy" diet and is indicative of the bioavailability of this compound. One study, in which the effect of genistein and genistein conjugates on the growth of human breast epithelial cells was measured, indicated that certain conjugates of genistein, *e.g.*, genistein-7-sulfate, were as effective as genistein in inhibiting cell growth [32]. Serum concentrations of total genistein (both free and conjugated forms) in the range of 0.2 µg/ml have been detected in humans consuming 50 mg of genistein and daidzein per day [33].

The results of experiments with both tumor cells and animal models provide some insight into the mechanism of chemoprevention. In HL-60 and MCF-7 cells, exposure to genistein resulted not only in the inhibition of cell proliferation, but also in an increase in cell death [34, 35, 36]. Further, in *ras*-transformed 3T3 cells, not only was growth inhibited, but the number of transformed foci was also significantly reduced [37]. No tumors were found in female rats fed a soy-based diet and the incidence of breast cancer was reduced in ionizing radiation-exposed female rats fed the same soybean diet [38]. The incidence of dimethylbenz[a]anthracene (DMBA)-induced mammary tumors was reduced in rats fed a genistein-containing diet and was accompanied by a decrease in the number of S-phase cells as determined by bromodeoxyuridine labeling [39, 40]. Replication indices in the bone marrow of DMBA-treated mice and the level of DNA adducts in mammary and liver tissue were reduced with genistein feeding [41]. The frequency of chromosomal aberrations in the bone marrow of male rats maintained on a soybean-containing diet increased significantly after three months of feeding, but returned to control levels with additional feeding. The nitrosamine-induced chromosome aberration frequency was reduced in male rats consuming this same diet [42]. These results suggest that the protective role of genistein is not in the prevention of DNA damage, but in the elimination of cells with damage from the population. Experimental evidence which demonstrates the increased rate of apoptosis in genistein-exposed cells supports this viewpoint [35, 43, 44].

Many of the biological effects of genistein result from its affinity for binding to a conserved amino acid sequence of certain receptors, such as the ATP binding site of the DNA synthesis-related enzyme, topoisomerase-II (topo-II) [45] and its tyrosine kinase inhibitory activity [46]. The binding of genistein to topo-II results in the stabilization of the "cleavable complex" and the formation of protein-linked double-strand breaks [37, 45]. DNA strand breaks have been detected in genistein-exposed HL-60 cells [47] as have protein-linked double strand breaks and a signature break in the pbr322 plasmid [37, 45, 48]. Although the more well-characterized topo-II inhibitor, *m*-amsa (reviewed in [49]), exerts its biological effects through intercalation into the DNA helix, exposure to *m*-amsa also results in the stabilization of the cleavable complex, the formation of protein-linked double strand breaks, chromosome aberrations and chromosomal mutations due to large-scale chromosome damage [50, 51, 52, 53, 54]. The formation of similar types of DNA damage

after exposure to either compound suggests that the mutagenic (or clastogenic) potential of *m*-amsa and genistein may be similar.

In order to evaluate the genotoxicity of genistein, the following experiments were conducted. First, the clastogenicity and mutagenicity of genistein was evaluated by the micronucleus assay and by the *tk/hprt* assay which detects both intragenic mutations and chromosomal damage. The effect of genistein on cell-cycle traverse was determined by flow cytometric (FCM) evaluation of bromodeoxyuridine (BrdUrd)-labeled DNA. The manner of cell death was determined by the flow cytometric-based FDA/PI assay with which the percentages of viable, apoptotic and degenerating cells were quantified. The AHH-1 *tk*^{+/−} cell line is heterozygous at the p53 locus due to a C → T mutation at codon 282 of exon 8 [23]. DNA sequence analysis did not detect this mutation in the L3 cell line; a sequence consistent with the wild-type was revealed. In order to address the role of p53 functional status in the response to genistein, these experiments were performed in both AHH-1 *tk*^{+/−} and L3. This is an especially important concept in evaluating the response to genistein exposure because the L3 line in which the p53 gene is wild-type may more accurately reflect the functional status of this gene in the population at large. AHH-1 *tk*^{+/−}, however, may be a more appropriate model for tumor cells in which p53 function is impaired or abrogated.

MATERIALS AND METHODS:

Cells and Cell Culture. AHH-1 *tk*^{+/−} cells (Gentest Corp., Woburn, MA) were derived from the AHH-1 human B-cell lymphoblastoid line originally described by [55]. AHH-1 *tk*^{+/−} cells are heterozygous at the *tk* locus, having undergone a two-step selection process with the frame-shift mutagen, ICR-191. The spontaneous mutant fraction in this cell line is approximately 3×10^{-6} at the *hprt* locus and 15×10^{-6} at the *tk* locus. Further, this cell line expresses the B-cell membrane antigens, CD19, CD20 and CD21, detectable levels of the Epstein Barr Virus latent membrane protein, LMP-1, and detectable levels of the apoptosis-inhibitory protein, bcl-2 [56]. Recent studies in our laboratory have found that the *p53* gene is heterozygous in this cell line with a C → T transition mutation at codon 282 of exon 8 [23].

The L3 cell line (generously provided by Dr. Bruce Penman, Gentest), was also derived from the AHH-1 cell line of [55]. Immunophenotyping revealed that the L3 line also expresses the human B-cell-related CD19, CD20 and CD21 membrane antigens previously detected in AHH-1 *tk*^{+/−} (SM Morris et al., unpublished results). This cell line was also subjected to a two-step selection process for heterozygosity at the *tk* locus. However, although the initial selection step was with the frame-shift mutagen, ICR-191, the second step was performed with benzo(*ghi*)perylene. The L3 line was utilized to develop the MCL-5 cell line in which the spontaneous mutant fraction at the *hprt* locus is approximately 3×10^{-6} and $3-5 \times 10^{-6}$ at the *tk* locus. Single-strand conformational polymorphism analysis and sequence analysis of exons 5 - 9 of the *p53* gene of the MCL-5 line revealed no differences from the wild-type sequence [23]. DNA sequence analysis of exon 8 of the *p53* gene was performed as described in [23] and a wild-type sequence was revealed.

Each of the cell lines was routinely cultured in medium RPMI 1640 (Gibco, Grand Island, NY) containing 10% iron-supplemented newborn calf serum (Hyclone, Logan, UT), 1% L-glutamine (Gibco) and 1% penicillin-streptomycin (Gibco). The cultures were maintained at a density of $3.5 - 5.0 \times 10^5$ cells per ml and the medium was replenished at 48 - 72 hours intervals. In order to reduce the spontaneous mutation frequency at both the *tk* locus and the *hprt* locus, the cells were cultured for three days in the presence of HAT (Gibco) followed by one day in culture with TH (Gibco). All cells were cultured in growth medium for a minimum of one week before use in a mutation assay. All cultures were maintained at 37°C in 5% CO₂ atmosphere with 100% humidity.

Chemical exposure. In order to expose either AHH-1 *tk*^{+/−} or L3 cells to the chemical, cells were seeded at a density of 5.0×10^5 cells per ml in complete medium. Genistein, obtained from Sigma (St. Louis, MO), was diluted in DMSO (Sigma). All exposure flasks (including the control) received an equivalent amount of DMSO. The individual cultures were exposed to increasing concentrations (0.0, 1.0, 5.0, 10.0 and 20.0 µg/ml) of genistein for 24 hours. At the end of the exposure period (designated Day 0), the cells were pelleted by gentle centrifugation, enumerated and seeded at a density of 3.5×10^5 cells per ml in

complete medium. Cultures were maintained at 37°C in a 5% CO₂ atmosphere with 100% humidity.

Mutation assay. The mutant fraction at both the *tk* locus and the *hprt* locus was determined as described in [54]. Briefly, cells were cultured in complete medium at a density of 5×10^5 cells per ml for 24 hours, diluted to a density of 3.5×10^5 cells per ml and maintained at this density for the length of the expression period (3 days, *tk*; 7 days, *hprt*). In order to establish selection plates for the *tk* locus, 10,000 cells were seeded into each well of a 96-well plate containing 0.2ml of medium 1640 supplemented with 10% heat-inactivated serum and 4.0 µg/ml of trifluorothymidine (TFT, Sigma). Three replicate plates were established for each concentration of chemical. The plates were maintained at 37°C in a 5% CO₂ atmosphere for 10 days at which time the plates were evaluated microscopically for the presence of positive wells (normal-growth clones). Each well was then supplemented with 22.0µl of complete medium with 40.0 µg/ml of TFT, returned to the incubator for 10 days, and then examined for the presence of additional positive wells (slow-growth clones). For the *hprt* locus, individual plates were seeded with 20,000 cells per well in 0.2ml of complete medium containing 0.6 µg/ml of 6-thioguanine (6-TG, Sigma). These plates were incubated for 13 days and then analyzed microscopically for the presence of positive wells.

In order to determine plating efficiencies, 2 cells in 0.2ml of complete medium without the selective agent (TFT or 6-TG) were seeded into the individual wells of a 96-well plate in 0.2ml. Duplicate plates were established for each concentration of chemical. The plates were incubated for 13 days and then evaluated microscopically for the presence of positive wells. Plating efficiencies were determined according to the methods of [57] and the mutant fraction according to [58]. Individual mutagenesis experiments were conducted three separate times.

Micronucleus Assay. The methods for the micronucleus assay were modified from those described by [59, 60, 61]. Briefly, at the end of the genistein exposure period, the medium was removed and the cells were recultured in complete medium at a concentration of 5.0×10^5 cells per ml for 48 hours. This was followed by a 24-hour incubation in 6.0 µg/ml Cytochlasin B (Sigma). At the end of the incubation period, the medium was removed by centrifugation, the cells were washed in complete medium and the cell pellet was incubated in a hypotonic solution (1:4, RPMI 1640:dH₂O) for 5 minutes. After centrifugation, the cell preparation was placed on slides and left to air dry for 24 hours. After a 15-minute fixation in methanol, the cells were stained in Acridine Orange (AO, Sigma) for 60 seconds (12.5mg AO/100.0ml Sorensen's buffer [Fisher, Pittsburgh], pH 6.8). The slides were washed twice in fresh Sorensen's buffer and then air-dried. Cover-slips were mounted with Sorensen's buffer and examined with a Zeiss fluorescence photomicroscope. Five hundred binucleated cells per slide were evaluated for the presence of micronuclei and three slides per concentration were examined unless excessive cell death had occurred. Individual experiments were conducted three times in each cell line, each at a different time.

Cell Viability. The effect of genistein exposure on cellular viability in each of the cell lines was determined by the fluorescein diacetate (FDA)/propidium iodide (PI) assay described in [54]. Briefly, cells were exposed to genistein as described above. At appropriate times, 1×10^6 cells per concentration were removed from the individual culture flasks. After the supernatant was removed, the cells were gently resuspended in 5.0 ml of complete medium with 100 μ l of a 1.0 μ g/ml FDA (Molecular Probes, Eugene, OR) staining solution and 50 μ l of a 1.0 mg/ml PI (Calbiochem, LaJolla, CA) staining solution. The cell suspension was incubated at 37°C for 10 minutes, the cells pelleted by gentle centrifugation, the supernatant removed, the cells washed once in phosphate-buffered saline (PBS, Gibco)/1% bovine serum albumen (BSA, Sigma), pH 7.4 and the cells resuspended in PBS. The cells were maintained on ice under an aluminum foil light shield for a minimum of 30 minutes until analysis by flow cytometry. Individual experiments were conducted three times in each cell line.

Cell Proliferation Assay. The effect of genistein exposure on the percentage of either AHH-1 *tk*⁺ cells or L3 cells in each of the cell-cycle phases, G0/G1, S-phase, and G2/M, was determined by the BrdUrd-DNA labeling procedure. Cells from each of the lines were exposed to increasing concentrations of genistein (0.0, 1.0, 5.0, 10.0, and 20.0 μ g/ml) as described above. After exposure, the cells were enumerated, reseeded at a density of 5.0×10^5 cells per ml for 24 hours and then reseeded at a density of 3.5×10^5 cells per ml for the duration of the culture. At intervals after exposure, aliquots of cells (5×10^6 cells per concentration) were removed and processed for FCM analysis as described in [62, 56]. Briefly, AHH-1 *tk*⁺ cells were labelled with 10 μ M BrdUrd (Calbiochem, LaJolla, CA) for 60 minutes in complete medium at 37°C, and then processed according to the instructions of the vendor (Becton-Dickinson, San Jose, CA) for the anti-bromodeoxyuridine monoclonal antibody (anti-BrdUrd). After processing, an aliquot (1×10^6 cells per concentration of genistein) of cells was resuspended in 50 μ l of 0.5% Tween 20 (Sigma)/PBS/1% BSA, pH 7.4 and 20 μ l of FITC-labelled anti-BrdUrd for 30 minutes at room temperature. After two rinses with PBS, 1.0 ml of a 5.0 μ g/ml PI staining solution was added to the cell suspension. The cell suspensions were maintained on ice for a minimum of 30 minutes before FCM analysis. Three individual experiments were conducted for each cell line, each on different days.

Flow Cytometry. Individual samples were analyzed on a FACSort (Becton-Dickinson) flow cytometer with an argon ion laser tuned to 488nm. The forward scatter (FSC) and side scatter signals were detected with linear amplification, triggering on the FSC signal. The FL1 (green, fluorescein) and FL2 (red, PI) signals were measured in logarithmic amplification (4-decade log scale) for the FDA/PI assays. For DNA analysis, the FL1-H (green, FITC) signal was collected in logarithmic amplification (4-decade log scale) and the FL2-H, FL2-A and FL2-W signals in linear amplification. The flow rate was 800 - 1,000 cells per second for FDA/PI analysis and 200 cells per second for DNA analysis. Electronic compensation was used to eliminate cross-signal detection. Lysis-II software (Becton-Dickinson) was used for data acquisition (25,000 events per sample) and for the

post-acquisition analysis of the list mode data. For DNA analysis, a singlet gate was established utilizing a FL-2A vs. FL2-W histogram. Percentages of cells in each of the three cell-cycle phases were determined from cells in the singlet gate.

Statistical Analysis. The effect of increasing genistein concentration on the mutant fraction, plating efficiency, and the frequency of micronuclei was determined by linear regression analysis. The slopes of the individual regression lines for mutation induction (*tk*-normal growth; *tk*-slow growth; *hprt*), plating efficiency (Day 0, Day 3, Day 7) and micronuclei were calculated and tested for differences from zero. The data from each of the two cell lines were analyzed separately. In subsequent analyses, the slopes of the individual regression lines for each of the cell lines were tested for differences from each other in order to evaluate the role of *p53* functional status on the induction of the specific endpoint.

Logistic regression analysis was utilized to analyze the cell viability and cell proliferation data (viable, apoptotic, G0/G1, S, G2/M) as described in [63]. Briefly, the proportion of cells within the region of interest (e.g., apoptotic, G0/G1) was determined as the ratio of the number of cells in that region to the number of cells analyzed. The test that the slope of the logistic regression line was equal to zero was performed to determine the effects of genistein concentration. The quasi-likelihood model was used to fit the logistic model and account for expected similarities between pools of cells as suggested by [64]. Individual cell lines were analyzed separately. In order to test for the effect of *p53* functional status on the induction of an endpoint, the slopes for the percent of cells within the region of interest were tested for differences from each other at each of the time points.

RESULTS

Mutant Fraction. The effect of exposure to genistein on the mutant fraction was determined in both cell lines. No significant effect of concentration of the induction of normal-growth *tk* mutants was detected in either cell line. In contrast, a significant increase in the recovery of slow-growth *tk* mutants was found in genistein-exposed AHH-1 *tk*^{+/−} cells with mutant *p53* ($p = 0.0006$), but not in L3 cells with wild-type *p53*. Further, the test for differences in slopes was significant ($p = 0.0122$) with an approximately four-fold greater number of recovered mutant clones in the AHH-1 *tk*^{+/−} cell line (Figure 1).

When the effect of exposure to genistein on the induction of mutations at the *hprt* locus was determined, a significant increase in the frequency of 6-TG-resistant clones was detected in both the AHH-1 *tk*^{+/−} ($p = 0.0023$) and L3 ($p = 0.0013$) cell lines (data not shown). No effect of *p53* functional status was detected as evidenced by the lack of a significant difference in the slopes of the concentration-response curves.

Cloning Efficiency. Linear regression analysis detected a significant decrease in relative cloning efficiency with increasing concentration immediately after exposure at Day 0 (Fig. 2A, AHH-1 *tk*^{+/−}, $p = 0.0001$; L3, $p = 0.0001$), at Day 3 (Fig. 2B, AHH-1 *tk*^{+/−}, $p = 0.0001$, L3, $p = 0.0001$) and at Day 7 (Fig. 2C, AHH-1 *tk*^{+/−}, $p = 0.0002$; L3, $p = 0.0291$) in each of the cell lines. No differences in the slopes of the concentration-response curves were found until Day 7; at that time, the slope for AHH-1 *tk*^{+/−} was significantly greater ($p = 0.0238$) than that for L3.

The L3 line (wild-type *p53*) consistently cloned at a lower rate than did AHH-1 *tk*^{+/−} (mutant *p53*). Thus, the intercepts of the concentration-response curves for the actual cloning efficiencies were tested for significant differences. At Day 0, no significant difference was detected although the actual cloning efficiency was lower in L3 (54% vs. 47%). At Day 3 and at Day 7, the differences were significant ($p = 0.009$ and $p = 0.0012$, respectively).

Micronucleus Assay. Exposure to genistein resulted in the induction of micronuclei in both cell lines (Figure 3). A significant effect of concentration was detected in AHH-1 *tk*^{+/−} ($p \leq 0.0001$) and in L3 ($p \leq 0.0001$). Differences in *p53* functional status affected the results as evidenced by the significant difference in the slopes of the two concentration-response lines ($p \leq 0.0001$). A higher percentage of micronuclei per 500 cells were detected in the L3 cell line than in AHH-1 *tk*^{+/−}.

Cell Viability. The effect of exposure to genistein on the proportion of viable cells, cells undergoing apoptosis and degenerating cells was determined by the flow cytometry-based FDA/PI assay. Viable cells are FDA^{pos}PI^{neg}, apoptotic cells are FDA^{neg}PI^{neg} and degenerating cells are FDA^{neg}PI^{pos}. The nature of these populations have been confirmed by electronic sorting of the individual populations and subsequent light, transmission

electron and scanning electron microscopic evaluation of the morphology of cells in the individual populations [63, 56]. In addition, DNA was isolated from cells sorted from these populations and examined for the presence of the "apoptosis-associated DNA ladders" by gel electrophoresis [56].

The results of the viability assay are shown in Figures 4A (L3) and 4B (AHH-1 *tk^{+/+}*). Measurements were made and analyzed statistically at multiple time points (-24, -16, 0, 8, 24, 32, 48, 56, 72, and 168 hours); however, for clarity only the data at 0, 24, 32, 48, and 72 hours are presented. A significant decrease in the percentage of viable cells was detected immediately after exposure at 0 hours ($p = 0.014$) and at 24 hours ($p \leq 0.0001$) in the L3 cell line. In contrast, a significant decrease ($p = 0.003$) in the proportion of viable cells was not detected statistically until 32 hours after exposure in the AHH-1 *tk^{+/+}* cell line. The delayed loss of viability, consistent with previous studies in this cell line [63, 54, 56] reflects the delayed onset of apoptosis associated with loss-of-function mutations in *p53*. Concentration effects were detected statistically at the 48 and 72 hour time points in both cell lines (all p -values were $p \leq 0.0001$). In a subsequent analysis, the slopes of the concentration-response curves were tested for significant differences between the cell lines. This was utilized as a measure of genistein-induced differences in cell viability as a function of *p53* status. Although the percentage of viable cells in the L3 cell line was consistently smaller in both control and exposed cultures, no significant differences were detected in the slopes of the concentration-response curves. This suggested that the effect of genistein on the reduction in cell viability was similar in both cell lines.

In both cell lines, the loss in viable cells was accompanied by a significant increase in the percentage of cells undergoing apoptosis (Figure 5A, L3; Figure 5B, AHH-1 *tk^{+/+}*). Measurements were made at multiple time points, but for clarity only the data for the 0, 24, 32, 48 and 72 hour time points are presented. As with the viability data, there were differences in the onset of a detectable concentration-response relationship. A significant slope was detectable at 24 hours in the L3 cell line ($p = 0.025$), but not until 32 hours in the AHH-1 *tk^{+/+}* cell line ($p = 0.002$). A significant concentration-dependent increase in the percentage of cells in the apoptotic compartment was detected at the 48 and 72 hour time points in both cell lines (all p -values were $p \leq 0.0001$). Similar to the results in the viability assay, the percentage of cells undergoing apoptosis was consistently elevated in both the control and genistein-exposed L3 cells compared to that observed in the AHH-1 *tk^{+/+}* cultures.

Cell Proliferation. The effect of exposure to genistein on the cell-cycle was evaluated both during the 24-hour exposure period and the subsequent culture period in both cell lines. Genistein affected the proportion of L3 cells in the cell-cycle phases during the exposure period. A significant loss (-16 hour, $p = 0.005$; 0 hour, $p \leq 0.0001$) of G1 cells was accompanied by a significant increase in the proportion of cells in S-phase (0 hour, $p = 0.02$) and G2/M (-16 hour, $p = 0.005$; 0 hour, $p \leq 0.0001$). At 24 hours post-exposure and after, no effect of concentration on the percentage of G0/G1 cells could be detected

statistically. The proportion of G0/G1 cells was consistently greater in L3 compared to AHH-1 *tk⁺* in both control and genistein-exposed cultures. A shift from an increasing proportion of S-phase cells to a decreasing one was detected at 8 to 24 hours in L3. The loss in S-phase cells was detected until 56 hours after exposure (all p-values were $p \leq 0.005$). From 24 to 56 hours, L3 cells arrested in G2/M (all p-values were $p \leq 0.0001$). The percentages of L3 cells in each of the cell-cycle phases are presented graphically in Figure 6A (G0/G1), 7A (S-phase), and 8A (G2/M). However, for clarity, only the data from the 0, 24, 48, and 72 hours time points are presented.

In AHH-1 *tk⁺*, there was a significant decrease ($p = 0.003$) in the percentage of cells in G0/G1 at -16 hours which could be accounted for by the significant increase ($p = 0.011$) in S-phase cells. The block in S-phase was also detected at the early time points after exposure as indicated by the significant increase (0 hour, $p = 0.012$; +8 hour, $p = 0.033$) in the proportion of S-phase cells. Those cells which were able to complete DNA synthesis entered the G2 phase of the cell-cycle as indicated by the significant increase ($p \leq 0.0001$) in the percentage of G2/M cells. However, at 24 hours and continuing through 72 hours, the effect of increasing concentration was to arrest cells in G2/M as evidenced by the significant increase in the percentage of cells in this cell-cycle phase (all p-values were $p \leq 0.0001$). The inability of cells to pass this restriction point was reflected in the depletion of cells in G0/G1 (all p-values were $p \leq 0.0001$) and S-phase (all p-values were $p \leq 0.0001$ with the exception of 72 hour, $p = 0.021$). The percentages of AHH-1 *tk⁺* cells in each of the cell-cycle phases are presented in Figure 6B (G0/G1), 7B (S-phase) and 8B (G2/M). For clarity, only the data from the 0, 24, 48, and 72 hour time points are presented.

Although exposure to genistein resulted in the arrest of cells in G2 in both cell lines, the percentage of cells that underwent G2 arrest was consistently lower in L3 than in AHH-1 *tk⁺* (Figures 8A and 8B). A comparison of the slopes of the concentration-response curves at each of the time points indicated that the differences were significant at 24 hours ($p=0.0118$) and at all subsequent time points from 32 to 72 hours after exposure (all p-values were $p \leq 0.0001$).

DISCUSSION:

Our laboratory is interested in defining the role of apoptosis in the recovery of mutant clones in mammalian cells exposed to DNA-damaging agents. Previously, we have demonstrated that chemically-induced mutant clones, utilized as markers for cells with DNA damage that retained viability and clonogenicity, were recovered under conditions where cell death occurred by apoptosis [54, 56]. In addition to the presence of cells with DNA damage, the onset of apoptosis was delayed and no G1 arrest could be detected [63, 54, 62, 56]. Because these characteristics are associated with the mutant *p53* phenotype, we sequenced the *p53* gene in AHH-1 *tk*^{-/-} and detected a C → T mutation at codon 282 of exon 8 [23]. In addition, the wild-type sequence was found in the *p53* gene of the closely-related lymphoblast line, MCL-5 and its parent line, L3. In order to further evaluate the role of apoptosis in mutagenesis, we have conducted experiments to determine if the phytoestrogen and topo-II inhibitor, genistein, induced clastogenic or mutagenic damage. We then sought to determine if *p53* functional status affected the recovery (viability and clonogenicity) of the damaged cells through its role in the apoptosis pathways.

Our initial objective was to determine if exposure to genistein resulted in an increase in the mutant fraction at either the *tk* locus or the *hprt* locus in either of the cell lines. In both AHH-1 *tk*^{-/-} and in L3, significant increases in the mutant fraction at the *hprt* locus were accompanied by the induction of micronuclei. A significant increase in the number of *tk* mutants with the slow-growth phenotype was detected in AHH-1 *tk*^{-/-}. Because of the genistein-induced inhibition of topo-II by binding to the ATP receptor site, stabilization of the "cleavable complex" and formation of protein-linked double-strand breaks [45], and our previous results in which slow-growth *tk* mutants were recovered after exposure to the topo-II inhibitor, *m*-amsa [54], the induction of slow-growth *tk* mutants was not unexpected. However, in contrast to our results with AHH-1 *tk*^{-/-}, we were unable to recover slow-growth *tk* mutants in the L3 cell line. These results might be explained by a lowered basal level of topo-II activity as has been described in mutant CCRF-CEM cells [44] reducing the available target sites. However, the induction of micronuclei and a small, but significant increase in the mutant fraction at the *hprt* locus indicated that clastogenic damage had also occurred in this line.

Because of the difference in the functional status of *p53* between the lines, this suggested that a *p53*-mediated pathway or pathways was important in the response to genistein-induced DNA damage. Evidence exists that *p53* functions in cell-cycle control by upregulating the synthesis of the p21^(waf-1/cip-1) protein which is important in establishing the G1 checkpoint after DNA damage [65]. *p53* also downregulates the apoptosis-inhibitory protein, bcl-2, and upregulates the apoptosis-promoting protein, bax, after exposure to DNA damaging agents [4]. The binding of the C-terminal domain of the *p53* protein to the ends of ionizing radiation-induced single-strand DNA in order to facilitate end-rejoining and strand-transfer reactions suggests a role for *p53* in DNA repair [66, 67]. A "loss-of-function" mutation in the *p53* gene may then result in an increase in the mutant fraction

after exposure to DNA-damaging agents due to the abrogation or impairment of these pathways.

Evidence which indicates the importance of *p53* functional status in determining the mutant fraction at the *tk* locus may be found in studies with the TK6 family of human lymphoblastoid cell lines. Exposure of either WTK1 (mutant *p53*) or WI-L2-NS (mutant *p53*) to ionizing radiation results in an increase in the mutant fraction at the *tk* locus [12, 13, 17, 18] or an increase in the chromosome aberration frequency [14] when compared to TK6 (wild-type *p53*). The increased recovery of chromosomal mutants in cell lines with mutant *p53* is not limited to the effects of ionizing radiation. Not only was the spontaneous mutation frequency greater in WTK1, but exposure of TK6 and WTK1 to the simple alkylating agents, ethyl methanesulfonate (EMS) and methyl methanesulfonate, resulted in an increased recovery of slow-growth *tk* mutants in WTK1 compared to TK6 [14]. Perhaps more relevant to this study, the higher spontaneous and dimethylnitrosamine (DMN)-induced mutation frequency in AHH-1 *tk*⁺ compared to MCL-5 (a derivative of the L3 line used in this study) can be attributed to the recovery of LOH mutants in AHH-1 *tk*⁺ [24, 68].

When dominant-negative *p53* mutations were transfected into TK6, the ionizing radiation-induced mutation frequency at the *tk* locus in the transfectants was comparable to that observed in the mutant *p53* cell line, WTK1 [69]. In that study and others, the increase in the recovery of *tk* mutants was accompanied by a delay in the onset or decrease in the rate of apoptosis. These findings provide support for the hypothesis that not only does impaired DNA repair contribute to the mutant fraction, but also the inhibition of or escape of damaged cells from apoptosis. Evidence towards this viewpoint comes from studies with cells derived from colon cancer with the mismatch mutator phenotype and defective in mismatch repair [70]. Loss-of-function mutations due to an unrepaired frame-shift mutation in a microsatellite sequence in the apoptosis-promoting protein, *bax*, compromise the apoptosis pathways contributing to the progression of genomic instability.

In the experiments of Cherbonnel-Lassere et al. [17], the genes for *bcl-2* and the *bcl-2*-related protein, *bcl-x_l*, were each transfected into TK6 and WTK1. Ectopic expression of either *bcl-2* or *bcl-x_l* in TK6 resulted in an increase in the mutant fraction at the *tk* locus due to both an increase in the survival of mutant clones and an increase in clonogenic survival. Because *bcl-2* is well-recognized to function as an apoptosis-inhibitory protein and, as yet, no DNA repair function has been associated with its expression, these results provide evidence that inhibition of apoptosis has a significant effect on the recovery of chromosomal mutants. Additional evidence which suggests a role for *p53*-modulated apoptosis in the recovery of ionizing radiation-induced mutants comes from the studies of Griffiths et al. [71]. The increase in the *hprt* mutant fraction in B-cells obtained from *p53*-mutant mice compared to those obtained from wild-type mice was attributed to an increase in the clonogenic survival of the mutant cells and was accompanied by a decrease in the percentage of cells undergoing apoptosis.

Molecular analysis of *tk* mutant clones indicated that cells which had undergone LOH due either to deletion or to somatic recombination at the *tk* locus were recovered in both WTK1 and TK6, but to a substantially greater extent in WTK1 [20, 21]. Chromosome painting experiments revealed that nonhomologous recombination and the resulting unbalanced translocations contributed significantly to the increase in the frequency of *tk* mutant clones derived from WTK1 [19]. These results suggest that defects in *p53*-mediated recombinational repair also contribute to the increased mutant fraction observed in *p53* mutant cell lines.

In previous studies, we proposed that the delayed apoptosis observed as part of the mutant *p53* phenotype in mutagen-exposed AHH-1 *tk*^{+/−} cells was an important factor in the ability to recover chromosomal mutants. In order to determine if differences in the rate or onset of apoptosis were factors in the differential recovery of genistein-induced mutants between AHH-1 *tk*^{+/−} and L3, we examined the kinetics of apoptosis and the level of clonogenic survival after exposure to genistein in each cell line. In these experiments, the basal rate of apoptosis was consistently higher and the actual cloning efficiencies were consistently lower in L3 than in AHH-1 *tk*^{+/−}. Both cell lines constitutively express P4501A1 and the increased rate of apoptosis in L3 suggests that functional *p53* is signaling cells with DNA damage to undergo apoptosis. The *p53*-mediated increase in the rate of apoptosis may account for the lowered actual cloning efficiency in L3 observed here and for the decreased recovery of spontaneous and DMN-induced LOH mutants in MCL-5 (derived from L3) compared to AHH-1 *tk*^{+/−} [24, 68].

When the kinetics of genistein-induced apoptosis were evaluated, the onset of apoptosis was detected immediately after exposure in L3, but was delayed in AHH-1 *tk*^{+/−}. The delay in the onset of apoptosis, characteristic of cells with mutant *p53*, may be accounted for by the arrest of cells in G2 that we, and others [35, 72] have observed after exposure to genistein. In contrast to the results with AHH-1 *tk*^{+/−}, a lower percentage of L3 cells arrested in G2 and was accompanied by an increase in the percentage of cells undergoing early apoptosis. Alternatively, the delay may reflect the altered expression of the *p53*-regulated proteins, *bcl-2* and *bax*, in AHH-1 *tk*^{+/−}. We are currently conducting experiments to determine the level of expression of the *bax* and *bcl-2* proteins in genistein-exposed cells.

On a mechanistic basis, genistein exerts many of its biological effects by competitive binding to the ATP receptor of both topo-II and tyrosine kinase. Both the DNA damage induced as a result of topo-II inhibition and the inhibition of tyrosine kinase activity may signal genistein-exposed cells to undergo apoptosis. Experiments which have attempted to partition the DNA damaging effects of topo-II inhibition from those associated with tyrosine kinase inhibition have not been conclusive. For example, CD4⁺CD8⁺ thymocytes undergo apoptosis after genistein exposure, but not after exposure to a different tyrosine kinase inhibitor, herbimycin A, suggesting a topo-II-related mechanism [43]. The tyrosine kinase inhibitor, tyrphostin AG82 does not bind to topo-II, in contrast to the tyrphostin

derivatives AG 786 and 213 [73]. The induction of apoptosis by tryphostin AG82, but not genistein, can be blocked by the tyrosine phosphatase inhibitor, vanadate [74], consistent with a role for topo-II-inhibition in genistein-induced apoptosis. Further, mutant CCRF-CEM cells with lowered expression of topo-II β are markedly resistant to the toxicity of genistein [44].

In certain signaling pathways, tyrosine phosphorylation is necessary to inhibit apoptosis and when tyrosine kinase activity is blocked, cells undergo apoptosis (reviewed in [75]). An example is the v-abl protein tyrosine kinase which has been shown to suppress drug-induced apoptosis after transfection into hematopoietic cells [76, 77, 78]. Several lines of evidence indicate that the inhibition of tyrosine kinase activity by genistein signals cells into apoptosis. When tyrosine phosphorylation, as measured by a phosphotyrosine antibody, was completely blocked by genistein, Jurkat cells underwent apoptosis in a concentration-dependent manner [79]. HL-60 cells, in which the *p53*/DNA damage/apoptosis pathway is impaired due to a "loss-of-function" mutation in the *p53* gene, undergo apoptosis in a concentration-dependent manner [35], consistent with a *p53*-independent pathway.

Although the onset of apoptosis was delayed in AHH-1 *tk*^{+/+}, once apoptosis was initiated, the rate of genistein-induced apoptosis was similar in both cell lines. Further, the genistein-induced reduction in actual cloning efficiencies was statistically indistinguishable between the two cell lines. That the similarity in the responses between the two cell lines is specific to genistein is suggested by preliminary experiments in our laboratory with the simple ethylating agent, ethyl methanesulfonate (EMS). When L3 cells were exposed to EMS and the resulting clonogenic survival values compared to those previously reported for AHH-1 *tk*^{+/+}, survival levels were markedly lower in L3 than in AHH-1 *tk*^{+/+}. The inability to detect a *p53*-related effect on genistein-induced apoptosis and clonogenic survival does not appear to be limited to the human lymphoblastoid cells utilized in this study. Differences in the level of apoptosis and survival of genistein-exposed gastric carcinoma cell lines could not be related to the functional status of *p53* [72]. In those experiments, *p53* expression was not detected in cells with wild-type *p53* leading to the suggestion that apoptosis occurred through a *p53*-independent pathway.

In summary, we clearly recognize the difficulties associated with the extrapolation of data obtained from cell culture models to animal studies. However, our results do indicate that the chemopreventative effect of genistein observed in human populations consuming a "high-soy" diet may be mediated in part through its ability to recruit cells with DNA damage into the pathways for programmed cell death. The increased rate of apoptosis and the lack of recovery of slow-growth *tk* mutants in the L3 cell line with intact *p53* tumor suppressor function compared to AHH-1 *tk*^{+/+} with mutant *p53* suggests that both a *p53*-dependent pathway and a tyrosine kinase-dependent pathway contribute to the destruction of cells with DNA damage. The recovery of genistein-induced slow-growth *tk* mutants and the induction of micronuclei in AHH-1 *tk*^{+/+} is consistent with the induction of clastogenic damage and chromosomal mutations due to the inhibition of topo-II. Further, this may

suggest that genistein-induced apoptosis is inefficient in cells in which tumor suppressor function, such as AHH-1 *tk^{+/+}* with mutant *p53*, is impaired or abrogated.

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Figure Legends.

Figure 1. The effect of genistein exposure on the frequency of TFT-resistant clones (*tk* locus) with the slow-growth phenotype in L3 cells (dashed line) and AHH-1 *tk*^{+/+} cells (solid line). Each data point represents the mean (\pm s.e.m.) of three individual experiments. The average mutant fraction in control cultures was 16.1 ± 6.1 in AHH-1 *tk*^{+/+} and 12.3 ± 2.1 in L3 control cultures.

Figure 2. The effect of genistein exposure on percent relative cloning efficiency in L3 cells (dashed line) and AHH-1 *tk*^{+/+} cells (solid line) at Day 0 (A), Day 3 (B), and Day 7 (C) after exposure. Each data point represents the mean (\pm s.e.m.) of three individual experiments. Actual cloning efficiencies in control L3 cultures were 47.2 ± 11.4 at Day 0, 40.0 ± 4.5 at Day 3, and 34.9 ± 5.7 at Day 7. In AHH-1 *tk*^{+/+}, control cloning efficiencies were 63.4 ± 15.0 at Day 0, 68.8 ± 18.0 at Day 3 and 68.6 ± 18.9 at Day 7.

Figure 3. Induction of micronuclei in genistein-exposed cultures of L3 cells (dashed line) and AHH-1 *tk*^{+/+} cells (solid line). Each data point represents the mean (\pm s.e.m.) of three individual experiments.

Figure 4. The effect of genistein exposure on the percentage of viable cells in the L3 cell line (A) and in the AHH-1 *tk*^{+/+} cell line (B). The percentage of viable cells was determined by flow cytometry utilizing the FDA/PI assay. Each data point represents the mean (\pm s.e.m.) of three individual experiments. The concentrations of genistein were 0 μ g/ml [■], 1 μ g/ml [▨], 5 μ g/ml [▧], 10 μ g/ml [▩] and 20 μ g/ml [⊞].

Figure 5. The effect of genistein exposure on the percentage of apoptotic cells in the L3 cell line (A) and in the AHH-1 *tk*^{+/+} cell line (B). The percentage of apoptotic cells was determined by flow cytometry utilizing the FDA/PI assay. Each data point represents the mean (\pm s.e.m.) of three individual experiments. The concentrations of genistein were 0 μ g/ml [■], 1 μ g/ml [▨], 5 μ g/ml [▧], 10 μ g/ml [▩] and 20 μ g/ml [⊞].

Figure 6. The effect of genistein exposure on the percentage of cells in the G0/G1 phase of the cell-cycle in the L3 cell line (A) and in the AHH-1 *tk*^{+/+} cell line (B). DNA content was determined by PI staining and BrdUrd incorporation by a FITC-labeled monoclonal against BrdUrd and subsequent flow cytometric analysis. The percentage of cells in G0/G1 was determined from the analysis of 25,000 cells per sample. The data presented are from three separate experiments and represent the mean (\pm s.e.m.). The concentrations of genistein were 0 μ g/ml [■], 1 μ g/ml [▨], 5 μ g/ml [▧], 10 μ g/ml [▩] and 20 μ g/ml [⊞].











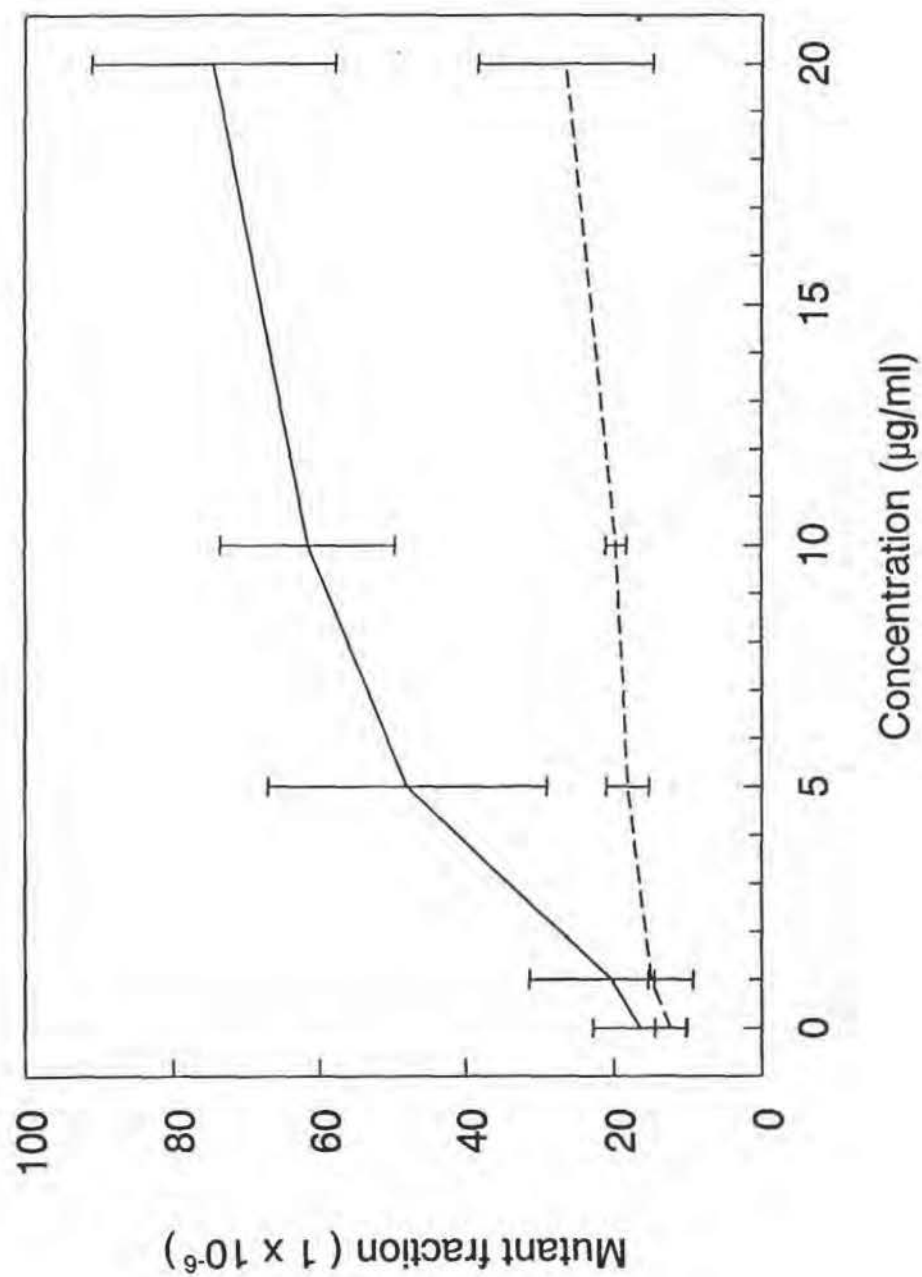
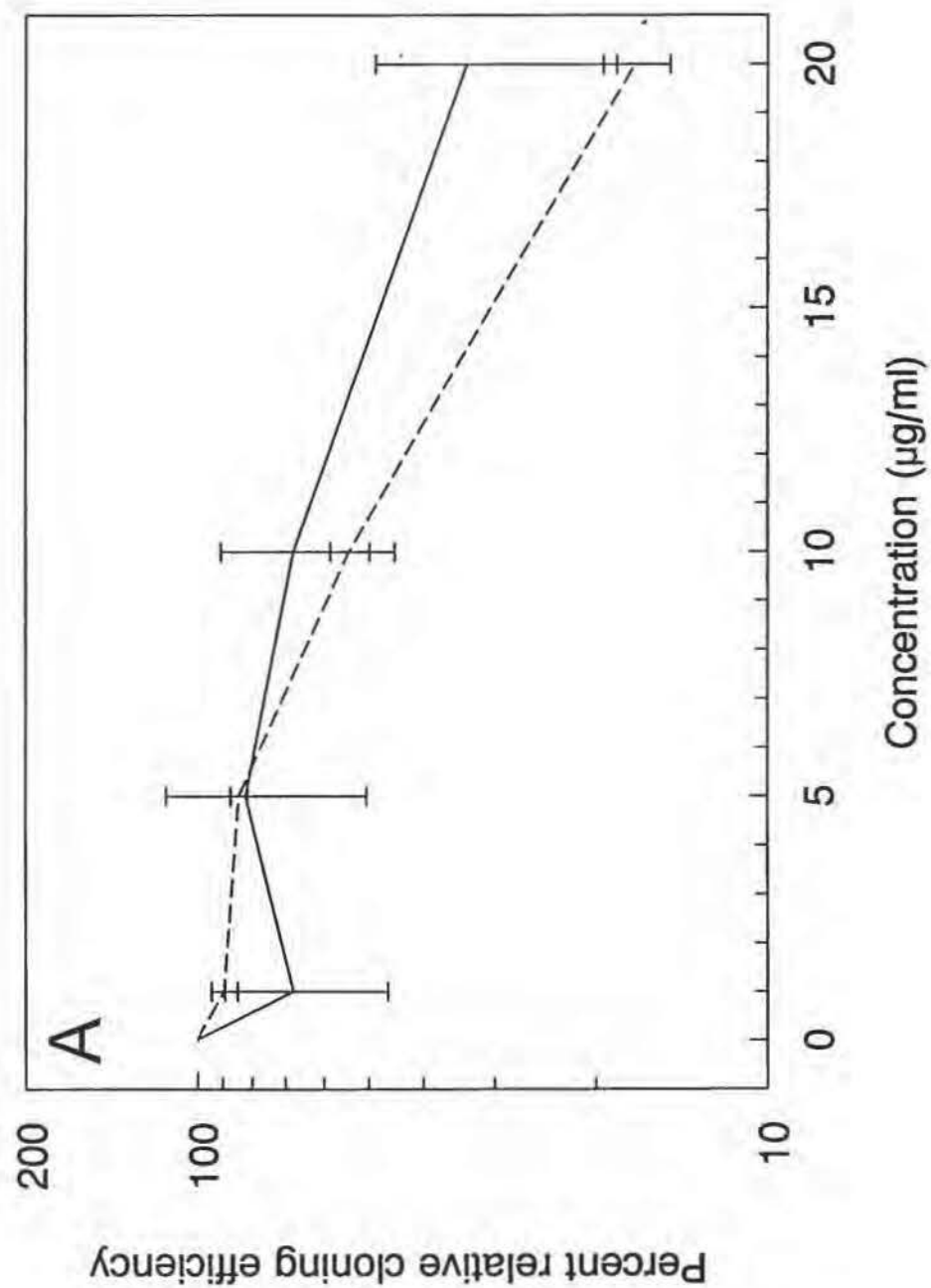
Figure 7. The effect of genistein exposure on the percentage of cells in S-phase in the L3 cell line (A) and in the AHH-1 *tk^{+/+}* cell line (B). Materials and methods described in the text. The data presented are from three separate experiments and represent the mean (\pm s.e.m.). The concentrations of genistein were 0 $\mu\text{g/ml}$ [], 1 $\mu\text{g/ml}$ [], 5 $\mu\text{g/ml}$ [], 10 $\mu\text{g/ml}$ [] and 20 $\mu\text{g/ml}$ [].

Figure 8. The effect of genistein exposure on the percentage of cells in G2/M in the L3 cell line (A) and in the AHH-1 *tk^{+/+}* cell line (B). Materials and methods described in the text. The data presented are from three separate experiments and represent the mean (\pm s.e.m.). The concentrations of genistein were 0 $\mu\text{g/ml}$ [], 1 $\mu\text{g/ml}$ [], 5 $\mu\text{g/ml}$ [], 10 $\mu\text{g/ml}$ [] and 20 $\mu\text{g/ml}$ [].



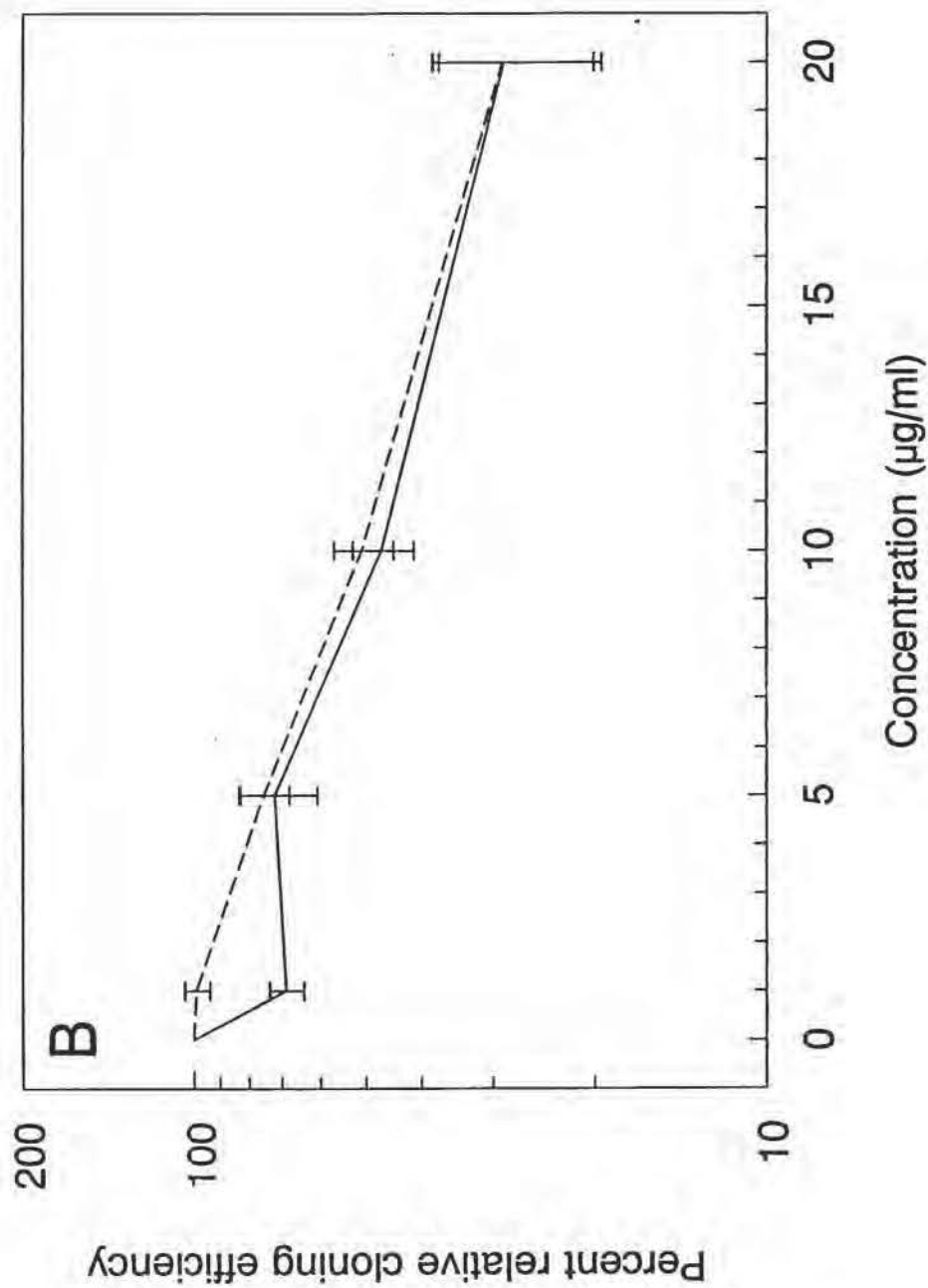
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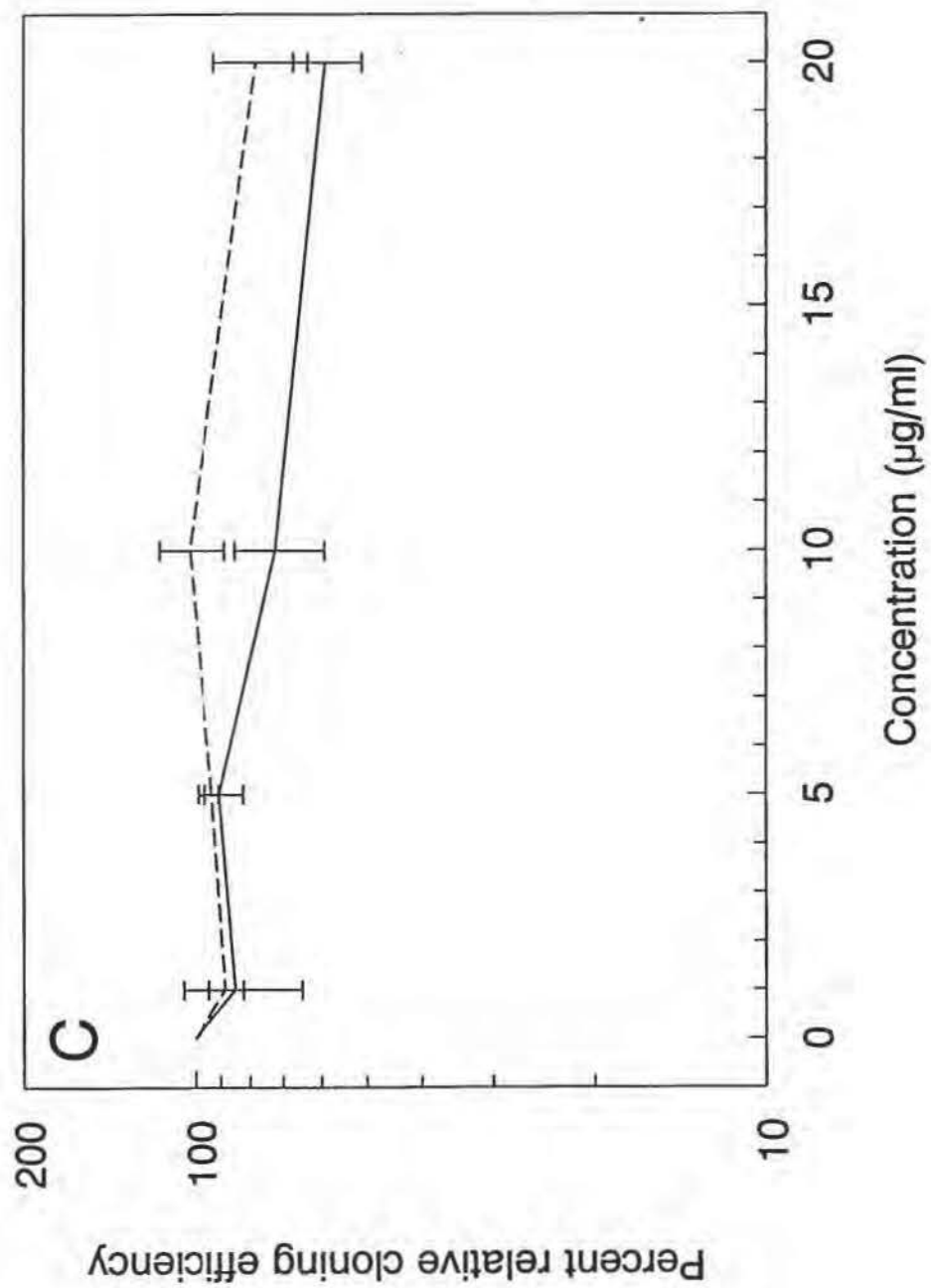


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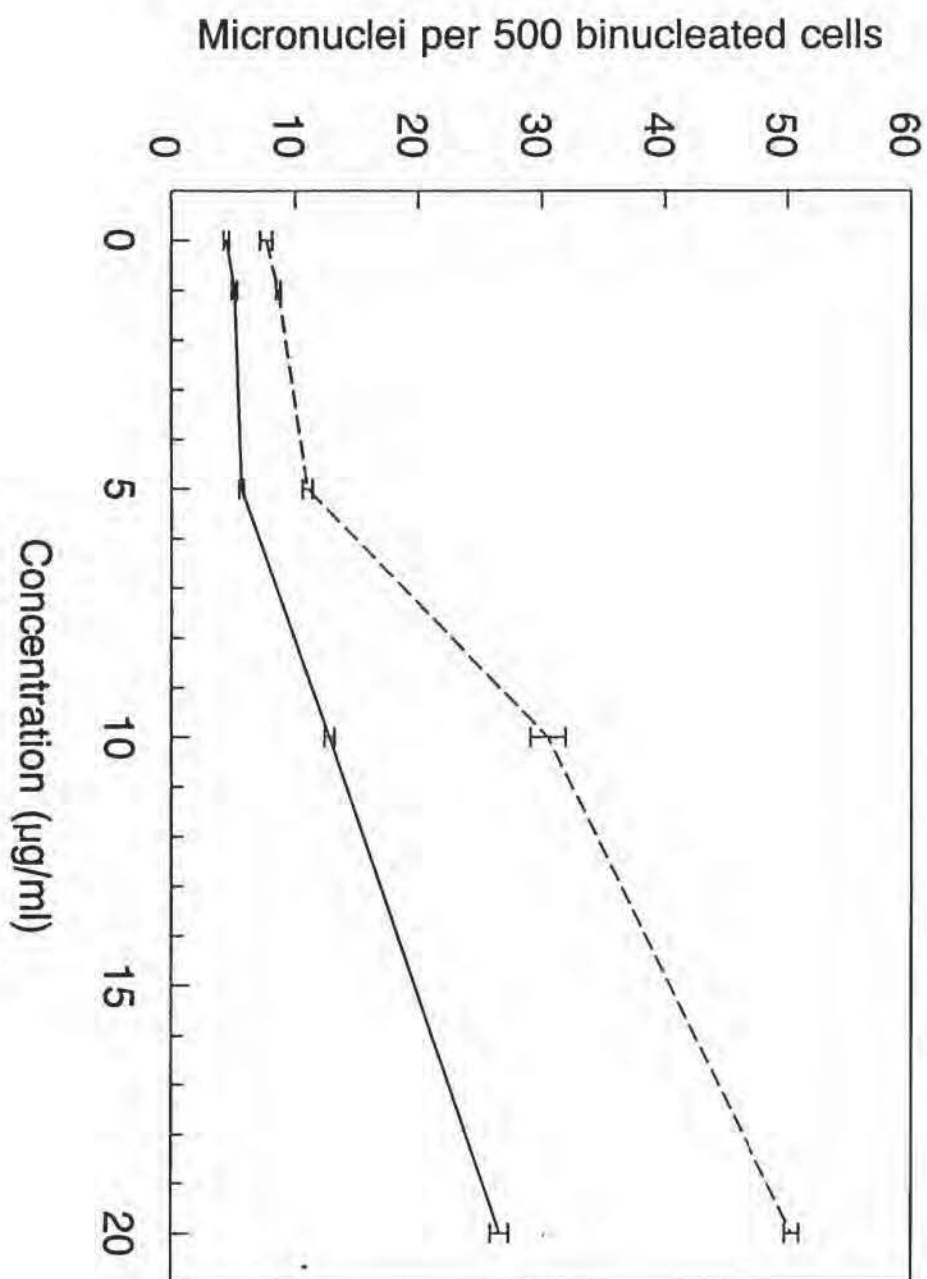
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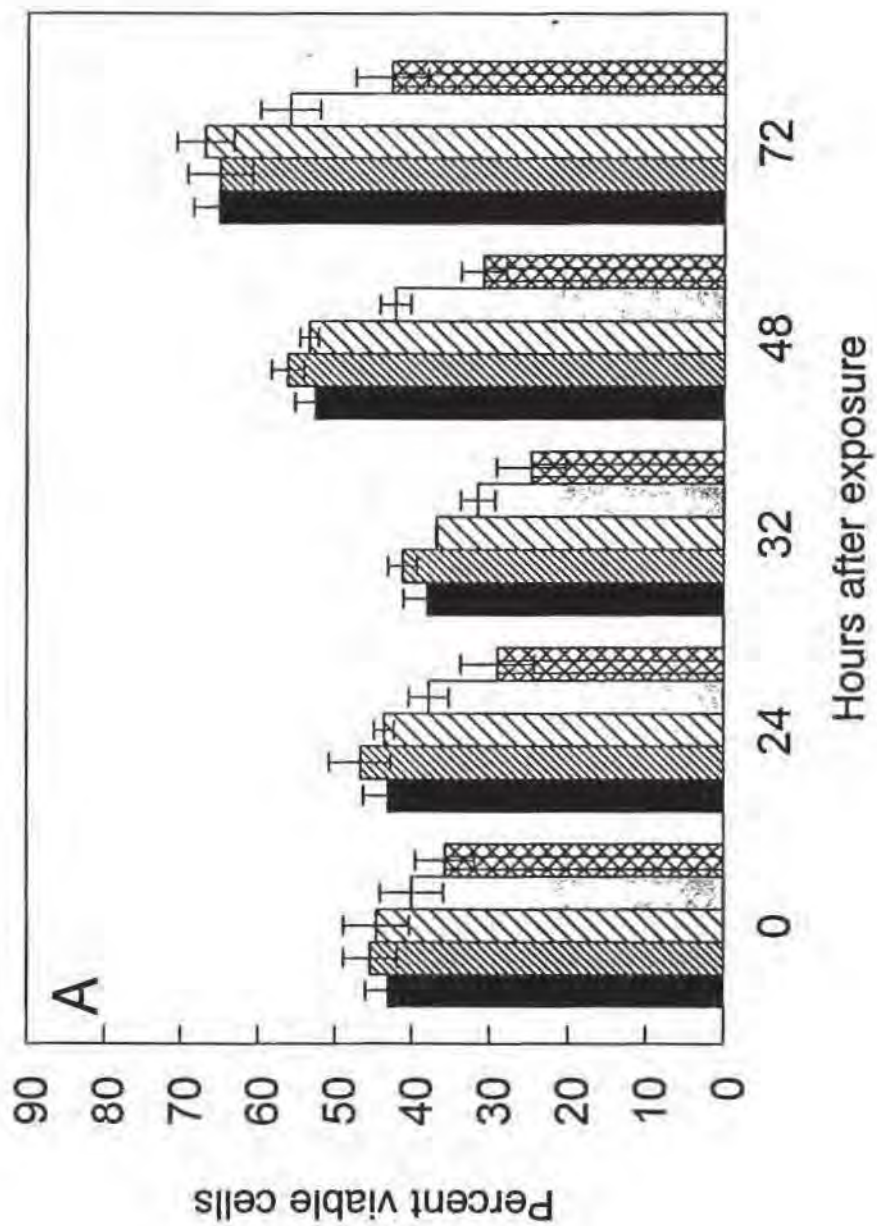


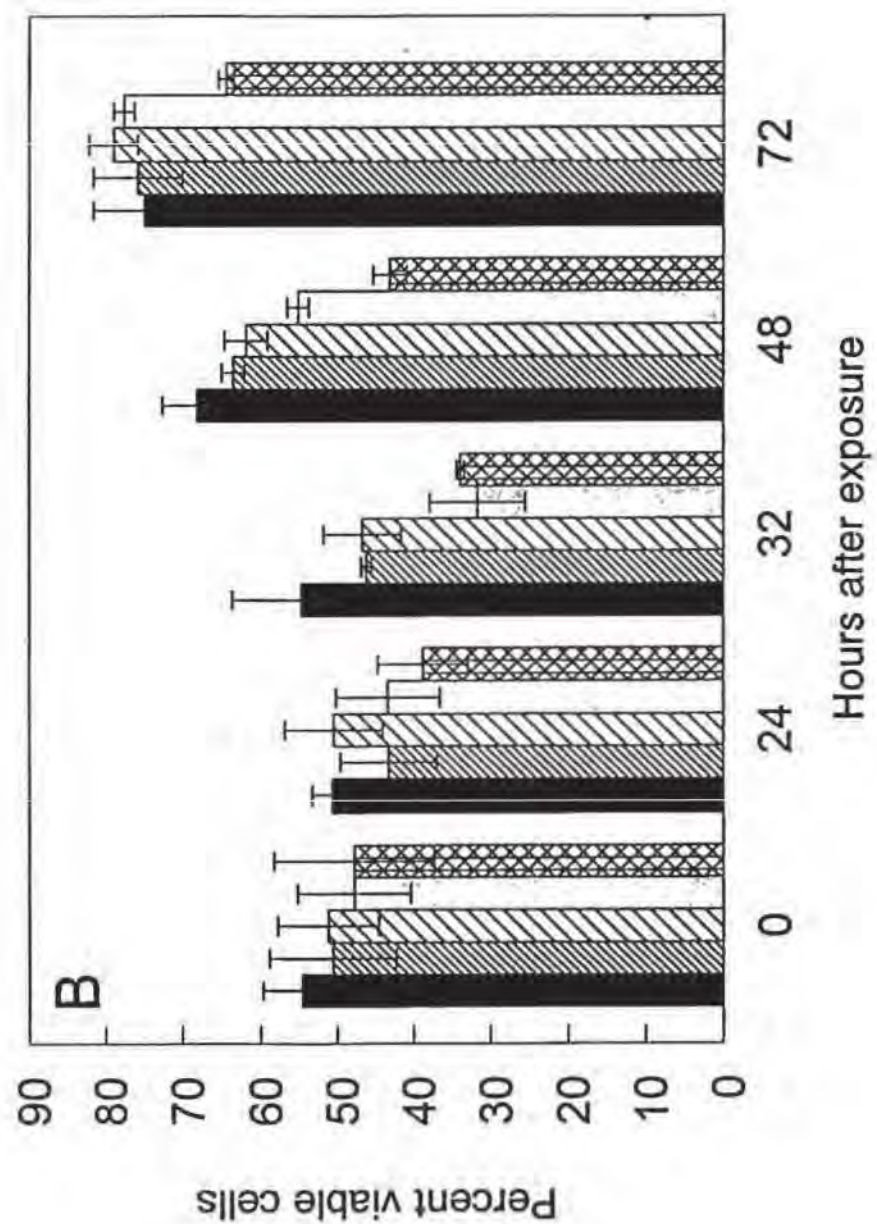
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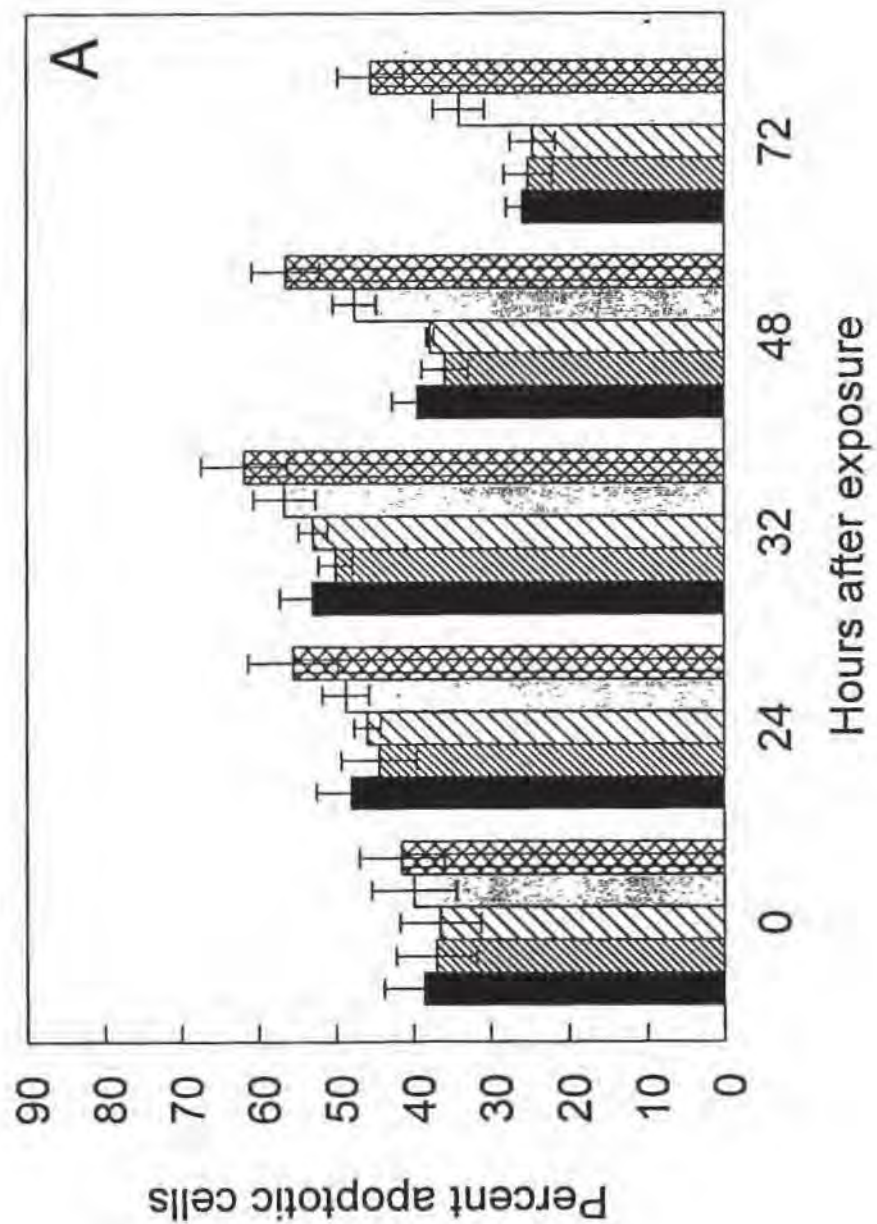






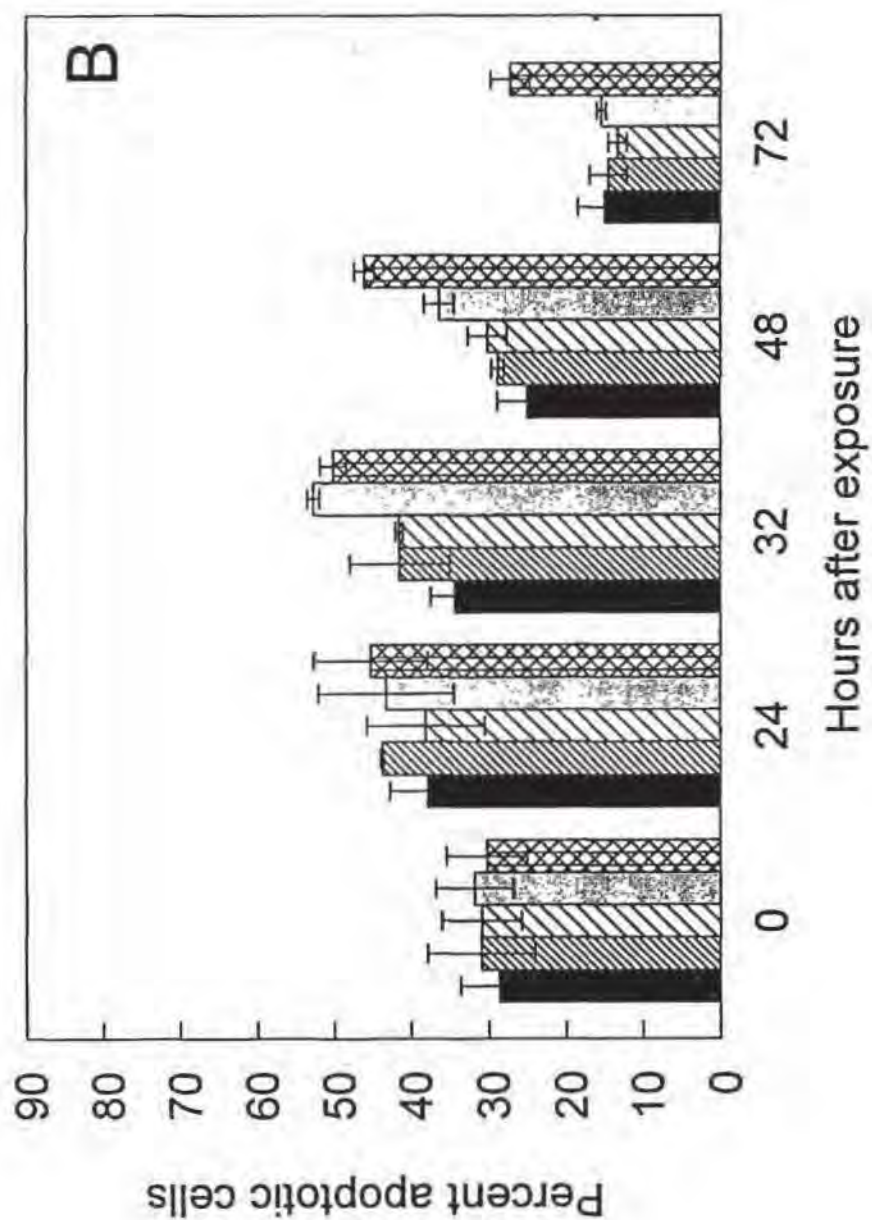
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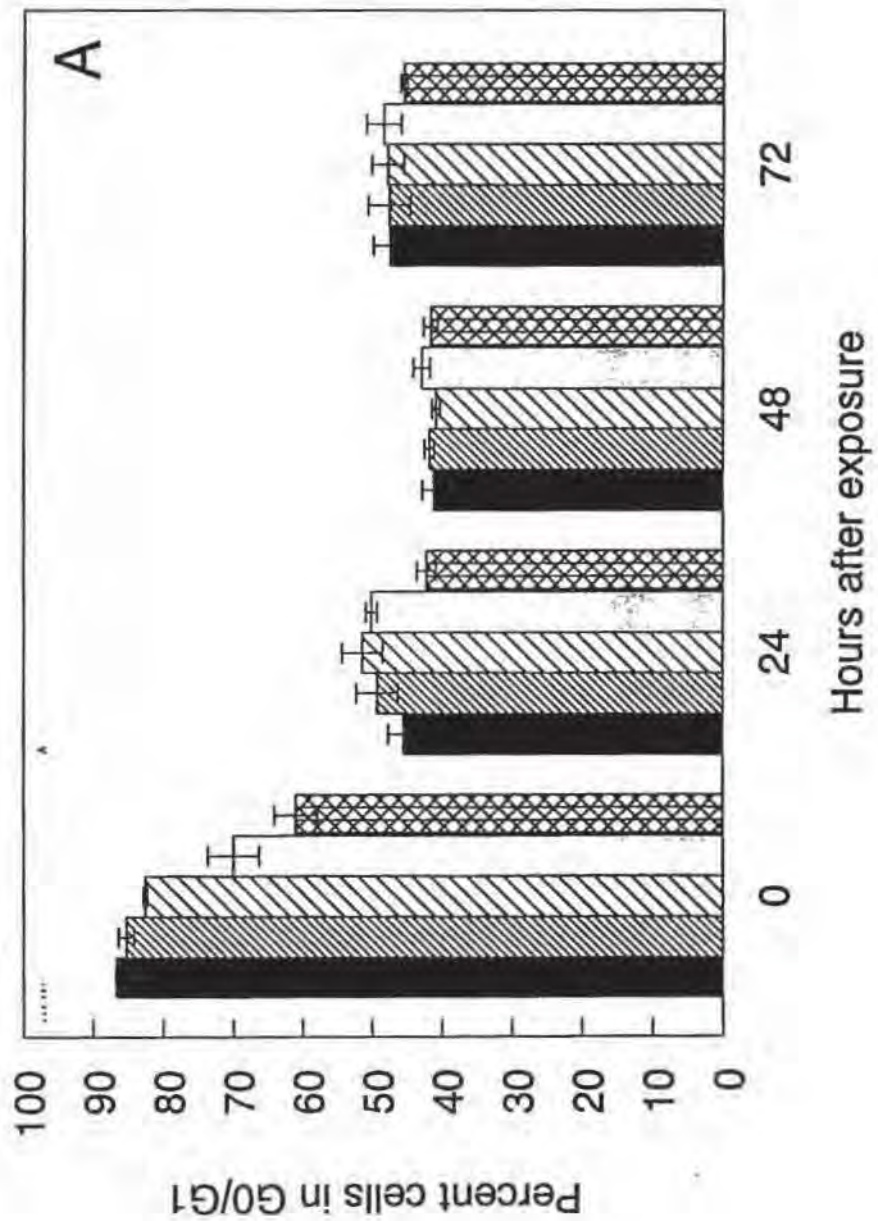
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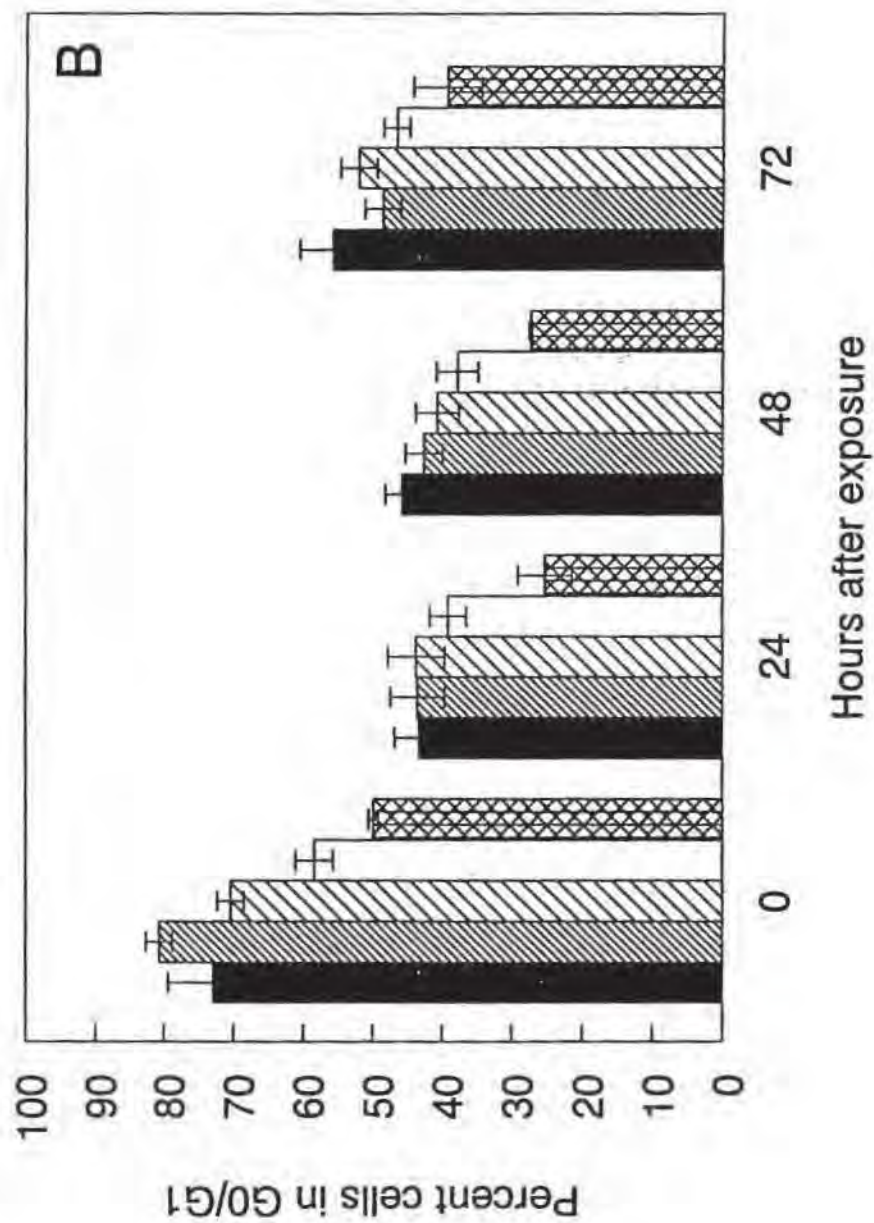
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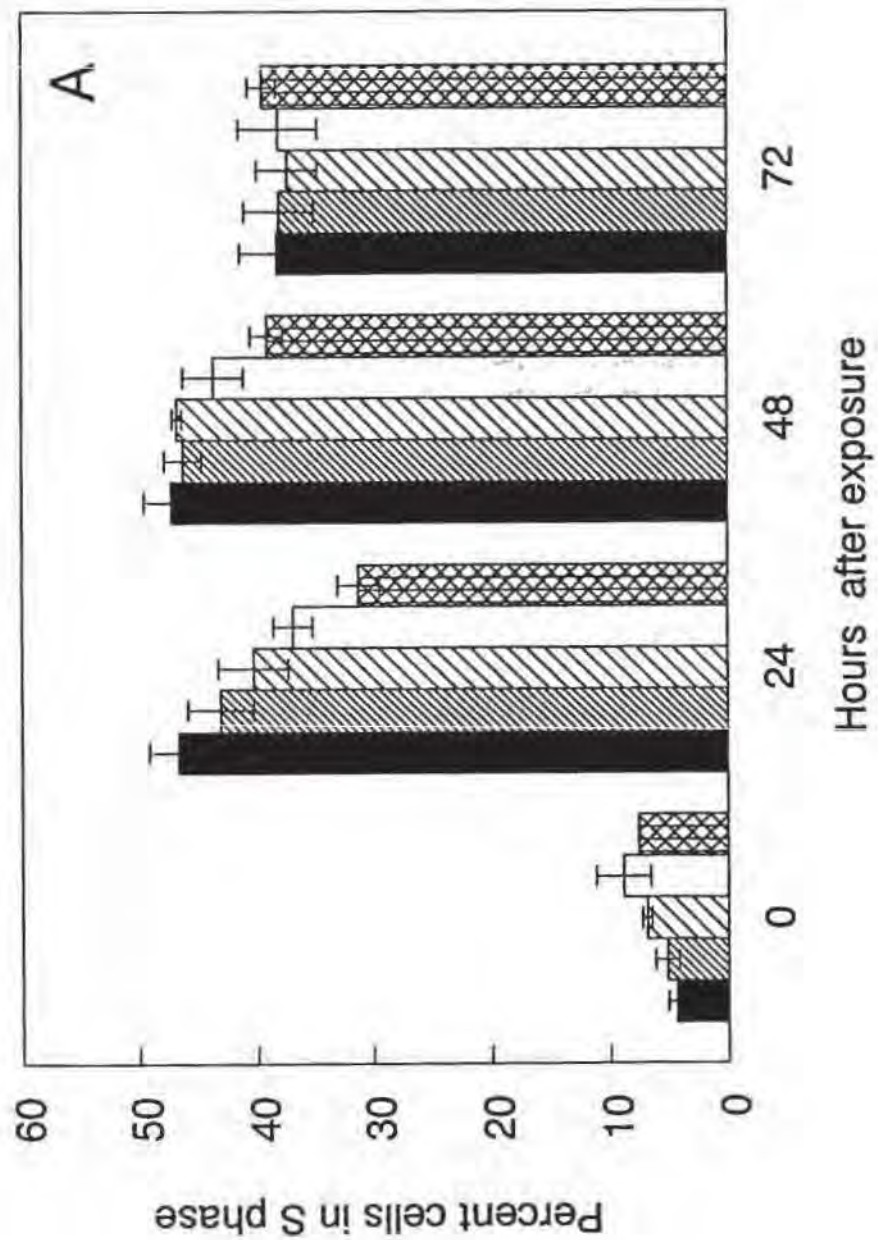
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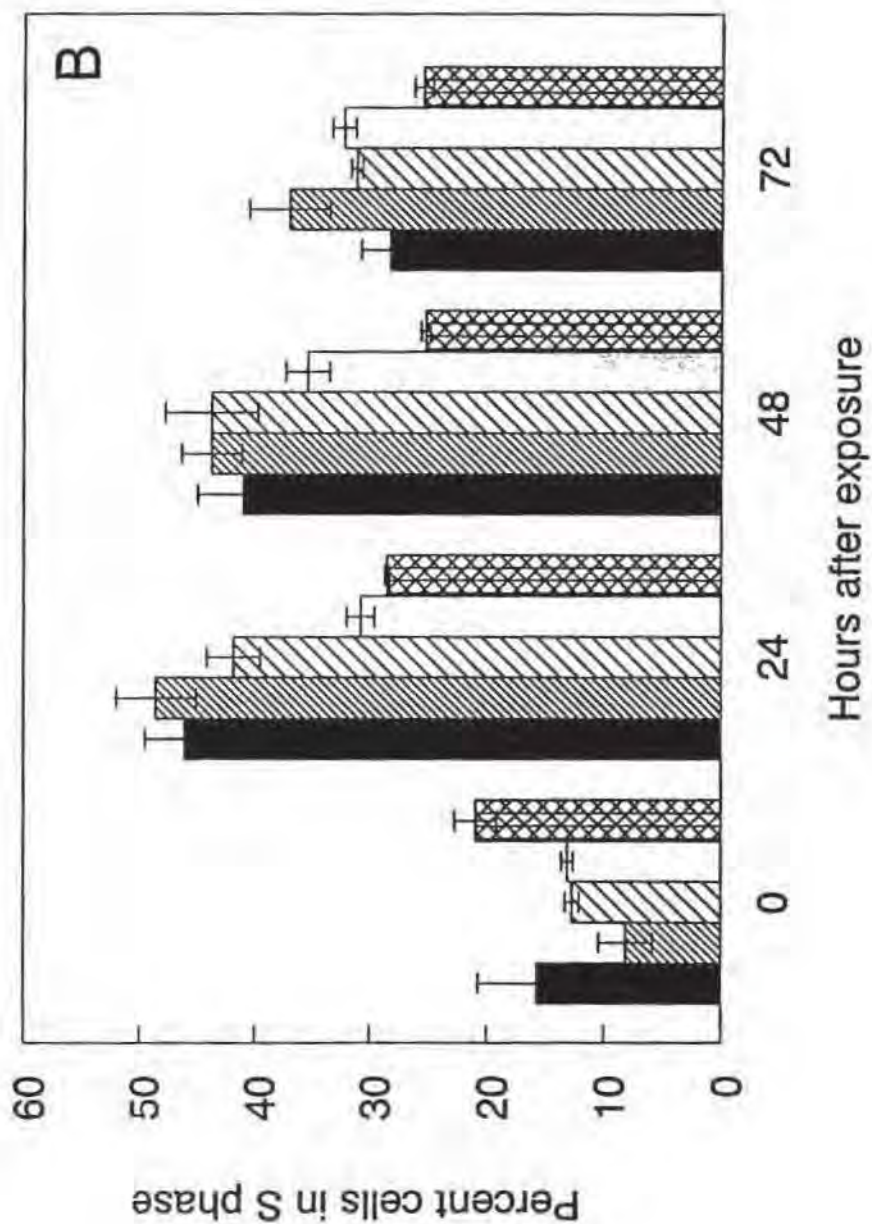
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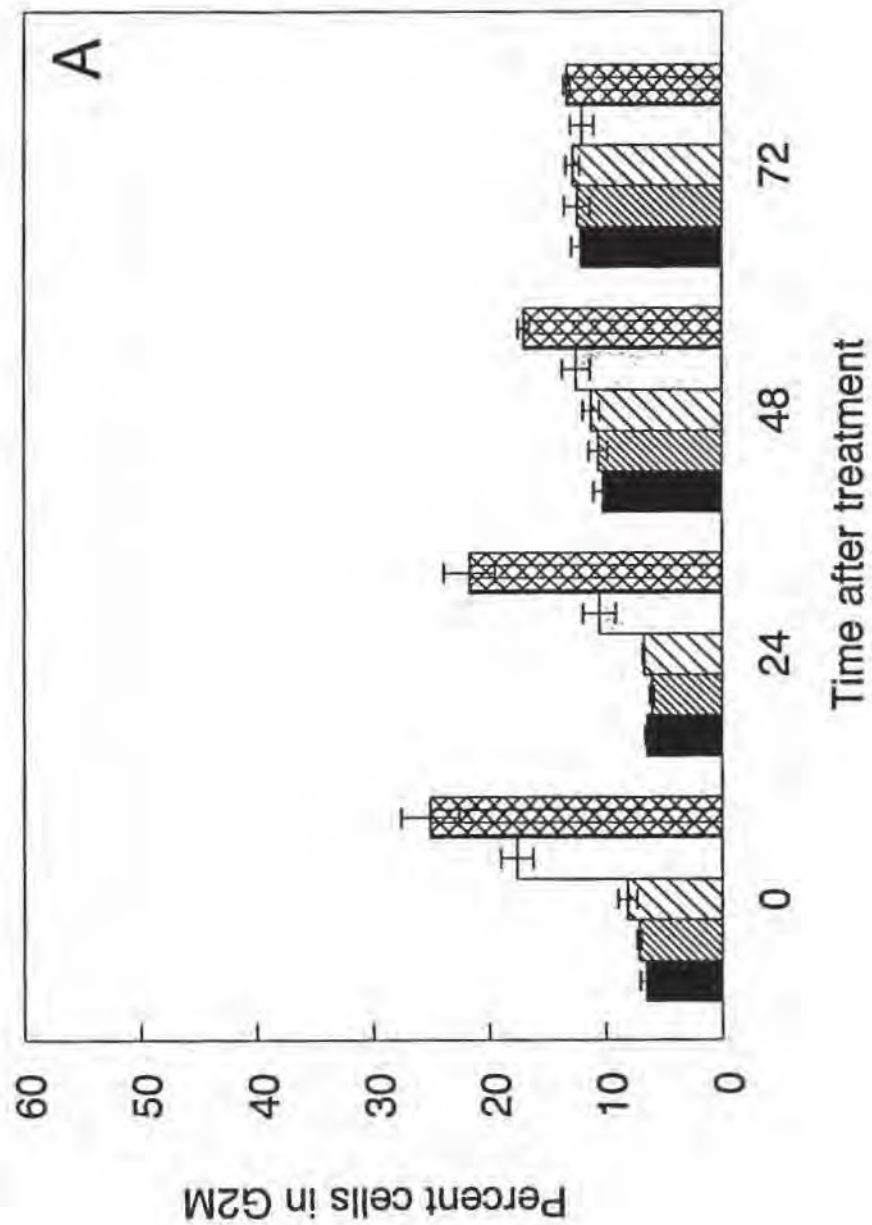
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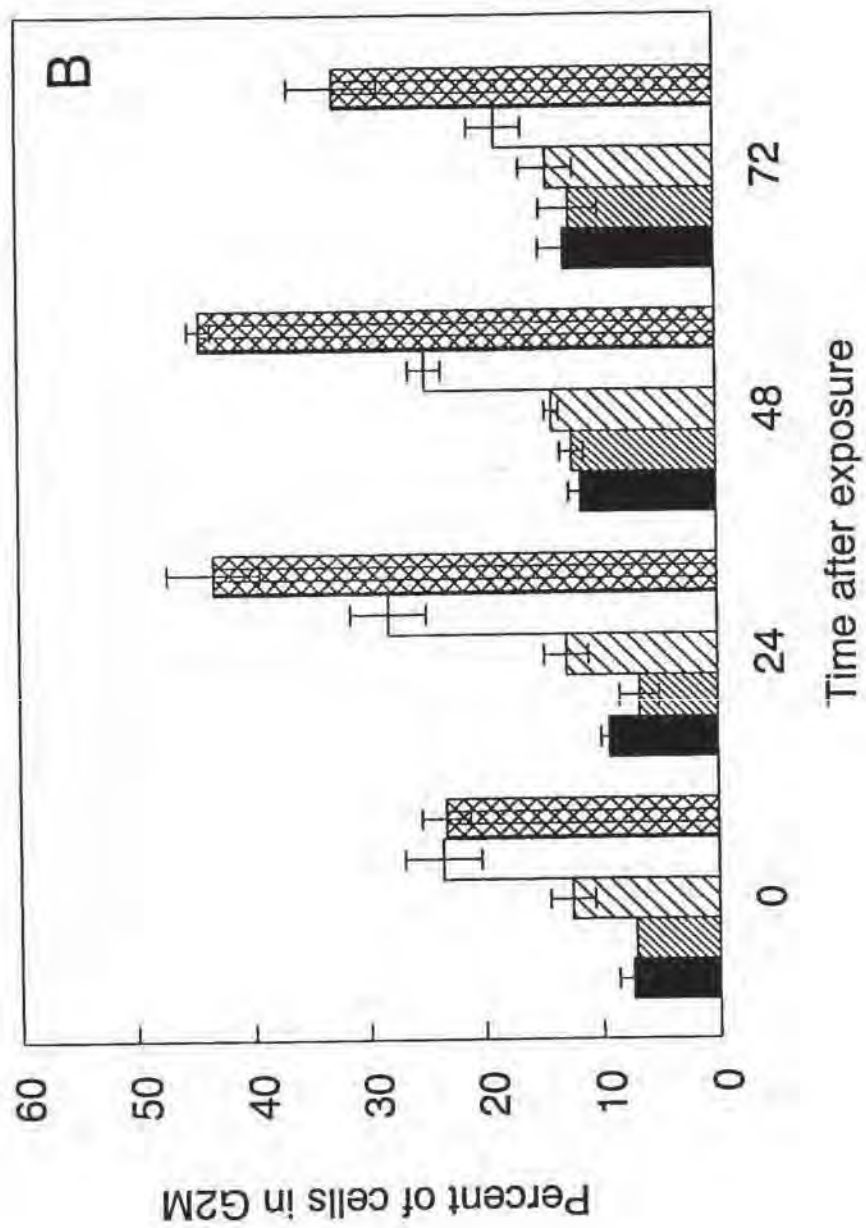
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Page 1 of 3

To
 Dr Linda Kahl
 Office of Premarket Approval - CFSA
 Food and Drug Administration HFS 206
 200 C Street SW
 WASHINGTON DC 20204
 801 (202) 418 3131

From ~~RICHARD T. SAUT~~
 R.D.H.
 WHANGAREI
 NEW ZEALAND
 JULY 9 1998

Dear Dr Kahl, GRN 0000021

Attached to this fax are pages 367 and 368 of a paper which I have been awaiting for some time.

Here it is: "GENOTOXICITY OF ESTROGENS": METZLER, KULLING, PREIFFER AND JACOBS.

The publication journal should show clearly at top left of page 367.

You will note that genistein, the soy isoflavone which is biologically active quantities in soy feeds for human consumption, figures prominently in this review.

There is no conceivable way that isoflavones could be determined to be safe.

Sincerely

~~RICHARD T. SAUT~~

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From ~~Bent~~ ~~Z. Lebensm. Unters. Forsch. A~~ 206: 367-373

Z. Lebensm. Unters. Forsch. A (1998) 206, 367-373

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REVIEW

Manfred Metzler · Sabine E. Kulling · Erika Pfeiffer
Eric Jacobs

Genotoxicity of estrogens

Received: 14 December 1997

Abstract Genotoxic effects of the endogenous mammalian estrogen 17 β -estradiol and the synthetic estrogen diethylstilbestrol have recently been demonstrated, e.g. the induction of numerical chromosomal aberrations (aneuploidy, i.e. the condition in which one or more whole chromosomes of a normal set are missing or present in more than the usual set of copies) and the formation of deoxyribonucleic acid (DNA) adducts. It is likely that the genotoxicity of the estrogens acts in concert with their hormonal activity to give rise to their carcinogenic effects. Many of the phytoestrogens that occur in plants and the numerous anthropogenic estrogens in our environment, which are ingested with food, have not yet been examined for their genotoxic potential. Recent studies have demonstrated that some but not all of these estrogens exhibit genotoxicity. The type and strength of the genotoxicity strongly depends on the chemical structure and does not correlate with estrogenicity. For example, coumestrol and genistein are clastogenic in cultured mammalian cells and lead in gene mutations, whereas biochanin-A and bisphenol-A have the potential to cause aneuploidy. Daidzein, enterolactone, enterodiol and certain bisphenols are devoid of genotoxic effects. The genotoxicity should be determined individually for each estrogen and taken into account in the assessment of its carcinogenic risk.

Key words Estrogens · Genotoxicity · Phytoestrogens · Bisphenols · Hormonal carcinogenesis

Introduction

According to an old but still valid definition, an estrogen is a substance that stimulates cell division in the tissues of the female genital tract, e.g. the uterus, vagina and mammary gland [1]. The most active mammalian estrogen is the

steroidal female sex hormone 17 β -estradiol (E₂; Fig. 1). However, it has long been known that estrogenic activity is not restricted to steroidal hormones. In 1938, the stilbene estrogen diethylstilbestrol (DES; Fig. 1) was synthesized and found to match or even exceed E₂ in terms of hormonal activity [2]. Later, numerous other compounds of quite diverse chemical structure were detected to exhibit estrogenicity [3, 4]. Among them are both natural substances such as endogenous plant constituents (phytoestrogens) and fungal metabolites (mycoestrogens), as well as a variety of man-made chemicals (termed xenoestrogens or, more precisely, anthropogenic estrogens). Some examples of phytoestrogens (coumestrol, genistein, daidzein, biochanin-A, enterolactone, enterodiol), mycoestrogens (zearalenone) and anthropogenic estrogens (bisphenol-A) are depicted in Fig. 1. Other xenoestrogens recently identified comprise certain halogenated pesticides, phthalates, as well as polychlorinated biphenyls and their hydroxylated metabolites [4]. A listing of estrogenic compounds would not be complete without mentioning those designed for medical purposes, e.g. 17 α -ethinylestradiol and its derivatives used in oral contraceptives.

Beneficial and adverse effects of estrogens

Estrogens are a two-sided sword meaning that they can be both beneficial and detrimental to an organism. The mammalian estrogen E₂ is essential for normal development and reproduction and has numerous other beneficial effects, e.g. on the bones, immune system and central nervous system [5]. On the other hand, there are epidemiological and experimental data associating E₂ with cancer of the mammary gland and uterine endometrium, and possibly also with prostate cancer [6, 7]. Breast cancer is the leading type of cancer in women of Western industrialized countries and prostate cancer is second only to lung cancer in men in the USA and Europe [8]. The synthetic estrogen DES, which was used mostly in the USA between 1952 and 1970 by pregnant women to prevent miscarriage, is now known to

M. Metzler (✉) · S. E. Kulling · E. Pfeiffer · E. Jacobs
Institute of Food Chemistry, University of Karlsruhe, P.O. Box 6980,
D-76128 Karlsruhe, Germany

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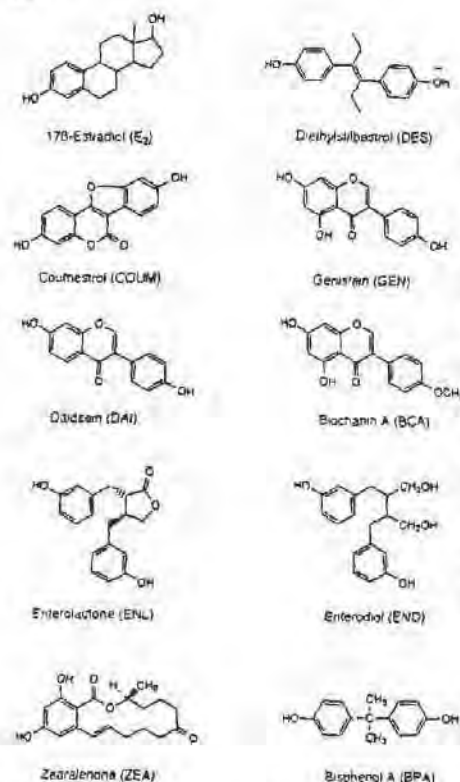


Fig. 1 Chemical structures of various substances with estrogenic activity

be the cause of vaginal and cervical tumors in the female offspring and testicular cancer in the male progeny of the treated women [9]. In animal studies, both E₂ and DES are clearly carcinogenic, for example, causing a 100% incidence of renal tumors in male Syrian golden hamsters [10, 11]. The observed transplacental carcinogenicity of DES in humans has also been observed to occur in mice [9].

The potentially beneficial and adverse effects of the other aforementioned types of estrogens are presently under vigorous discussion. Phytoestrogens, although two to four orders of magnitude lower in estrogenic potency than E₂, have reportedly led to abortions and male infertility in livestock [12]. Similar reproductive problems were noted after the feeding of moldy corn or hay with high zeaxanthone levels [13]. Irregularities of the menstrual cycle were observed in women fed diets rich in phytoestrogens [14]. On the other hand, there is a substantial body of epidemiological data suggesting that populations with a high intake of isoflavone phytoestrogens, e.g. from soy products in

Asia, have a much lower incidence of mammary and prostate cancer [15]. Similar data exist for those fed diets containing phytoestrogens of the lignan type, e.g. from flaxseed [15]. Experimental studies of rodents have shown that isoflavones have protective effects against tumors induced by chemical carcinogens in adult animals [16]. Prenatal exposure of rats to genistein appears to protect against chemically induced mammary gland cancer [17].

Anthropogenic estrogens in the environment enter our body as contaminants of our food. They are blamed for a plethora of adverse effects both on wildlife and humans, for example, abnormal sexual development in alligators and sea gulls as well as feminization of male fish have been proposed to be associated with environmental estrogens [18]. Because of these disturbances of the hormonal system, xenoestrogens in the environment have also been termed endocrine disruptors. In humans, the apparent decline in venison quality over the past few decades as well as increases in breast cancer in women and testicular and prostate cancer are linked to environmental xenoestrogens [18]. Although there is some support from animal studies, these hypotheses remain highly speculative at the present time in view of the small amounts of xenoestrogens and their low estrogenicity in comparison with endogenous estrogens and phytoestrogens. Nonetheless, in view of the profound health implications, the hypothetical effects of xenoestrogens in humans and wildlife merit further clarification. This is particularly true for the implication of estrogens as carcinogens.

Possible mechanisms of estrogen-mediated carcinogenesis

Interest in estrogens and cancer has been stimulated by the observation, mentioned above, that DES is a transplacental carcinogen in humans. Several laboratories have attempted to elucidate the mechanisms by which DES and also E₂ can lead to tumor formation. As a result of these efforts, different mechanisms listed in Table 1 have been proposed, which are not mutually exclusive but may act in concert [19].

As both E₂ and DES are highly potent estrogens and cause tumors in estrogen-responsive tissues, it is likely that their ability to stimulate cell proliferation is important for their carcinogenicity. In an animal model of estrogen carcinogenesis, the kidney of male Syrian golden hamsters, only potent estrogens [11], but not necessarily all of them [20], are carcinogenic. For example, 17α-ethinyl-E₂ and 2-

Table 1 Mechanisms of estrogen-mediated carcinogenesis [19]

1. Hormonal stimulation of cell proliferation
2. Heritable reprogramming of cellular differentiation
3. Induction of genetic changes in target cells by
 - a) Induction of nondisjunction and aneuploidy via microtubule alterations
 - b) Induction of mutations following activation to DNA reactive intermediates
 - c) Modulation of endogenous or dietary mutagenic agents

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PAGE 1 of 2

OU

~~RICHARD J. JAMES~~

R-D 4

WHANEAREI N.Z.

JULY 13 1998

DR LINDA KAHLE,
Office of Pre Market Approval, FAX 64. 9. 4340564
C.F.S.M. HFS 206,
200 C ST WASHINGTON DC 20204

001 (202) 418 3131

Dear Dr Kahle

GRN 000001

As you are aware, performing a thorough check of the Archer Daniels Midland G.R.A.S. determination for Isoparones has been quite slow because of the time it takes to obtain material from the archives.

We now have these documents from the Dept of Commerce.

: P.B. 241954 } all are evaluations of
P.B. 260773 } soy protein isolates
P.B. 300717 } or T.V.P. as foods

Each one raises some serious questions which seem not to have been further addressed.

As a result, the record shows that none of these products were ever decreed to be safe as a human foodstuff.

Should there not be somewhere a something which resembles a high school diploma and says "I (whois) by virtue of the powers vested in me by (what'sis) having examined the evaluations (of thingammy) hereby determine that thingammy is etc. etc."

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or alternatively (which would correspond with some of my diplomas)--- "should go back to the drawing board and try harder."

Page 2 of 2

This affects procedurally how we approach this:

Since it seems G.R.A.S. status may never have been achieved at all, would one submit a petition to have G.R.A.S. determined now; or petition to have G.R.A.S. simi....

We need help now please, to make quite sure we have our facts right before we move on. If you can find a "determination" we would like it faxed to us

We rather feel that there never was a determination, since none of the normal concerns (e.g., but not limited to, protease inhibitors, lectins, saponins, B-sitosterol, genistein, daidzein, phytates, nitrates,) appearing in the literature, and the 1971 edition of Smith & Circle were never adequately addressed at all. Not only did these concerns appear in the text book, but the National Academy of Sciences Food Protection Committee had also expressed grave concern in 1966 and again in 1973.

Moreover, at the same time the W.H.O (Farnsworth et al) was examining the use of soy as an anti-fertility agent as a substitute for contraceptive pills.

This fax is copied to Tamara Turk at N.T. 1-5, in the hope she may be able to locate a "determination".

~~Respectfully,
R. S. Jones~~

000730

copy Tamara Turk : 001 (703) 321 7308



~~RECEIVED 7 JUL 1998~~
 R.D. &
 WHANARET IVZ
 July 16 1998

Dr Linda Kahl
 Office of P.R. Market Approvals
 C.F. SAN

Fax 001 (252) 418 3131

Dear Dr Kahl

GRN 000001 etc
 By copy of this fax, I am also asking Mary
 Hodge at the F.O.I.A. office for help.

I have the three evaluations of soy protein isolate
 and T.V.P. from the Dept of Commerce.

However, there should surely be some auxiliary
 material such as:

- Memos setting the scope and parameters for the evaluations;
- Requests for proposals, and award and terms of contract;
- Receipts of proposals, time for performance etc;
- and then: Transmissions of the evaluations;
- Consideration of the Evaluations;
- Recommendations for action;
- A record of the action taken.

At present, the only conclusion I can draw is
 that the evaluations were never considered, and
 that these products never were determined as GRAS
 Please clarify.

Sincerely

~~RECEIVED 7 JUL 1998~~
 copy. Mary M Hodge
 001 (301) 443 1726

000731



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Phone: (870) 543-7372

FAX: (870) 543-7136

National Center For
Toxicological Research
Jefferson, AR 72079-9502

September 23, 1998

Dr. Linda Kahl
FDA/CFSAN
Office of Premarket Approval (HFS-206)
200 C Street, SW
Washington, DC 20204

Dear Dr. Kahl:

I have enclosed for your information a copy of the protocol for the genistein multigeneration study that is currently underway at NCTR. This work is being conducted under an Interagency Agreement between FDA/NCTR and NIEHS/NTP. The protocol contains a summary of results obtained in the dose range finding studies. More detailed reports on the range finding studies are in preparation.

If you have any questions about the protocol or require more information, please don't hesitate to contact me.

Sincerely,

K. Barry Delclos, Ph.D.
Division of Biochemical Toxicology (HFT-110)
e-mail: bdelclos@nctr.fda.gov

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Isoflavones in Soy-Based Infant Formulas

Patricia A. Murphy,* Tongtong Song, Gwen Busman, and Kobita Bazua

2312 Food Science Building, Food Science and Human Nutrition Department, Iowa State University, Ames, Iowa 50011

Six of the major soy-based infant formulas marketed in the United States were assayed for their isoflavone levels. Samples were taken from the east coast, midwestern, and west coast regions of the United States. Isoflavone levels were variable across brands probably due to different amounts of soy isolate used in product formulation. Total isoflavones ranged from 214 to 285 $\mu\text{g/g}$ of dry formula or ≈ 25 –30 $\mu\text{g/mL}$ of reconstituted formula.

Keywords: Phytoestrogens; genistein; glycitein; daidzein

INTRODUCTION

Soy-based infant formulas have been marketed in the United States for over 60 years as safe and important feeding options for infants. These formulas have been fed to millions of infants with no evidence of harmful effects. However, there has been no systematic evaluation of isoflavone levels in these soy-based foods. There are only four studies in the literature (Sethell et al., 1997; Sethell and Welsh, 1987; Lu et al., 1995; Nguyele et al., 1995) with any analysis of isoflavone levels in infant formula. We have been involved in the development of a database of isoflavone levels in foods, commercial ingredients, and soybeans. Soy-based infant formulas will be a component of that database.

A number of phytochemicals possess cancer prevention properties that may inhibit tumor initiation, prevent oxidative damage, and/or affect steroid hormone or prostaglandin metabolism to block tumor promotion (Caragay, 1992). There is a growing literature on the health protective benefits gained by consumption of soy foods. Isoflavones have been implicated in the prevention of breast, prostate, and colon cancers (Messina and Barnes, 1991), in lowering of the risk of cardiovascular disease (Anderson et al., 1995), and in the improvement of bone health (Bahram et al., 1996). The interest in soybean components has been so strong that two international conferences have been convened to report the state of knowledge in the field (Messina, 1995; Sethell, 1997). The beneficial effects of isoflavones may be due, but only in part, to the weak estrogenic activity of the isoflavones. Genistein, daidzein, and their glucosidic forms, daidzin and genistin, are 100 000 to 500 000 times less potent estrogens than estradiol (Farnakalidis and Murphy, 1985). In adults, plasma concentrations of isoflavones do not exceed 13 μM (Barnes, 1995a) even on a high soy isoflavone diet. Japanese adults are estimated to consume up to 12 mg/day (Mukutake et al., 1996). In contrast, Americans consuming a traditional Western diet may consume only 1–3 mg/day (Barnes et al., 1995).

The three isoflavones in soybeans and soy products, genistein, daidzein, and glycitein, occur in four possible forms: the free phenolic form, the glucoside, the malonyl glucoside, and the acetyl glucoside (Wang and Murphy, 1994a,b). The bioavailability of the isoflavones is ap-

parently affected by the gut microflora of the consumer (Xu et al., 1994). There does not appear to be a difference in the bioavailability of the glucosides or free forms (Farnakalidis and Murphy, 1985; Xu, 1995). The effects of processing alter the distribution of the forms and can result in the loss of some isoflavones through leaching and removal of undesirable fractions. The distribution of the isoflavone yields a picture of the processing history of a particular soy product (Wang and Murphy, 1996). We have developed a routine HPLC method to evaluate isoflavones in soy products (Song et al., 1997a) and have employed it to examine the isoflavone levels in commercially available soy-based infant formulas.

METHODS AND MATERIALS

Samples. Infant formulas were purchased in the Baltimore, MD, metropolitan area, Ames, IA, and San Francisco, CA, in 1995. Soy infant formulas obtained were Mead-Johnson's ProSoy, Gerbers, Ross Laboratories' Enamil, Wyeth's Nursoy, Mead-Johnson's Enfamil, and Carnation's Asoy and were different lot numbers per each brand (n = 3). All formulas were obtained as the dry instant formula except Wyeth's Nursoy from a west coast location, which was available only in liquid form. The liquid formula was freeze-dried prior to sampling and analysis. Enfamil was not available in the Baltimore area.

Standards. All chemicals were of reagent grade from Fisher Scientific unless noted otherwise. The internal standard, 2,4,6-trihydroxydeoxybenzoin (THB), genistein, and daidzein were synthesized with resorcinol, 4-hydroxyphenylacetic acid, boron trifluoride etherate, N,N-dimethylformamide (DMF), and methanesulfonyl chloride from Sigma Chemical Co. (St. Louis, MO). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used.

Seven isoflavone standards were used in the calibration curves. Genistein (Gen) was synthesized according to the method of Chang et al. (1994). Daidzein was synthesized according to our modification of Chang's method: 4 mL of BzF₃-Et₂O was added to a beaker containing 200 mg of THB in 10 mL of DMF, and the mixture was heated in a microwave oven (Kemo U88) for 30 s at medium energy. Methanesulfonyl chloride (4 mL) was then added to the mixture, and it was heated for an additional 70 s at medium in the microwave oven. Four hundred milliliters of cold H₂O was added to the reaction mixture, giving a light yellow precipitate. The precipitate was washed with water and recrystallized from methanol. Genistin (Gin), daidzin (Din), and acetylgenistin (AGin) were from previous work in our laboratory (Wang and Murphy, 1994a). Glycitin (Glin) was purified using the method of Farnakalidis and Murphy (1985) from soybean (Schuten USA). Glycitein (Glein) was purified from a 0.1 N HCl

* Author to whom correspondence should be addressed (telephone (515) 294-1970; fax (515) 294-8181; e-mail pmurphy@iastate.edu).

hydrolysis of soy germ (98 °C for 2 h) by adding acetonitrile (ACN) and filtering. The filtrate was dried by rotary evaporation and redissolved in 100% ethanol and chromatographed on a Sephadex LH-20 (Sigma) column (L × 50 cm) with 50% ethanol as the eluent. The glycitein peak was collected and freeze-dried. The identity and purity of glycitein was confirmed by HPLC retention times in combination with UV spectral analysis, melting point determination, and mass spectrum analysis. Isoflavone standard solutions were prepared on the basis of UV maximum absorbance and molar extinction coefficients (Ollis, 1962). The purity of the standards was based on the percentage peak areas according to the Beckman System Gold software, and final concentrations were adjusted based on these purities. For isoflavones without pure standards, the malonyl forms, and acetyl daidzin and acetyl glycin, the appropriate standard curves were adjusted on the basis of molecular weight differences as confirmed by our previous work with most of these standards (Wang and Murphy, 1994a,b).

Internal Standard. The internal standard, THB, was synthesized according to a modification of the procedure of Chang et al. (1994). Resorcinol (2.9 g) was added to a mixture of 4-hydroxyphenylacetic acid (2 g) and boron trifluoride etherate (4.5 mL). This mixture was refluxed for 10 min, cooled, and treated with 30 mL of saturated sodium acetate and 15 mL of saturated sodium bisulfate. The yellow precipitate was filtered and washed with water and then methanol and dried to give yellow crystals. The crystals were redissolved in 100% ethanol and chromatographed on Sephadex LH-20 with 50% ethanol as the eluent. A single peak was collected giving >99% THB.

Quality Control. Quality control measures included the following: analysis of authentic standards each day; food samples were analyzed, recovered GIn, Gen, DIn, and THB on five different soy food matrices were conducted monthly; and analysis of four reference materials weekly over the lifetime of this project (24 months). Precision and accuracy estimates were made on the basis of these quality control measures. Any significant deviations (>5%) from baseline of these reference materials would lead to system inspection and correction before any further samples would be analyzed. All data were corrected for recovery based on internal standard.

Isoflavone Analysis. Dry infant formulae were extracted as 2 g samples in 10 mL of acetonitrile, 2 mL of 0.1 N HCl, and 7 mL of water in a 125 screw-top Erlenmeyer flask with stirring for 2 h at room temperature. The extracts were filtered and taken to dryness on a rotary evaporator at <30 °C. The residues were dissolved in 80% HPLC grade methanol. An aliquot was filtered through a 0.45 µm PTFE filter (Alltech Associates, Deerfield, IL) and analyzed by HPLC. A Beckman System Gold chromatograph with a Model 507 autosampler, a Model 126 dual pump, a Model 168 photodiode array detector, and an IBM 486 computer using Beckman System Gold HPLC data processing software (version 8, 1993) was used. A YMC pack ODS-AM-303 column (6 µm, 25 cm × 4.6 mm) was employed for chromatographic separations and maintained at 34 °C with a column heater. A linear gradient was composed of A (0.1% acetic acid in water) and B (0.1% acetic acid in acetonitrile). The gradient profile was modified from that of our previous paper (Wang and Murphy, 1994a) to reduce analysis time. After injection of a 20 µL sample, the system was maintained at 15% B for 5 min, then increased to 29% in 31 min, and then to 35% in 8 min. The system was recycled to 15% B at the end of 45 min. The flow rate was 1.0 mL/min for the first 5 min, then increased to 1.5 mL/min for the next 40 min, and returned to 1.0 mL/min for recycle. The UV absorbance was monitored from 200 to 350 nm. UV spectra were recorded and peak areas were integrated using Beckman System Gold software.

Statistical Analysis. Midwestern sample analyses were replicated in triplicate. Statistical evaluation was conducted using the General Linear Models procedure and Student's *t* test with SAS package (version 6.03, 1996, Cary, NC). Differences were considered significant if $p \leq 0.05$.

RESULTS AND DISCUSSION

Figure 1 presents a typical chromatogram obtained in soy-based infant formula analysis resulting in baseline resolution of all soy isoflavones and the internal standard, THB. All data reported in Tables 1 and 2 were corrected for recovery with internal standard (Song et al., 1997a). To evaluate the analytical precision of isoflavone analysis, triplicate analyses of the midwestern-purchased infant formulae were done. The results of these analyses are presented in Table 1 for total isoflavones. The largest percent coefficient of variation was an average of 5% for total genistein for all formulae. These data supported our decision to analyze only one sample from each lot of infant formula for the national survey and still obtain representative data.

An analysis of variance revealed that there were only minor differences in isoflavone concentrations within a brand among sampling locations. The differences were in total genistein, acetyl genistein, and glycitein. There were no other differences in the other 13 formulae. Total isoflavone content (the sum of total genistein, total daidzein, and total glycitein) was not different among sampling locations.

Total isoflavone levels were quite similar across brands: 29% daidzein, 59% genistein, and 12% glycitein. Table 2 presents the results of isoflavone analysis for the individual formulae of each isoflavone and the total isoflavones adjusted for their molecular weight differences and expressed as the free isoflavone formulae (i.e., totals of genistein, daidzein, or glycitein). Simple addition of isoflavone concentrations without this correction will overestimate true isoflavone aglycon concentrations by almost a factor of 2 (Wang and Murphy, 1994a, 1996). Since only the free formulae of the isoflavones are absorbed by the gut and exert their potentially protective effects, the concentrations of isoflavones in food products must be expressed in this manner to avoid erroneous conclusions and labeling.

An analysis of variance showed small but significant differences across brands for individual isoflavone formulae. The effects of processing to produce soy isolate from soybeans is evident from the distribution of the different formulae. The malonyl glucosides are moderately reduced in these formulae compared to being the highest concentrations of isoflavone formulae found in raw soybeans (Wang and Murphy, 1994a,b, 1996). The glucosides, daidzin, genistin, and glycytin, are higher than the malonyl formulae, indicating the product, either at soy isolate manufacture and/or infant formula formulation, has undergone heat processing. There are appreciable concentrations of the acetyl glucosides, indicating some intensive heat treatment has occurred during processing such as spray drying. Little hydrolysis of the glucosides appears to have occurred since the genistein, daidzein, and glycitein concentrations are quite low as expected (Wang and Murphy, 1996; Farnalkalidis and Murphy, 1985).

The variation among soy infant formula brands observed here may be due in part to the difference in the percent soy isolate used in each brand's formulation. Within a single infant formula brand, it is probable that the isolate used was produced by a single supplier. Some or all of the soy isolates used in preparing these infant formulae may have come from a common supplier. The percent soy isolate in these infant formulae ranges from 14.6 to 21%. When total isoflavone concentrations were adjusted on a percent soy isolate basis (as reported on product label), they were 1271 (A soy),

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Figure 1. HPLC chromatogram of infant soy-based formulas with isoflavone and internal standard peaks resolved. Axis represents UV absorbance at 254 nm: DfIN, daidzein; GLY, glycytein; GfIN, genistein; MDfIN, malonyl daidzein; MGLY, malonyl glycytein; AcGfIN, acetyl genistein; AcDfIN, acetyl daidzein; AcGLY, acetyl glycytein; MGfIN, malonyl genistein; DEfIN, daidzein; GLYfIN, glycytein; THfIN, internal standard; GEfIN, genistein.

Table 1. Precision of Isoflavone Analysis in Infant Formulas from Midwestern Sample Set^a

Formula	n	total daidzein			total genistein			total glycytein		
		X	s	CV	X	s	CV	X	s	CV
Prosoy	3	57	1	2	132	7	5	29	0	0
Gerber	3	62	1	2	140	5	4	29	0	0
Isomil	3	56	3	6	127	7	6	27	1	4
Nursy	3	63	5	8	139	10	7	28	1	4
Enfamil	3	66	1	2	140	9	6	29	2	7
Alsoy	3	67	2	3	141	6	4	27	0	0

a) n = number of replicates; X = average; s = standard deviation; CV = coefficient of variation (%).

1329 Gerber), 1338 Nursy), 1356 Enfamil), 1487 (Isomil), and 1535 (Prosoy). These isoflavone concentrations were higher than we have reported for commercial soy isolates ranging from 450 to 1100 (g/g) (Wang and Murphy, 1994a). Yearly variation in the isoflavone content of soybeans can be different by a factor of 5 (Wang and Murphy, 1994b).

There are only four studies in the literature on isoflavone content of infant formulas (Setchell et al., 1997; Setchell and Welsh, 1987; Lu et al., 1995; Nguyen et al., 1995) for four of the products we have analyzed: Alsoy, Nursy, Prosoy, and Isomil. None of these papers report data on glycytein levels except for that of Setchell et al. (1997), who give only an average for five brands. Given the structural similarity of glycytein to the estrogenic isoflavones, genistein and daidzein, we should anticipate this minor isoflavone will contribute to the total phytoestrogen activity of soy foods. We have preliminary evidence that glycytein is

as potent an estrogen as daidzein (Song et al., 1997b). Our total genistein compares well with the total reported for the same products in Setchell and Welsh (1987) and Lu et al. (1995). However, our total daidzein concentrations are approximately half of those reported by Setchell and Welsh (1987) and Lu et al. (1995). Nguyen et al. (1995) used an isocratic HPLC system to evaluate isoflavones in a variety of soy foods but did not resolve the acetyl glucosides from other isoflavones, nor did they consistently resolve them as acetyl glucosides from the glucosides. They claim no acetyl forms were present in their products. Others have consistently shown acetyl forms in any heat-processed soy product for which all isoflavone forms are resolved by gradient HPLC (Wang and Murphy, 1994a, 1996; Coward et al., 1993; Song et al., 1997a).

Total isoflavones ranged from 214 to 267 (g/g) of dry formula or 25–30 (g/g) of reconstituted formula. Our values are lower than Setchell's (1997), but it is not clear how he calculated the total isoflavone levels. An increase in addition of various glucosidic forms with free forms will overestimate isoflavone concentrations by 50–100% due to the molecular weight difference of the glucosidic moiety. Since only the aglycon is apparently absorbed, perhaps isoflavone levels in foods should be reported on a molar basis. On a ready-to-drink formula basis, our data represent 8.82, 19.10, and 3.54 (g/g) L daidzein, genistein, and glycytein, respectively. Depending on the volume consumed by infants, which will be dependent on age, the child may consume from 5 to 12 mg of isoflavone (kg of body weight)⁻¹ day⁻¹. The infant formula isoflavone amounts are greater than typically

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Table 2. Isoflavone Distribution in Soy-Based Infant Formulas

formula	% SPI	µg of isoflavone/g of dry formula												total		
		Din	Gln	Gln MDin	MGln	MGln	ADin	AGln	AGln	Den	Gen	Glen	Glen	Den	Gen	Glen
Alsoy	21	37 ^B	83 ^{AB}	17 ^{AB}	48 ^A	92 ^A	19 ^A	40 ^A	84 ^A	14	10 ^{BC}	11 ^{CD}	0	76 ^A	154	27 ^A
ProSoy	17	40 ^B	94 ^{AB}	18 ^{AB}	39 ^A	75 ^A	19 ^A	37 ^{AB}	87 ^{AB}	13	10 ^{BC}	12 ^{BC}	3	73 ^{AB}	151	30 ^A
Infamil	18	76 ^A	85 ^{AB}	19 ^{AB}	34 ^A	57 ^{AB}	17 ^A	37 ^{AB}	71 ^{AB}	9	12 ^A	15 ^A	3	70 ^{AB}	135	28 ^A
Gerber	17	38 ^B	68 ^B	18 ^{AB}	30 ^{AB}	66 ^{AB}	18 ^A	32 ^{AB}	71 ^{AB}	12	11 ^{AB}	13 ^{AB}	3	67 ^{AB}	126	29 ^A
Isomil	15	31 ^B	75 ^{AB}	17 ^{AB}	30 ^{AB}	68 ^A	16 ^A	34 ^{AB}	71 ^{AB}	11	9 ^C	10 ^D	3	63 ^{AB}	130	27 ^A
Nursy	16	57 ^{AB}	134 ^A	21 ^A	10 ^B	33 ^B	5 ^B	15 ^B	51 ^B	0	10 ^{BC}	11 ^{CD}	3	57 ^B	138	17 ^B
LED	36	64	3	24	36	8	23	30	18	1	2	8	17	37	9	43

^aValues in a column with the same superscript are not significantly different at $P < 0.05$ LSD, test significant difference; % SPI, percentage soy isolate reported on label; Din, daidzein; Gln, genistein; Gln MDin, malonyl daidzein; MGln, malonyl genistein; MGln, malonyl genistein; ADin, acetyl daidzein; AGln, acetyl genistein; AGln, acetyl genistein; Den, daidzein; Gen, genistein; Glen, glycitein; total Den, total moles of daidzein \times molecular weight of daidzein; total Gen, total moles of genistein \times molecular weight of genistein; total Glen, total moles of glycitein \times molecular weight of glycitein; total total sum of total daidzein, total genistein, and total glycitein.

consumed by adult soy foods consumers. However, in the > 60 years of use of soy-based infant formula, there is no evidence of any difference in growth and development of children compared to those children consuming cow milk formula or human milk. Infants are exposed to small amounts of these compounds in the range of 1–2 µM isoflavones in breast milk if the mother is consuming soy foods. It is likely that the potential health protective effects attributed to soy isoflavones may be an advantage to infants consuming soy formulas.

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000533

Dear Dr Kahl
F.Y.I
~~[Signature]~~

ou |



RICHARD F. JAMES
RD4
Whangarei
NEW ZEALAND

Dr Barry Locke
Office of Chief Medical Officer & Commissioner
Food and Drug Administration
3500 Fishers Lane H.F7
Rockville Md 20852
U.S.A.

23.11.1998

Boy Based Bait 1998

...relating to processing the information ... to complete the picture of the
... approach to questions of safety ...

TRYPSIN INHIBITANT FORMULAS

... 1984 ... very misleading ...
... the protocols, the parameters and results of the study. The study
... to work ... treatment adequately removed ... inhibition at all. It
... study of the effect of ... of soy trypsin inhibitor.

... even more puzzling to find that the study was terminated in 1987 after 38
... just as the pigs' pancreases began to show cellular damage, it was even
... puzzling to find that the project was only signed off and audited in 1994 (six
... months after my enquiry and eight years after the work); and most puzzling of all:
... were new paragraphs typed up ex post facto in 1994 and substituted for the
... original?

... asked Patricia Gee to try to locate the original pages which were
... from the study report. I doubt whether Dr Barthoff will still have these
... but it will be a good bet that the original report can still be found at the
... Dept. of Agriculture's office at Belleville.

I have attached ... sheet of BFG 229.7 to ...

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HORMONE DISRUPTION BY SOY INFANT FORMULAS

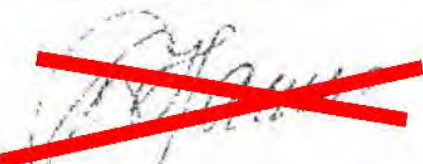
I have still not received any notes from the meeting at N.I.C.H. of May 17, 1997. This meeting has been variously described to me as a brainstorming session, a symposium, a meeting having described isoflavones as a toxicant looking for a disease, a meeting having formally resolved not to investigate.

A meeting was attended by industry representatives from as far away as Switzerland or scientists associated with the industry, and seems to have reached a fairly momentous conclusion involving, potentially, the lifetime health of many thousands of past and future children, yet there are no records. Where, as a minimum, are the notes that Nicolas Duy said had been located last May?

F.D.A. personnel attended, and some even made verbal presentations. Where are the speech notes?

Your prompt help would be appreciated please, as you can see from the length of time I have been searching for answers that none of us is getting any younger. What I find almost unbelievable is that the U.S. law requires the manufacturers of infant formulas to notify the F.D.A. after they have been informed of possible health hazards. Warnings of health hazards inherent in these products are being issued in many countries, yet the F.D.A. has made no effort to take a public stance; indeed, if the circumstances of the N.I.C.H. meeting are any guide, it has tended to conceal the problem. One wonders whether the Inspector-General should be asked to examine the adequacy of the F.D.A.'s stance.

Yours sincerely


Richard James

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Page 2 of 2

Dear Dr Kahl.
I may it you might
like to be kept
informed

OU



RICHARD F. JAMES
RD-4
Whangarei
NEW ZEALAND
PHONE: 64.9.4340564
FAX: 64.9.4340567

January 4 1999

The Manager
Documents Management Branch
(F.A. 305)
Food and Drug Administration
5630 Fishers Lane, Rm 1061
Rockville Md 20852
U.S.A.

Dear Sir/Madam

**DOCKET # 98P-0883 : PETITION FOR HEALTH CLAIMS
BY PROTEIN TECHNOLOGIES INTERNATIONAL**

As at this date, the four volumes of the petitioner's Health Claim Petition have not arrived in New Zealand. Therefore my comments are preliminary, and address only the comments by the F.D.A. evaluator at page 62978 Federal Register 63(217) of November 10, 1998, and not the actual words of Protein Technologies International (P.T.I.), which purport to satisfy Sec 10.1.14(b)(3)(ii) as to the requirement for prior safe and lawful use.

Additionally, I would like to address the allegation that soy protein isolates (this includes soy protein concentrates and textured vegetable protein) were in common use prior to 1958.

In general, the "safe use" has already been refuted by submissions to the F.D.A. in March and April 1998. The occasion was the examination of soy protein isolates for the petition of Archer Daniels Midland Corp; the file number was GRN 090001; the Office of Premarket Approvals; the officer in charge is Dr. L. J. G. F. (HFS 206). I respectfully request that all documents on the subject be included in, and form part of, my present comments.

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A. SAFETY OF USE
A1. NATURAL TOXIN RESIDUES

It is indisputably established that the modern mass production methods of processing do not remove any of the natural toxicants of the soybean from the finished product.^{1,2,3,4} P.T.I. itself concedes there are remaining levels. It is also well established in the scientific literature that certain human subgroups⁵ of the population are at health risk from a number of the natural toxins remaining in soy products after processing depending on the quantity consumed and duration of exposure, for example:

- Infants and young children;
- High soy consuming vegetarians;
- Pregnant women.

All of these population groups are presently at some level of health risk from residue levels of natural toxins in soy products already available for human consumption.

In 1987 an investigation into the deaths of over 100 cheetahs in zoos revealed that they died of liver cancer, but were in the main sterile.⁶ Those that weren't sterile had weakling babies that largely died before maturity. A very similar situation to the laboratory rats 40 years earlier.⁷ At the last page of the research into the cheetah deaths from liver failure, which revealed that the soy isoflavones at a low dietary level had done the damage, the authors issued a strong warning of the possibility of deleterious effects occurring in humans. An analogy was drawn with similar reproductive damage caused to the children of women who were administered diethylstilbestrol, an estrogenic drug which was teratogenic (i.e. produced deformities in the next generation). Around 30,000 cases emerged. Yet despite that, no safety precautions have been instituted to protect human consumers from the same potential results of high soy consumption.

In 1997, an U.S.F.D.A. risk assessor⁸ identified as "oral hazards" uterine hypertrophy, reproductive failure, infertility, testicular atrophy, menstrual cycle disruption and impaired reproduction as hazards associated with consumption of soy isoflavones. Has P.T.I. conducted safety tests to ensure these ailments do not occur in humans?

Health experts in the U.K., Switzerland, Australia and New Zealand have already expressed concern over the presence of isoflavone toxins in foods for infants.^{9,10,11,12} Isoflavones have been shown to disrupt the reproductive and hormonal systems of rats, pigs, mice, cattle, sheep, parrots, cheetahs, quail, fish, adult U.S. women, adult English women and adult Japanese men.^{13,14,15,16,17,18,19,20} Even the possibility of their entry into the human food chain was viewed with dismay by the Committee for Food Protection, National Academy of Sciences in 1973.²¹ The American Chemical Society devoted a chapter on

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plant-derived carcinogens, including the isoflavones, in its treatise on carcinogens.²²

There is no doubt that adverse biological effects have been shown to be caused by soy isoflavones in humans; for instance a comprehensive review of the possible physical and psychological effects of these toxicants was published in 1998 as they may affect humans who were exposed in infancy.²³

In many of the referenced effects on animals the dietary intake is known or can be estimated (for instance the quantity that caused the female piglets to mature sexually prematurely is very similar to that in soya infant foods which are fed to baby girls). A good guide for calculation of dietary intakes is reference (3) which examines the quantities in soy based human foods.

In addition, examination of the processing of soy protein derivatives has raised various questions of health hazards from artifacts manufactured during the extraction and gas-fired drying processes.

A2. MANUFACTURED CARCINOGENS

In 1979, the Life Sciences Committee of F.A.S.E.B. examined the processes by which soy protein was isolated from the soy bean feedstock. They commented: "The Federation (F.A.S.E.B.) recognises that the safety of G.R.A.S. substances is of national significance, and that its resources are particularly suited to marshalling the opinions of knowledgeable scientists to assist in these evaluations."²⁴ One should remember that this was 1979, and the evaluation assumed a per capita (man, woman, child) consumption of soy in the U.S.A. as only between one eighth and one third of a gram per day. By no stretch of the imagination could this be interpreted as "common use" in 1970, let alone in 1958. That level was not deemed to pose a health hazard, though the evaluators pointed out that infants were consuming up to 27 grams per day; that is, 80 times higher than the population rate, whilst increasing adult consumption could pose health hazards.

The areas of particular concern were nitrosamine formation, lysinoalanine formation, and excessive nitrite levels. The safety specifications decreed by the evaluators were never instituted, and G.R.A.S. status as a human food was never achieved or modified. One could also legitimately argue that this negative 1979 safety evaluation superseded and nullified the earlier limited approvals for specific uses as well.

A2.1 NITROSAMINES

Despite the adamant direction by the Life Sciences evaluators that precautions be taken to exclude nitrosamine formation, none appear ever to have been instituted. The only published analysis shows a level of 1.5 ppb of

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N Nitrosodimethylamine in a food containing 20% soy protein, giving a probable level of 7.5 ppb in the soy protein itself.²⁶ In the context, this would constitute a health hazard.

A2.2 Lysinoalanines

The Life Sciences Evaluation of Soy Protein Isolates in 1979:

"It is essential (sic) that specifications for food grade soy protein isolates be established including acceptable levels of lysinoalanine, nitrite and nitrosamines."

In 1975, Sternberg²⁶ et al had found 1980 ppm of lysinoalanine in soy protein hydrolysed for 24 hours at 110°C. Subsequent research showed that those levels which caused renal toxicity in rats (100 ppm) may quite easily be administered to human infants, based on the Sternberg quantifications.²⁷ This was the reason for the concerns expressed by the Life Sciences Evaluators, and there is no evidence in the F.D.A. records that these recommendations were ever addressed further by the industry, or that safety specifications were ever prepared, or a quality control programme ever instituted. P.T.I. are presumably familiar with the regulations governing their industry and should be asked for evidence of compliance.

A2.3 NITRITE

The Life Sciences evaluators (page A18) accepted that soy protein generally contains around 50 ppm of nitrite. The amount in bacon and preserved meats was generally accepted to be hazardous at around 120 ppm.²⁸ When one considers how little bacon and how much soy protein an infant consumes, it is no wonder the Life Sciences evaluators were concerned! P.T.I. should be asked for evidence of compliance with this simple processing safety precaution during the past nineteen years. Here is what a special task force employed by the Life Sciences Committee had to say:²⁴

"The portion of the Newberne study dealing with nitrite involved a total of 1953 rats (Table 9 in his report), a very respectable number. Examination of the results of the study without reference to experimental design or statistical methods supports Newberne's conclusion that "Despite the somewhat less than convincing case that nitrite is lymphomagenic in Sprague-Dawley rats, one cannot escape the distinct impression that nitrite does affect the lymphoreticular system of the rat ... While these observations require some consideration, the data are only suggestive and the biological significance of nitrite associated lesions of the lymphoreticular system is unclear. There are suggestions, however, of sufficient magnitude and the study used sufficient animals to raise

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questions about the widespread use of relatively high concentrations in our food supply."

As I have already noted, the Life Sciences Committee's evaluation that the per capita consumption in 1970 was estimated to be no higher than one-third of a gram per day, and quite probably as low as one-eighth of a gram per day. By no stretch of the imagination do the historic records in the F.D.A. itself support the notion that any soy protein isolate ingredients were in "common use" prior to 1958. Even at the minute levels adopted by the Life Sciences Committee, it was deemed to harbour a health hazard (except as a cardboard sealant). Contrary to P.T.I.'s contention ("self determination"), the only G.R.A.S. codification which is currently in force is as a cardboard sealant. All other uses, presumably, should be individually authorised under Pre-Market Approval procedures of C.F.-SAN. No use as an infant food has ever been codified.

B. LAWFULNESS OF USE

B1. REGULATORY COMPLIANCE

Soy protein isolate powder was first used as the protein source for infant formula in about 1964. At that time it was known to the product developers that the natural toxins were not removed from the product.⁴ Isoflavones, phytosterols and saponin were specifically named, and it was apparent, in the light of the mouse studies, that the dietary levels per kg of body weight being fed to human infants exceeded the dose levels per body weight that damaged the mice reproductively.¹⁴

Yet no acute or chronic toxicity studies on humans have been revealed. The isolated soy proteins had never been approved as a safe human food for infants when they were introduced into infant formula around 1964. Prior to 1958 some human uses were "prior sanctioned" on an ad hoc basis. Infant and baby foods were never included. After 1958 specific G.R.A.S. status should have to be determined and codified into law. For a list of the approved uses see Appendix A of the 1979 Life Sciences Evaluation effective in 1979.

For the whole time span from 1964 up to 1979, the use of soy protein isolates - known to contain natural toxins at active levels in the dietary amounts being offered - was experimental for babies, with no risk of chronic toxicity or hormonal damage ever being assessed or revealed. There is no record that the use of this material as an infant food was ever lawful at all. In the light of what has now become known of the systemic disruption becoming evident in later life in children^{29,30} as infants, were fed soy formulas, to adhere to this attitude is extremely foolhardy.

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B2. COMPLIANCE WITH STATUTORY DUTIES

In the specialist area of infant foods, safety and lawfulness of use should be paramount. Yet it appears that the soy protein isolate manufacturers, and their clients, the infant formula manufacturers, have consistently ignored the law of the United States of America. Section 106.100(K) of the Food Drugs and Cosmetics Act requires a manufacturer to record, make available to the F.D.A., and notify the Center for Food Safety and Applied Nutrition of any complaint of a possible hazard to health by infant formulas.

Notifications of the existence of the possible health hazard in this context were:

June 1994	by Allan Aspell and Associates directly to the manufacturers;
Dec 1995	the presentation of Professor C. Irvine's Research at a Conference before Infant Formula Manufacturers: Little Rock, Arkansas; ²³
July 1996	by the British Department of Health; ⁹
May 1997	by the Swiss Federal Office of Public Health; ¹⁰
July 1997	by Dr D.M. Sheehan. ³⁹

Yet not one manufacturer complied with this legal requirement, according to the F.D.A. itself.⁴⁰

B3. CLAIMS OF G.R.A.S. STATUS

The F.D.A. evaluator has alluded to claims by P.T.I. that the U.S. Department of Agriculture and the Association of American Food Control Officials have asserted support for G.R.A.S. status. My research has been unable to confirm this. Of course, neither of these organisations has jurisdiction in the area; P.T.I. should be asked, under oath and penalty of perjury, to support their assertions.

B4. INTERNATIONAL APPROVALS

Enquiries at the New Zealand Ministry of Health³² confirm that F.A.O. have never evaluated soy protein as a human foodstuff. My enquiries have gleaned me from Geneva, copies of F.A.O. guidelines G.L. 4-89 and 175-89. The information I have is summarised here.

Despite the abundant evidence of reproductive toxicity in numerous species of animals, fish and birds, no acute or chronic toxicity studies have been furnished to F.A.O./Codex. Codex guideline GL4/89^{32a} provides: "Prior history of safe use may be taken into account in evaluation of a novel V.P.P. proposed for general consumption, but this is not necessarily sufficient to preclude adequate pre-clinical testing by currently available, more objective laboratory animal feeding studies, and where applicable, studies using human volunteers." To my knowledge the only three human volunteer studies which have been reported and

which would meet these criteria are, first, where 60gr of soy protein per day within 30 days (45mg isoflavone) depressed the hormone levels necessary for ovulation in adult women,¹⁶ the second, where intermittent consumption of soy protein caused estrogenic effects in adult females' breasts³³ and the third, where consumption of heat treated, pickled soy bean had marked depressive effects on the thyroid function of both young and old Japanese men.²⁰ None of the published studies meets the definition of a chronic or sub-acute study.

Subsection 2.1.2 of Codex Guideline GL4-89 requires examination of potentially hazardous solvent residues. P.T.I. should be asked to produce evidence that it has ever complied with this F.A.O. guideline.

Subsection 2.1.1.3 requires similar disclosure of toxicological significance of metals or minerals. The Australian College of Paediatrics has been concerned about aluminium content of soy protein baby foods.¹¹ When this came up for discussion with a soy vendor in 1994, in my presence the company concerned joked that the isoflavone toxicity would be forgotten, just like the aluminium one. "If we ignore it, it will go away," they said. They were wrong. But this attitude to customer safety is pervasive in the soy industry. P.T.I. is the principal supplier of soy protein isolate to infant formula manufacturers, and should be asked for details of safety precautions in respect of aluminium contamination.

Subsection 2.1.1.1 requires determination of nitrogenous components to be obtained. Since this goes right to the heart of the reasons (excess nitrite, and nitrosamine formation) why soy protein isolates did not pass the 1979 U.S.A. Life Science evaluation, P.T.I. should be asked to produce evidence that this determination has been met in respect of F.A.O./Codex guidelines.

Section 2.4.1 requires sub-acute toxicity studies. None have been provided to F.A.O.

Section 2.4.2 requires an evaluation of chronic, reproductive, teratogenic and mutagenic studies which could be necessary following an appraisal of the source of manufacture. Since the action of the isoflavones, which are abundant in all processed soy products, meets all these criteria, P.T.I. should produce evidence that it has complied with the F.A.O. requirements.^{33,34,35,36}

It has been conclusively established that the modern processing does not destroy the natural soy bean toxins. It has also been recognised for many years that the levels of toxins in the basic meal are highly variable, depending on strain, growing conditions, process quality control and storage.³ Because the product is not completely cooked an almost infinite combination of biologically active toxins is possible. For example, two competent analytical laboratories identified analogues of isoniazid in soy protein products in 1993.³⁷ Isoniazid is an anti-tuberculosis drug, and similar compounds seemed to have been created from

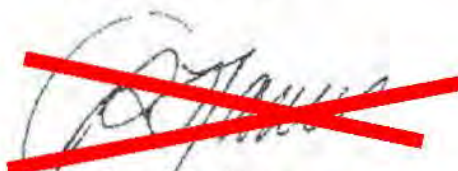
heat and steam application to Niacin in the soy protein, in the opinion of scientists who detected these compounds.

C. ANOTHER POTENTIAL RISK - Glyphosate Residues

The discussion of the Life Sciences Evaluation above, highlighted the seriousness with which carcinogens such as nitrosamines, as processing artifacts, were regarded by the G.R.A.S. Evaluation Committee. How then would that same F.A.S.E.B. committee have regarded the possible creation³⁸ of N-Nitrosoglyphosate and similar compounds from the permission of increased glyphosate residues being advocated by soy protein processors. They would surely have dictated the same approach: to insist on safety specifications to ensure N-Nitrosoglyphosate compounds are excluded. The application of gas fired heat in the spray drying of the soy protein creates favourable conditions for the creation of these carcinogens. Is P.T.I. prepared to follow the Directive of the 1979 F.A.S.E.B. Committee in this respect, in 1999 and beyond?

Since several of the assertions made by P.T.I. appear to be incorrect and since it can be assumed that this large transnational corporation with a market value in 1998 of \$1.5 billion is fully conversant with its own industry and its own products it would seem that, if similar applications are made in the future, they should be accompanied by an affidavit of accuracy: i.e. sworn under oath and subject to the law of perjury.

Yours sincerely


Richard F. James

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Valerie A. James
Rural Delivery 4
Whangarei
NEW ZEALAND

January 24 1999

The Manager
Dockets Management Branch (HFA - 305)
Food & Drug Administration
5680 Fishers Lane Rm 1061
Rockville
M.D. 20852
U.S.A.

**COPY FOR YOUR
INFORMATION**

Dear Sir/Madam

DOCKET 98P 0683 - HEALTH CLAIMS LABELLING FOR SOY PROTEIN

I wish to comment and add information re the above, in addition to the submission I made dated January 18th 1999.

1. *"Evaluation of the Health Aspects of Soy Protein Isolates as Food Ingredients"* 1979 Contract # F.D.A. 223-75-2004, states that, "Edible soy protein isolates for food uses appeared about 1957 as a major article of commerce" (p.8). The estimated per capita daily intake was estimated to be approximately 150mg in 1970 (p.9). The Food Drug and Cosmetics Act (21 USC 321 s.) indicates that G.R.A.S. substances are exempt from the pre-marketing clearance that is required for other food additives. The recognition of safety by experts is required for G.R.A.S. status to be granted for "those substances used in food prior to January 1, 1958 on a reasoned judgement founded in experience with common food use" etc. (p.1). It seems that G.R.A.S. status for soy protein has not been granted.

I conclude that there was very little "experience" and neither was it "common" prior to January 1, 1958. Further it is indicated that "conclusions will need to be reviewed as new or better information becomes available." My search of records reveals no evaluation later than that; it concluded, "It is essential that specifications for food-grade soy protein isolates be established including provisions for acceptable levels of lysinoalanine, nitrite and nitrosamines." (p.32) and that, "Specifications should be developed for food-grade soy protein isolates which exclude nitrosamines." (p.31).

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2. It is important to distinguish between the different sources of soy protein and extraction, concentration or isolation techniques employed in preparation or manufacture. "Biological and Physiological Factors in Soybeans" Rackis, J. Am. Oil Chemist's Soc: Jan. 1974 indicates that there are a number of differences between characteristics of soy protein isolates and other sources of soy protein i.e. "a deficiency of certain essential nutrients and the interaction of phytic acid with protein, vitamins and minerals during processing are the primary factors responsible for the poor nutritive value of soy isolates." (p.161A) and, "In contrast to soy bean meal, a different spectrum of nutritional and biological factors is associated with the feeding of soy isolates. As the source of protein in a diet, the isolates increase the requirement for vitamins E, K, D₂, D₃, and B₁₂. Phosphorus is utilized poorly. Soy isolates also create deficiency symptoms associated with decreased availability of calcium, magnesium, manganese, molybdenum, copper, iron and zinc. Availability of zinc is affected the most."

In respect of iron deficiency, please find enclosed "Iron Deficiency in Monkeys Fed Diets Containing Soybean Protein" Fitch et al, and note the conclusion that, "These studies show that, in the Rhesus monkey, diets containing isolated soy bean protein decrease the absorption of iron and may induce iron deficiency anemia". Studies in humans also show that iron absorption is impaired by soy protein consumption i.e. Cook et al "The Inhibitory Effect of Soy Products on Non-heme Absorption in Man" Am. J. Clin. Nutr. 34, 2622 (1981) and Hurrell et al "Soy Protein, Phytate and Iron Absorption in Humans" Am. J. Clin. Nutr. 56, 573 (1992). The Department of Agriculture, Food and Nutrition Service, 7 CFR Parts 210-225 and 226, Final Rule on Vegetable Protein Products; used in Child Nutrition Programs (p.776) recognises that some concern was expressed about reduced bioavailability of iron from meals using vegetable protein, by limiting the incorporation to "up to 30% soy substitution for several meals per week (which is) justifiable if there are adequate amounts of meat, fish, poultry and ascorbic acid in the diet."

It should be noted therefore, that since it is "the substitution of soy protein for animal protein" which "produced significant decreases in serum cholesterol values" (Anderson et al, "Meta-analysis of the Effects of Soy Protein Intake on Serum Lipids" New Eng. Journal of Med. P.277, Vol 333 No.5, (1995)) information needs to be included that mineral and/or vitamin supplementation will be necessary for those choosing to eliminate animal protein from their diets. Further, "the process employed in the isolation of the protein .. resulted in elimination of most of the Vitamin K" Fomon, *Nutrition of Normal Infants* 1991. Thus, in infants, cases of Vitamin K deficiency were reported: Goldman and Deposito 1966; Morgan 1969;

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Williams et al 1970; Schneider et al 1974; Committee on Nutrition 1971, 1980. The problems were eliminated when products were fortified with vitamin K. Those substituting animal protein with soy protein need to be made aware of the need for dietary fortification with Vitamin K

A most important concern is that soy protein products can cause reduced zinc plasma levels. I enclose a copy of Lonnerdal et al "*Nutritional Aspects of Soy Formula*" Acta Paediatr. Suppl. 402, 105-8 (1994) which demonstrates "low bioavailability of zinc from soy formula in human adults" (p.106) and also a copy of Prasad et al, "*Serum Thymullin in Human Zinc Deficiency*" J Clin. Invest. 1202-1210, Vol.82, (1988) which reports the manifestations of a moderately zinc-deficient state resulting from the substitution of animal protein with a soy protein based diet. The conclusion is that, "In as much as one may expect hormone and immunological changes to occur in human subjects even if the deficiency of zinc is only mild, it is important to recognise this condition so that corrective measures may be undertaken." Because the activity of thymullin is dependant on the presence of zinc in the molecule and because thymullin is an important hormone in the normal functioning of the immune system, autoimmune diseases and primary or secondary autoimmunodeficiencies may result from low levels of zinc, and thymullin deficiency.

Fort et al report that two autoimmune diseases are related to soy-formula feeding in infancy i.e. "*Breast Feeding and Insulin-Dependent Diabetes Mellitus in Children*" Jour. of the American Coll. Of Nutr. Vol.9 No.2 439-441 (1986); "*Breast and Soy Formula Feeding in Early Infancy and the Prevalence of Auto Immune Thyroid Disease in Children*" Jour. of the American Coll. Of Nutr., Vol.9(2) 164-167 (1990).

3. Dr Fraser Scott, of Health Canada, has researched the association between food and the incidence of diabetes in the B.B. Rat. A copy of "*Food-induced Type 1 Diabetes in the B.B. Rat*", Diabetes/Metabolism Reviews Vol.12, No.4 341-359 (1996) is enclosed Please note that:
- (p.349): "The finding that soy is a diabetogenic is supported by several studies using B.B. d p rats";
 - (p.350): "The possibility that soybeans contain diabetes-inducing agents that are decreased but not abolished by current processing techniques has major implications for infant feeding practices, food processing in general and may shed light on the pathogenesis of type 1 diabetes";
 - (p.350): "We have data showing that soy protein isolates retain significant diabetogenic activity";

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- (p.355): "Dose and timing studies suggest that the process is not attributed to a single triggering event but rather there is a requirement for frequent exposure, as one would get with common dietary constituents";
- (p.356): "These data suggest the autoimmune disease to which many B B d p rats succumb is to a large extent plant food-induced diabetes".

It is because of these concerns that the American Academy of Paediatrics recommend (Vol.94, No.5 pp.752-754, (1994)) "The substitution of soy-based formulas for milk-based formulas is not advised for either general or high-risk infant feeding practices because of animal studies linking the ingestion of soy protein intake to the development of diabetes."

Because Diabetes Mellitus etiology is linked to frequency of exposure, caution is needed before increased consumption of soy protein isolate is recommended.

The Australian College of Paediatrics has also noted (Jour. Paed. and Child Health 34: 318-319 (1998)) immune deficiency as a risk factor of soy protein infant feeding, basing its position on a report in Lancet July 2 1983 "Diet and Anti-Body Response to Vaccinations in Healthy Infants" by G Zoppi et al. Also, based in part on the two Fort studies, the Swiss Health Service has warned against the use of soy formulas containing isoflavones. Therefore, I believe that, if Protein Technologies claims benefits from insulin reduction in subjects over-endowed with insulin, it should also disclose risks of diabetes in the normal population, especially the very young.

4. The claim is made (P62978, Docket No.98P-0683) that allergic reaction to soy "is rarely seen in children more than 4 years old or adults. Many children out-grow food allergies and soy and seafood allergies are among those likely to be outgrown in contrast to allergies to milk, egg white or peanuts." This claim is incorrect. In fact, peanut allergens extensively cross react with soy allergens (Eigenmann et al, "Identification of Unique Antigenic Fractions of Soy and Peanut", (Abstr.) J. Allergen Clin. Immunol, 1, (3), 582 (1996/97). This may cause possible severe reactions at the first exposure including fatal and near fatal anaphylaxis (Sampson H.A. et al, "Fatal and Near Fatal Anaphylactic Reactions to Food in Children and Adolescents" N.Eng. J Med, 327, 380-4, (1992).) Communications from the Swedish National Food Administration to the New Zealand Ministry of Health (copy enclosed) describe the suddenness, severity and incidence of allergic reactions to soy, some of which were life threatening when children were known to be allergic to peanut and also suffering from asthma. This may be because food allergy can be a trigger in asthma. Moroz et al, "Medical Intelligence", The New Eng. Journal of Medicine, (1980)

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documents . sudden near anaphylatic reaction to soy by a woman, including one which occurred within minutes of tasting her infant's soy formula.

Adverse reactions to soy, are studied and reported by Gunn et al, "Gastrointestinal Illness Associated with Consumption of Soy Protein Extender" Jour. Food Protection, Vol. 43, 525-529, (1980). It is probable that the illnesses were triggered by allergic responses on an epidemic scale in Los Angeles. Descriptions and explanations of the soy allergens are discussed in length in "Recent Advances of Research in Antinutritional Factors in Legume Seeds" EAPP publication 1993. I commend a review of this book which also documents many problems with soy feeding experienced by domestic animals.

5. P.62978 of Docket No. 98P-0683, (#2, Background) lists "naturally occurring soy constituents." All of these have been considered by experts and reviewed extensively especially at the first conference about antinutritional factors in legume seeds, 1988. I enclose copies of some of the published conference notes "Recent Advances in Research in Legume Seeds", Conference notes, Pudoc, Wageningen, 1989 (The soybean lectins are included in these reports although not mentioned in the above docket). P.17 (Biological Effects of Dietary Lectins) states, "lectins may affect immunity, the endocrine system and general metabolism". Further information about soybean lectins is available in "The Lectin", Sharon et al, and "Plant Lectins" A. Pusztai. Any evaluation of the health effects of soy protein ingestion needs to include consideration of all "naturally occurring soy constituents" therein.
6. P.62979 of Docket No. 98P-0683, states that "soy isoflavones have been hypothesized as a protective factor against breast cancer in populations that consume large amounts of soy protein". However, recent analysis indicates that Asian soy protein ingestion and isoflavone exposure has been significantly overestimated. (Fukutake et al, "Quantification of Genistein and Genistin in Soybeans and Soybean Products" Food Chem. Tox. 34, 457-461, 1997. Therefore, I agree (Murkies et al, "Clinical Review 92 Phytoestrogens" Jour. Clin. Endoc. And Metabol. Vol. 83 No.2, 297-302) that "it is naïve to try to extract a single component of a total lifestyle from a community such as Japan, where several other significant lifestyle factors are operative, and expect to see a distinct correlation with disease". An examination of the claimed benefits for cancer risk reduction by the American Institute for Cancer Research (1997) indicates: "The evidence suggests that isoflavones and lignans may decrease the risk of breast cancer but is, as yet, insufficient".

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7. The "controlled human trial" (Ref.25 F.R. P62979) indicated changes in the menstrual cycle of women treated but there is debate as to whether the effects are "favourable" or adverse. While the lengthened cycle was hypothesised as beneficial, it is now reported that "a pattern of short or long menstrual cycles during reproductive life may be a risk factor for breast cancer." (Whelan et al, "Menstrual Cycle Patterns and Risk of Breast Cancer" American Journal of Epidemiology, Vol.140, No.12, 1081-1090). The original study, from Cambridge University, by Cassidy reported that:

"The steroid hormones secreted by the ovary, in combination with the action of the pituitary and hypothalamus, provide the cyclical changes for a normal ovulatory cycle to occur." "A daily dietary intake of 25.08 +/- 0.31mg of daidzein and a 19.85 +/- 0.43mg of genistein over a single menstrual cycle resulted in significant biological effects. The mid cycle surges of both lutenizing hormone and follicle stimulating hormone were significantly suppressed on the soy protein diet." ... "In many respects, the hormonal effects observed in the present investigation resembles the pathological changes reported in the subclinical, temporary ewe infertility syndrome (Lightfoot and Worth 1974). In 1983 Adams and Martin confirmed that the surge of L.H. (Lutenizing Hormone) was reduced in infected ewes."

Therefore, Cassidy concluded that, "the mechanism for ovulation was impaired." Robertson, "Phytoestrogens: Toxicology and Regulatory Recommendation" Proceedings of the Nutrition Soc. of N.Z. Vol.20, 35-42 (1995) warns in respect of Cassidy et al (1995) that "These results would also appear to have adverse implications for fertility in these young women."

8. I agree that soy protein is devoid of cholesterol and that will be a benefit for those who need to avoid cholesterol. Others will benefit from following the guidelines of the New Zealand Ministry of Health (personal communication) e.g.:

"The Ministry publishes food and nutrition guidelines for different age ranges. The aim of the guidelines is to help individuals make choices about what to eat to keep healthy. The guidelines do not claim that by eating a certain food a specific health problem will be corrected, but that eating habits based on moderation and variety can help maintain and improve health."

rather than increasing the consumption of one type of protein at the expense of another. Also, if benefits are claimed, risks should be

revealed. The only true claim is that soy protein does not contain cholesterol. Nor does any vegetable protein.

Yours sincerely

Valerie James

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24/01/99 Ltr DMBFDA/Docket 98P

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OU



25 January 1999

Our Ref:80353

Centre for Food Safety and Applied Nutrition (HFS-465)
Food and Drug Administration
200 C St. SW
Washington DC 20204
UNITED STATES OF AMERICA

COPY

Attention: Dr SM Pilch

Dear Dr Pilch

Re: Docket No. 98P-0683

In response to a petition filed by Protein Technologies International, the Food and Drug Administration (FDA) has advised that it is proposing to authorise the use, on food labels and in food labelling, of health claims on the association between soy protein and reduced risk of coronary heart disease.

The FDA has tentatively concluded that, based on the totality of publicly available scientific evidence, soy protein included in a diet low in saturated fat and cholesterol may reduce the risk of coronary heart disease.

In response to the FDA's advice as detailed in Docket No. 98P-0683, I would like to submit the attached information to your office for your consideration. I understand that submissions are to be made to the Dockets Management Branch but I did not have a fax number for that office.

Yours faithfully
KINGETT MITCHELL & ASSOCIATES LTD

Mike Fitzpatrick PhD MNZIC

45DA PLATA
FRED THOMAS DRIVE
P.O. BOX 27 849 TAKAPUNA
AUCKLAND, NEW ZEALAND
TELEPHONE (09) 876 8068 FAX (09) 866 9272

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1. Introduction

In response to a submission by Protein Technologies International (PTI), the FDA has tentatively concluded that, based on the totality of publicly available scientific evidence, soy protein included in a diet low in saturated fat and cholesterol may reduce the risk of coronary heart disease.

Should PTI's health claim petition be granted it would pave the way for labelling such as "25 grams of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease".

My submission does not address the issue of the health claim that soy protein reduces the risk of coronary heart disease *per se*. Rather, it opposes the claims by PTI that soy protein is a GRAS foodstuff and that there are no risks associated with the daily consumption of between 25 g and 100g of soy protein.

In fact, the FDA has never granted soy protein GRAS status. I would submit that the proof required to make such a claim is lacking. Also, there are very real risks associated with consuming soy protein. These risks appear to have been ignored by PTI and it is essential that the FDA give their full attention to the exposures to isoflavones and nitrosamines that will occur on daily exposure of up to 100g of soy protein.

In this regard, FDA must fully disclose to consumers the possible risks of soy protein as well as the possible benefits.

2. Safety for human use

PTI describe soy protein as a safe and lawful food and claim GRAS status by self-determination based on common use in food before January 1 1958. PTI also claim that the "FDA has recognised soy protein products as having GRAS status" and argue that the reason soy protein is not listed as GRAS is because "it is impractical for FDA to list all substances that are GRAS for their intended use".

Firstly, one must note that the self-determination of GRAS status of soy protein by the soy industry is meaningless and such self determinations should not be given any credence. In order to protect consumers only independent bodies, or those with recognised legal status, can perform legitimate determinations on the safety of consumer products.

Secondly, PTI's claim that products such as soy protein isolate were in common use in food before January 1 1958 is incorrect. The Select Committee of GRAS Substances (SCOGS) provided an independent evaluation of soy protein in the form of isolated soy protein in its 'Evaluation of the Health Aspects of Soy Protein Isolates as Food Ingredients' (1). The SCOGS committee found that at the time of their review (some 20 years after 1958) the use of soy protein was uncommon. In their determination of the likely average dietary exposure to soy protein

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isolate, the SCOGS committee reported a maximum per capita daily intake of soy protein isolate of about 150 mg from food items and a negligible amount due to migration from packaging.

Thirdly, and quite simply, soy protein is not legally a GRAS foodstuff, as the FDA has never codified it as such. In their review of soy protein isolates the SCOGS committee noted that such products were initially developed as binders in paper coatings. Soy protein isolates were GRAS under the provisions of the Code of Federal Regulations as substances migrating from paper and paperboard products used in food packaging because it was assumed that only very small amounts would be subject to human ingestion.

The SCOGS committee concluded that there was no evidence that soy protein isolates were a hazard when consumed at levels typical at that time (or levels that might reasonably be expected to be in future use) provided acceptable levels of lysinoalanine, nitrite and nitrosamines were established. To this end the committee recommended that food-grade soy protein isolates have specified acceptable levels of lysinoalanine, nitrite and nitrosamines.

To date, acceptable levels of lysinoalanine, nitrite and nitrosamines have not been established for food-grade soy protein isolates and one might conclude that soy protein isolates have not been granted GRAS status for this reason.

To claim that the FDA has not codified soy protein isolate as GRAS because it is impractical to list all substances that are GRAS for their intended use is inconsistent with the fact that other food items that the SCOGS committee found were GRAS have subsequently been codified as such. Had the SCOGS committee been satisfied that soy protein isolate was safe for intended use, there is little reason to suggest that GRAS status would not have been codified. Quite simply, soy protein was not granted GRAS status because the SCOGS committee was not convinced it was GRAS.

The findings of the SCOGS committee still have particular relevance to the PTI health claim petition. The PTI petition recommends a daily intake of 25 to 100 g of soy protein and it is quite evident that at this level of intake is far greater than the SCOGS committee ever anticipated when they were conducting their GRAS review. Hence, the concerns expressed by the SCOGS committee relating to exposure to lysinoalanine, nitrite and nitrosamines take on additional significance. For example, a daily consumption of 25 to 100 g of isolated soy protein may result in nitrosamine exposures that exceed established No Significant Risk Levels (NSRL's). This is discussed further in Section 3.2.1.

There are also several other issues which were not examined by the SCOGS committee during their review that are further reasons why soy protein isolates should not be granted GRAS status. These issues relate to the isoflavone content of isolated soy proteins and are discussed in Section 3.2.2.

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Until the safety issues discussed in Sections 3.2.1 and 3.2.2 are adequately addressed soy protein still cannot, and should not, be viewed as GRAS.

3. Risks associated with soy consumption

3.1 Recommended daily intake

PTI state that the "effective daily level of intake of soy protein associated with a significant effect on blood lipids and lipoproteins is 25 grams". According to PTI, this amount of soy protein could be obtained from 46 to 48 g of steam treated soy flour (52 to 54% protein) or 27 to 28 g of isolated soy protein (90 to 92% protein).

PTI also state that the "upper level of soy protein intake" should be "100 g of protein on a daily basis" although they imply that a diet of up to 155 g of soy protein per day is unlikely to cause harmful effects. In their submission of support of PTI health claim, the American Soybean Association (ASA) recommends a maximum daily level of daily intake of 100 g of soy protein. According to PTI this amount of soy protein could be obtained from 185 to 192 g of steam treated soy flour or 109 to 111 g of isolated soy protein.

However, there appears to have been little thought given to the level of exposure to potentially toxic factors, such as isoflavones, that would result from such diets. It behoves PTI and the ASA to fully determine the risks associated with the exposure to isoflavones that could occur when soy protein is consumed at the levels they have determined as appropriate to result in a reduction in the risk of coronary heart disease. However, neither PTI nor the ASA have performed any analysis of this kind and by not doing so have neglected their duty to consumers and cast doubt on their ability to act as a responsible producers of food commodities. This oversight also makes a mockery of the claims by PTI and the ASA that their petitions are "representative and balanced"

The manner in which PTI dismiss the very real risks that factors such as isoflavones pose is of considerable concern. In stating that "most domesticated animals and fowl are fed soy based rations and fertility is not a reported problem" PTI enter a new realm of deceitfulness.

3.2 Recommended daily exposure level and exposures to harmful compounds

3.2.1 Isoflavones

The potential for biological effects in humans due to soy isoflavones has been clearly identified and includes changes in the function of sex glands, the central nervous system, the thyroid, and behavioural patterns (2-7).

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Soy flour may contain up to 3080 µg/g total isoflavones (8) and isolated soy protein may contain up to 2500 µg/g total isoflavones (9). Therefore, daily ingestion of the qualifying amount of soy protein (25 g) could result in a daily intake of up to 148 mg isoflavones if the soy protein were derived from soy flour or up to 70 mg isoflavones if the soy protein were derived from isolated soy protein.

The maximum recommended level of daily intake of soy protein (100 g) could result in a daily intake of up to 591 mg isoflavones if the soy protein were derived from soy flour or 278 mg isoflavones if the soy protein were derived from isolated soy protein.

By recommending at least 25 g, but not more than 100 g, per day of soy protein to achieve a reduction in the risk of coronary heart disease PTI and the ASA are effectively sanctioning the ingestion of up to approximately 600 mg of isoflavones per day.

The effects of large acute and/or chronic doses of isoflavones in humans have not been established. However, even at modest doses, isoflavones are biologically active in humans. For example, a study of dietary intake of 45 mg total isoflavones (measured as total genistein and total daidzein) per day for 30 days in pre-menopausal women resulted in significant biological effects (10). These effects were a reduction in mean mid-cycle levels of LH and FSH to 33% and 53% respectively of the levels observed when the women were fed control diets that did not contain soy. Some individuals responded to the isoflavones less than others, however, in one individual LH and FSH levels were reduced to 17% and 32% of normal levels respectively.

In the Cassidy study all of the women still ovulated but the effects of the isoflavones continued for three months after the diet ceased. Clearly there is potential for women who are exposed to dietary isoflavones to suffer sufficient reduction in LH and FSH levels that they might become anovulatory.

PTI also cite the Cassidy study and note that "the changes in menstrual cycle length and hormone levels observed in these women were similar to those reported in response to treatment with Tamoxifen". One must assume that PTI have little understanding of the likely effects of exposing Tamoxifen *en masse* to the general population at more than ten times a biological dose. Although isoflavones are typically thought of as having the potential to combat hormone related disease, there will be doses at which they are acutely and chronically toxic.

Prominent isoflavones researchers have warned about the dangers of "mega-dosing" on these compounds (11) although to date a "mega-dose" has not been defined. A daily intake of 600 mg of isoflavones is more than one order of magnitude greater than the level that produced a biological effect during the Cassidy study and represents a dose that could readily result in adverse effects. To be certain to maintain a diet containing no more than a biological dose of isoflavones (45 mg) it is

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advisable that women consume no more than approximately 15 g of soy flour or 18 g of isolated soy protein per day.

Other effects on the sex steroid hormone status of women and men are quite possible. *In vitro* the soy isoflavones are potent inhibitors of 17- β -hydroxysteroid oxidoreductase (12-13) and, therefore, can modulate the synthesis and metabolism of oestradiol and other steroid hormones (14).

There have also been reports of soy isoflavones increasing nipple fluid secretion (15) and, although the study was not conclusive, consumption of soy was identified as a significant positive association in an increased occurrence of premature thelarche in Puerto Rico (16).

Contrary to PTI's claim, the reproductive and developmental toxicity of isoflavones has been demonstrated in several species of animals including domestic animals and fowl (17-21). In fact it was the toxicity of dietary levels of isoflavones to animals that first raised the awareness of the scientific community to the fact that soy isoflavones were endocrine disruptors (22). Reproductive effects, infertility, thyroid disease or liver disease due to dietary intake of isoflavones had been observed for several animals including mice (20), cheetah (21), quail (23), pigs (24), rats (25), sturgeon (26) and sheep (27).

As well as direct reproductive system effects, there are other biological effects of soy isoflavones. Like many endocrine disruptors, the soy isoflavones cause thyroid dysfunction in humans. Several papers from the 1960s reported that infants fed soy-based formulas developed goitre although the goitrogenic factors were not identified at that time (28-32). More recent reports have identified the actual and potential toxicity of soy on the thyroid (33-36) and the active factor in soy has been identified as the isoflavones. *In vitro* these compounds are potent inhibitors of thyroid peroxidase; more potent, in fact, than common anti-thyroid drugs (37).

If FDA approve the PTI health claim they will effectively endorse the consumption of up to 600 mg isoflavones per day. Such exposures to very large quantities of anti-thyroid compounds should be avoided at all costs. Chronic exposures to other anti-thyroid flavonoids in millet have resulted in endemic goitre in certain regions of Africa. Such exposures to anti-thyroid agents, regardless of whether or not iodine intake is sufficient, have the potential to induce thyroid disease (38).

3.2.2 Nitrosamines

The potential for soy products to contain nitrosamines was discussed at length during a GRAS evaluation of isolated soy protein (1). A discussion of the toxicity and mechanism of formation of nitrosamines is outside the scope of this submission; it is sufficient to note that:

- nitrosamines are among the most carcinogenic compounds in existence.
- nitrosamines are not naturally-occurring in soybeans but form during processing of soy products by reaction of nitrite with amines.

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There is little information on the levels of nitrosamines in soy products although given the manner in which products such as isolated soy protein (which includes acid washing and spray drying steps) or steam treated soy flour are prepared it is likely these compounds will be present. A single report found that a diet comprised of 11.9% isolated soy protein contained 1.5 ng/g of N-nitrosodimethylamine (39). This data suggests that consumers that ingest the maximum recommended amount of isolated soy protein (111 g) could be exposed to up to 1.4 µg of N-nitrosodimethylamine per day.

The California Environmental Protection Agency Office of Environmental Health Hazard Assessment (OEHHA) has established NSRL's for nitrosamines (40). These levels range from 40 ng per day for N-nitrosodimethylamine to 80 µg per day for the least potent nitrosamine listed (N-nitrosodiphenylamine).

As discussed in Section 3.1 the maximum daily intake of soy protein recommended by Protein Technologies (100 g) is equivalent to 109 to 111 g of isolated soy protein. Therefore, consumers of soy protein could be exposed daily to levels of N-nitrosodimethylamine that are 35 times greater than the NSRL.

For the level of intake of soy protein recommended by PTI, the NSRL's determined for N-nitrosodimethylamine will be exceeded if it is present at a level in excess of 0.20 ng/g (parts per billion) in steam treated soy flour or 0.36 ng/g in isolated soy protein. The NSRL's determined for the least potent OEHHA listed nitrosamines will be exceeded if they are present at levels in excess of 0.42 µg/g (parts per million) in steam treated soy flour or 0.72 µg/g in isolated soy protein.

In accordance with the SCOGS committee review, PTI must establish that their products contain acceptable levels of nitrosamines before they recommend daily intake levels for soy protein. It must also be understood that the NSRL's discussed in this submission are those defined by OEHHA for a 70 kg adult male. Lower NSRL's are defined, for example, for adult women and teenagers.

3.3 Disclosure of risk

The risks associated with consumption of up to 100 g of soy protein per day have been discussed in Section 3.2. In the event that FDA approve the PTI health claim petition regarding the possible benefits of soy protein, FDA are duty bound to also fully disclose these risks.

4. Summary

PTI have claimed that a diet of soy protein may reduce the risk of heart disease. PTI also claim that soy protein is a GRAS foodstuff and that there are no risks associated with the consumption of up to 100g of soy protein.

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nt by: KINGETT MITCHELL

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In response, my submission is that soy protein is not a GRAS foodstuff and that the evidence required in order to make such a claim is still lacking.

My submission has noted that there are very real risks associated with consuming soy protein at the levels PTI have recommended (up to 100g of soy protein per day). These risks relate to the potential exposure to high levels of isoflavones (up to approximately 600 mg per day) and to nitrosamines.

The FDA must be willing to disclose to consumers the possible risks as well as the possible benefits of a diet of up to 100 g of soy protein per day.

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000908

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000910



Valerie James
McLeod Bay
RD4
Whangarei
NEW ZEALAND

August 31st 1999

Documents Management Branch (H.F.A. 305)
Food and Drug Administration
5630 Fishers Lane, Rm 1061
Rockville MD 20852
U.S.A.

Dear Sir/Madam

**PROPOSED HEALTH CLAIM LINKING CONSUMPTION
OF SOY PROTEIN AND CORONARY HEART DISEASE:
63 FED.REG.62977 (Nov 10, 1998)**

I have been informed that Protein Technologies Inc., has provided supplemental material (July 9th 1999) for consideration by the Dockets Management Branch in respect of the above proposed health claims. New studies have also been submitted by the American Soybean Association.

I assume that all new evidence presented by industry will be made available, by public notice, for public comment. If not I request that prior to publishing a final ruling, the F.D.A. includes in its evaluation a recently released ANZFA (Australia and New Zealand Food Authority) 40 page document especially the following statements:

"Phytoestrogens" ANZFA document, March 1999 (pp 4,5,29,28)

"Phytoestrogens appear to be able to alter the thyroid hormone levels in adults and in infants. The mechanism of action may be a direct competitive inhibition of thyroid peroxidase which is essential for the production of thyroid hormones, and also may be an indirect reduction of circulating free thyroxine levels. In normal individuals these effects may be compensated by the existing homeostatic mechanisms, but for individuals in whom iodine intake is low or the thyroid function is compromised, phytoestrogens are a potential hazard."

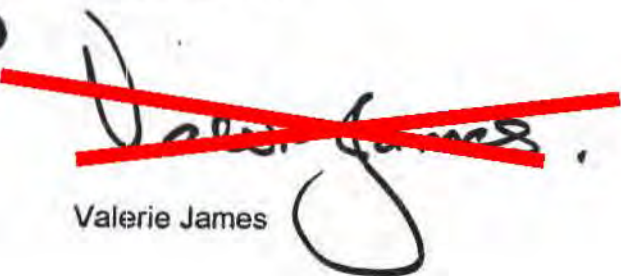
"While it is clear that phytoestrogens pose a potential hazard to the consumer of soy foods, they are also suggested to have benefits. The hazard consists of the potential changes in hormonal levels in adults and infants, and includes effects arising from oestrogen agonism; antioestrogenic activity; reduction in the thyroid function and alteration of sex-specific patterns of early development."

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001015

The ANZFA assessment was prepared by Dr Luba Tomaska, Principal Toxicologist, ANZFA, with the assistance of an expert panel consisting of:
Dr F. Cumming; Mr F. Dalais; Dr P. Hartmann; Prof. J. Mann; Dr P. McVeigh;
Dr Pat Tuohy; Prof. M. Wahlquist.

Yours sincerely


Valerie James

cc Susan Pilch
Linda Kahl

001016

001016

Dear Dr Kahl.

This copy for your "Make yourself heard at the
FDA" file

OU



Richard F. James MBA LLB
Rural Delivery 4
Whangarei
NEW ZEALAND

September 3 1999

The Manager
Dockets Management Branch - HFA 305
Food & Drug Administration
5680 Fishers Lane Rm 1061
Rockville Md 20852
U.S.A.

Dear Sir/Madam

**DOCKET 98P 0683 - FOOD LABELLING HEALTH CLAIMS:
SOY PROTEIN AND CORONARY HEART DISEASE**

I note your publication at pages 45932-45937, August 23, 1999, Vol 64 #1621 of the Federal Register. I believe it is improper to allow only 30 days for submissions. The law requires 60 days, and this is even more difficult as it is summer vacation time.

I would like to address two of the statements in that notice:

1. "When Congress enacted the 1990 amendments, it sought to ensure that the rules pertaining to health and nutrient content claims would be enforceable (see H Rept. 538, 101st Cong. 2d sess. 8,9 (1990)). Health and nutrient content claims are intended to make the consumer aware of the nutritional attributes of the labelled food. Because these claims are meant to help consumers maintain healthful dietary practices, it is of the utmost importance that they accurately reflect the nutritional composition of the labelled food. (See 136 Congressional Record, H12953, October 26, 1990, statement of house floor managers: "There is a great potential for defrauding consumers if food is sold that contains inaccurate or unsupportable health claims." (emphasis added))
2. "Ensuring the accuracy of claims was an overriding concern of Congress in passing the 1990 amendments. Congress envisioned that, under the Act as amended, "only truthful claims may be made on foods" (136 Congressional Record H12953, October 26, 1990, statement of Representative Waxman).

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A manufacturer who places a health or nutrient content claim in food labelling must have knowledge that the food qualifies to bear the claim. Congress expected that manufacturers would have to ascertain the nutritional attributes of their food products, through laboratory analysis or otherwise, in order to label those products properly. FDA has stated previously that a food manufacturer is responsible for the accuracy of its food labels (38 FR 2079 at 2163 and 2165). Indeed, a claim in food labelling that calls the consumer's attention to the food's nutritional characteristics is a representation that the manufacturer has evidence that the food meets the requirements for the claim. Thus, making a claim without such a basis would be misleading, in violation of section 403(a) of the Act." (emphasis added)

- 1 Dr Michael Bolger, Risk Assessor, Washington Office
Reproductive failure;
Uterine hypertrophy,
Infertility;
Impaired reproduction
His report is dated September 3, 1997
- 2 Dr D.M. Sheehan, FDA Senior Reproductive and Genetic Toxicologist Jefferson, Arkansas:
Toxicity in the thyroid;
Toxicity in tissues sensitive to estrogen,
Risk of abnormal brain development;
Risk of breast cancer;
Evidence of thyroid abnormalities;
Evidence of Autoimmune Diseases: Thyroiditis and Type 1 Diabetes;
Evidence of brain atrophy;
Evidence of dementia.
Report dated February 19, 1999
3. Also, the Australia/New Zealand Food Authority (ANZFA) has assessed potential risks in a March 1999 Assessment. Excerpt from page 19:

"Phytoestrogens appear to be able to interfere with the thyroid hormone homeostasis in adults and in infants. In normal individuals this effect may be compensated by the existing homeostatic mechanisms, but for individuals in whom iodine intake is low or the thyroid function is compromised, phytoestrogens are a potential hazard."
4. The Life Sciences (FASEB) Evaluation of Soy Protein as a Human Foodstuff (SCOGS - 101) of August 1979 found that the risk of nitrosamine formation in the processing of soy protein posed a health hazard. G.R.A.S. determination was withheld. In February 1999, Dr D.M. Sheehan, in his letter cited above, called for complete safety studies of soy protein. It is imperative that these studies be done before any health claims petitions can be granted.

I draw your attention to the Home Page introduction of the F.D.A.'s own Internet Site

"The Nation's Foremost Consumer Protection Agency"

The mandated duty of the F.D.A. is consumer protection as its first priority. Approval of dubious health claims is a distant second. In the light of the opinions of the F.D.A.'s own highly qualified experts, it is unlikely that a Federal Court would view the approval of these claims as a legitimate exercise of that primary duty.

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001018

Yours sincerely



For: Linda Kahl, Ph.D.,
Division of Product Policy,
H.F.S. - 205,
F.D.A.
200 "C" St SW
Washington D.C.

From: ~~Harold James~~ New Zealand.

This is a copy for your information

Regards, ~~Harold James~~

001019
001019

Refer to Hard Copy
For
Image



Computer Technology Services, Inc.

001019.001



Valerie James
McLeod Bay
RD4
Whangarei
NEW ZEALAND

September 16th 1995

Documents Management Branch (H.F.A. 305)
Food and Drug Administration
5630 Fishers Lane, Rm 1061
Rockville MD 20852
U.S.A.

Dear Sir/Madam

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A I refer to the statement (in CFR Part 101, Docket No 98P-0683) dated 23, 1994 that "if the agency issues a proposed regulation on a health claim petition, the agency is to complete the rule-making within 540 days of the date the agency receives the petition." Therefore the F.D.A. finds that there is good cause under 21CFR 101.40(b)(2) to provide 30 days rather than 60 days for public comment on this proposal."

I wish to appeal the reduced petition time. The only reason given for the truncated petition time (from 60 days to 30 days) was that the document above was not filed earlier in a timely fashion. When the F.D.A. called for public submissions on the original proposal the "cut-off" date was (at the latest) the end of January. No new submissions or evidence after that date other than that of F.D.A. origin (or from published scientific documents accessible to the F.D.A.) was acceptable. I can find no good reason why the public's right to know of and comment on, the revised proposals should be curtailed because of late filing by the F.D.A. It is the F.D.A.'s responsibility to file in a timely fashion to enable U.S. citizens domiciled abroad, or interested overseas consumer representatives to have the time to air say, otherwise citizens' or consumers' rights are diminished without due cause.

The F.D.A. invites comments on specific technical points (1 through 4). I shall submit comments (3) as follows:

30- [unclear] [unclear] [unclear]

"When the petition is accepted, the information is necessary for the proper performance of F.D.A.'s functions, and it is likely that the information will have practical utility..."

- (a) The F.D.A. requires food bearing health claims must be authorized by the F.D.A. in response to a petition. They also advise that the process for petitioning the agency is described in Section 101.70(a), and information that the petition must include is described in Section 101.70(f). Please note the conditions required below (emphasis added):

001020

"A. Preliminary requirements. A petition must include a statement of how the substance conforms to the requirements of 101.14(b) (21 CFR 101.14(b)). For petitions where the subject substance is a food, as defined in 101.14(b)(1), the petitioner should complete a comprehensive list of the specific..."

supply the substance in the food bearing the health claim. For each such ingredient listed, the petitioner should state how the ingredient complies with the requirements of S.101.14(b)(3)(ii) e.g. that its use is generally recognised as safe (GRAS), listed as a food additive or authorised by a prior sanction issued by the agency, and what the basis is for the GRAS claim, the food additive status or prior sanctioned status.

Substance means a specific food or component of food regardless of whether the food is in conventional food form or a dietary supplement that includes vitamins, minerals, herbs or other similar nutritional substances. ...

The claim is limited to describing the value that ingestion (or reduced ingestion) of the substance, as part of a total dietary pattern, may have on a particular disease or health-related condition.

If the substance is to be consumed at other than decreased dietary levels

(i) The substance must regardless of whether the food is a conventional food or a dietary supplement, contribute taste, aroma, or nutritive value, or any other technical attribute listed in S.170.3(d) of this chapter, to the food and must retain that attribute when consumed at levels that are necessary to justify a claim, and

(ii) The substance must be a food or a food ingredient or a component of a food ingredient whose use at the levels necessary to justify a claim has been demonstrated by the petitioner to the satisfaction of the agency to be safe and lawful under the applicable food safety provisions of the Federal Food, Drug and Cosmetic Act

(c) Scientific requirement FDA will promulgate regulations authorising a health claim if the petitioner determines, based on the totality of publicly available scientific evidence (including evidence from well-designed studies conducted in a manner which is consistent with generally recognised scientific procedures and principles), that there is significant scientific agreement among experts qualified by scientific training and experience to evaluate such claims, that the claim is supported by such evidence.

Analytical data that show the amount of the substance that is present in representative foods that would be candidates to bear the claim should be obtained from representative samples, using methods from the Association of Official Analytical Chemists (AOAC), where available. If no AOAC method is available, the petitioner shall submit the assay method used and data establishing the validity of the method for assaying the substance in food. The validation data should include a statistical analysis of the analytical and product variability.

- (b) Protein Technologies International Limited (PTI) identified the substance as a specific component of specific foods as "isoflavone containing soy protein". The petitioner specifically excluded forms of soy protein which do not contain isoflavones. Scientific documents were presented in support of the proposition that "isoflavone containing soy protein" was implicated in lowering total serum cholesterol levels. Other forms of soy protein were not capable of doing so. For ease of review, the following quotes (emphasis added):

PTI petition

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... Data establishing that isoflavone containing soy protein (soy protein concentrate and soy flour) conform to S.101.14(b) in that they are commonly consumed foods that are GRAS; Based on common use in food prior to 1958

Summary of scientific evidence ... based on the totality of publicly available scientific evidence (including evidence from well-designed studies conducted in a manner which is consistent with generally recognised scientific procedures and principles), there is significant scientific agreement among experts qualified by scientific training and experience to evaluate such claims, that the claim is supported by such evidence. ... relationship exists between the consumption of certain isoflavone containing soy products and a reduced risk of coronary heart disease."

001021

(4) Preliminary Requirements

The Substance of This Petition is Soy Protein with Naturally Occurring Isoflavones.

The petitioner submits that the substance of this petition be defined specifically as:

Soy protein containing the sum total (in aglycone units) of all 12 isomers of naturally-occurring isoflavones in amounts of no less than 2 mg's of soy protein.

The summary will also establish the basis for the threshold level of soy protein and the accompanying level of isoflavones required to achieve the biological effect of cholesterol-lowering that has been associated with a reduction in risk of coronary heart disease.

Sox protein with naturally-occurring isoflavones fully conforms to the definition of "substance" as described in 21 CFR 101.14(b)(2) which states that to be eligible for a health claim, the substance must be a food or a component of a food and that, in accordance with 101.14(b)(3)(v) the substance must achieve its effect through its use as a food or component of food, e.g. through its nutritive value, which is retained at the time consumed to justify the claim.

Other sources of soy protein commonly consumed in the diet may or may not contain sufficient amounts of naturally-occurring isoflavones to effectively lower levels. These include the traditional fermented and nonfermented soy tempeh and miso.

or food ingredients are also derived from soybeans. Some of these do not contain protein, others may contain protein, but without sufficient amounts of naturally-occurring isoflavones to have a cholesterol-lowering effect.*

Scientific Summary

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only soy isoflavones retained will be cholesterol-lowering

Specificity of Isoflavones

2. To specify the protein containing naturally-occurring isoflavones as the substance in each claim petition, evidence is provided to support the position that the claimed protein containing naturally-occurring isoflavones is the substance having the beneficial effect observed with ingestion of food sources of soy protein can be obtained from a protein containing naturally-occurring isoflavones and is independent of changes in other components within the diet when soy protein is consumed.

20. In isoflavones, there is no convincing experimental evidence to link any one of the others of these non-nutritive components

the amounts of isoflavones found in different
regions. Consequently, a minimum level of isoflavones
the 300 mg fraction in order to obtain the full benefit of
isoflavones.

ΔP was ...
... of the amount of sediments
($p < 0.05$)

...the effect of soy protein it is also necessary to ...
...is also necessary to ...
...from ingestion of soy protein. The ...
...study in humans indicate that this level ...
...metoprolol ... (Graham et al., 1987) ...
...of total ...

between soy isoflavones and the magnitude of the lipid response was also established in this study. Consequently, the effective daily level of intake of isoflavones in aglycone units can be calculated based on linearity to be approximately 2.0 mg/g of soy protein. At this level, 2.0 mg/g protein, a 3.2% decline in total cholesterol (95% CI = -0.8 to -5.6, and a 4.5% decline in LDL cholesterol (95% CI = -1.5 to -7.4) would be expected (Figures 1 and 2). For individuals with LDL cholesterol concentrations >160mg/dL, 25g soy protein with 2.0mg total aglycone isoflavones/g protein could be expected to produce a 7.7% decline in total cholesterol (95% CI = -4.8 to -10.7; and a 5.5% decline in LDL cholesterol.

Qualifying Amounts of Soy Protein to Permit Claim

It is proposed that the amount of soy protein required to qualify an individual food to bear the soy protein and heart disease health claim be established at 8.25g per reference amount customarily consumed (RACC). This proposed qualifying amount was derived by dividing the effective daily level of intake of 25g of soy protein by a factor of 4 to reflect the four eating occasions: three meals and a snack, defined as the typical dietary pattern of most Americans.

At an intake of 8.25g of soy protein, a minimum of 12.5mg of total aglycone isoflavones should also be present per RACC. It must be emphasized that the qualifying amounts of isoflavones are based on levels present naturally within the soy protein fraction.

The FDA, in evaluation of the health claims made by P.T.I. stated (Nov 1998) that the FDA was not persuaded that the isoflavone component of soy protein is a relevant factor in the heart-disease relationship.

Therefore, the narrow issue of the method FDA will use to verify that foods bearing the "qualified amount of soy protein" is irrelevant to the petition of P.T.I. Example: that the "substance" is "isoflavone containing soy protein" and only those foods containing "qualifying amounts of isoflavones" will result in the beneficial effects claimed.

The FDA has no mandate to consider and evaluate only the claims made for the petitioned substance. It does not have the mandate to consider or evaluate any other "substance". The FDA has ruled that the "substance" identified as the isoflavone component of soy protein is not a relevant factor. And yet this is the substance which is the basis of P.T.I.'s petition. The FDA has therefore in effect dismissed P.T.I.'s petition. The FDA has no mandate to substitute (under regulation) a different substance (i.e. soy protein per se) and suggest that a health claim to be enforced by only a minimum of soy protein per se in foodstuffs could be relevant to the health claims proposed by P.T.I. as specific to just one component of soy protein foods. Thus I submit that the proposed collection of information will have no practical utility in the enforcement of the petitioner's submission which is for "certain isoflavone containing soy protein". Furthermore, since

the FDA has determined that the substance is not a relevant factor, 5101.9 or 5101.35 FDA will not be able to enforce the health claim for the substance.

The FDA's decision to deny the petition and its subsequent denial of due process potentially gives legal approval to two additional soy protein isolates and soy protein (isolate) which currently, and for good cause, do not have GRAS status.

Comments were invited on:

001023

Comments should be submitted to the FDA by the date indicated on the label.

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- (c) The F.D.A. is required to ensure the claim is complete. A search of literature will indicate that P.T.I. did not accurately include summaries of adverse effects and so was incomplete, and was also misleading. The utility of the information arising from a diligent search of scientific literature, would result therefore, in the F.D.A. returning the petition to P.T.I. and in denying approval. Any petition found to be "incomplete" should be returned to the petitioner (Sec.101.70) and should be denied.
- (i) I include below, in part, a recent decision of the Broadcasting Standards Authority of New Zealand, which, having the force and authority of a court decision, will illustrate that the P.T.I. petition was in fact misleading, because of its omission of "significant disagreement among the experts"

IN THE MATTER of the Broadcasting Act 1989
AND
IN THE MATTER of a complaint by

R.F. JAMES
 of Whangarei

Broadcaster
TELEVISION NEW ZEALAND LTD

DECISION

Turning to standard G6 the Authority notes TVNZ's claim that the item was essentially a cooking demonstration and that such a programme would not be expected to outline the health risks of ingredients being used. This may well be so. However, when making claims about health benefits of ingredients which are themselves a matter of controversy, then the Authority considers that the broadcast should at least acknowledge the existence of that controversy. In this instance, the cooking demonstration involved the use of soy, and the claimed benefits of the product were promoted extensively. Those claimed benefits are a matter of contention and there is controversy. The Authority concludes that this raises questions of a broadcaster's duty to which standard G6 applies.

Authority notes that no effort was made to present or discuss the Authority considers that the broadcast has breached

on the programme to point out that there is significant disagreement among the experts about the claimed health benefits of soy. As these criticisms were not made, the programme lacked impartiality and balance, and it

above reason, the Authority upholds the complaint that a segment on Good Mornings on 3 May 1990, broadcast by Television New Zealand, breached standards G1 and G6 of the Television Code of Practice.

also refer the F.D.A. evaluators to Figure 1, p 279 "Meta-analysis of the Effects of Soy Protein on Serum Lipids." Anderson et al, New England Journal Medicine August 3, 1990. The net change for individuals in serum LDL cholesterol (from 3 trials) indicates that for some individuals, especially those subjects with initially normal levels, soy protein ingestion increased LDL levels. The complete study is published in the submission provided by P.T.I. as a part of their submission.

Yours sincerely,

BEST ORIGINAL COPY

001024

The F.D.A. required by statute, to ensure that

- (b) The subcommittee on the Committee on Labor and Human Resources of the Senate, in its report on the bill, stated that the bill should address the following questions:

1. Is there an optimum level of the particular substance to be consumed beyond which harm would be expected?
2. Is there any level at which an adverse effect from the substance or from foods containing the substance occurs for any segment of the population?
3. Are there certain populations that must receive special consideration? And, if so, what are the health factors (both positive and negative) which are relevant to consuming the substance?

The bill also requires that any scientific data shall include a detailed analysis of the effect of the use of the proposed claim on food consumption, specifically, any significant alterations in eating habits and corresponding changes in food intake. It also requires that such changes in food consumption be evaluated. The latter item shall include the effect on the intake of nutrients that have beneficial and adverse effects on the total diet.

The bill also requires that the level as determined in this specific provision shall be:

...adverse consequences to any segment of the population.

...by the person and is evaluated by the F.D.A.

That

the bill is "substantive" in nature.

...to the bill.

...to the bill.

the bill is "substantive" in nature, and the bill is "substantive" in nature. The bill is "substantive" in nature, and the bill is "substantive" in nature.

Therefore the F.D.A. must determine the exact alternative "substance" before it can make a determination. Only then will "truthful claims" be made. (125 Congressional Record 11, 12553, Oct 26, 1990, Statement of Senator Max Baucus)

- (c) Section 403(b) of the Food, Drug, and Cosmetic Act, which is a health claim authorized by regulation and is a claim for the agency.

001025

The F.D.A. is not a "substantive" in nature, and the bill is "substantive" in nature. The bill is "substantive" in nature, and the bill is "substantive" in nature. The bill is "substantive" in nature, and the bill is "substantive" in nature.



Please add
to GRN 000001.
Thank you

~~I include~~

Oct 6 1999

Dear Dr Kahl.

I have received the attached
copy from a contact in
London.

Please add it to your
"let your voice be heard
in the FDA" file

Sincerely
~~[Signature]~~

001026

THE FOOD COMMISSION

Publisher of The Food Magazine

22 September 1999

Dockets Management Branch (HFA-305)
Food and Drug Administration
5630 Fishers Lane, Rm 1061
Rockville MD 20852
USA

Dear Sir/Madam

Docket No 98P-0683 Food Labeling: Health Claims; Soy Protein and Coronary Heart Disease

The Food Commission is responding to the consultation regarding measurement of soy protein in relation to the above proposed rule (Federal Register August 23, 1999). We wish our comments to be considered in conjunction with our previous comments made on 25 January 1999.

We note that there is currently no reliable, accurate analytical method for measuring soy protein to verify compliance with the proposed health claim and that this is a particular problem for measuring soy protein in foods which may contain other protein sources. We further note that in the absence of such a method, the FDA is proposing to require that companies keep records and supply these to the FDA on request.

Truthfulness of claims

As the FDA makes clear 'Ensuring the accuracy of claims was an overriding concern of Congress in passing the 1990 amendments. Congress envisioned that, under the act as amended, "only truthful claims may be made on food" '.

As we have previously stated, the Food Commission is not convinced that there is adequate scientific data to support the proposed claim and PTI has failed in its submission to include information on the potential risks of recommending increased soy consumption for the general public. In response to the current consultation, we consider that a reliable, accurate analytical method for measuring soy protein would be essential to ensure that food companies comply with the requirements of any such claim. We do not believe that record keeping is a proper substitute for analytical methods which would be necessary to independently assess the truthfulness of claims.

001027

Our concerns over the truthfulness of the proposed claim extend beyond the methods of measuring soy protein in foods. As we stated in our previous submission, neither the FDA nor Protein Technologies has attempted to identify, quantify or explain the mode of action of the

Please note our new address - The Food Commission (UK) Ltd, 94 White Lion Street, London N1 9PF. Tel: 0171 837 2250 Fax: 0171 837 1141

Campaigning for safer, healthier food

The Food Commission (UK) Ltd, 94 White Lion Street, London N1 9PF. Tel: 0171 837 2250. Fax: 0171 837 1141
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The Food Commission (UK) Ltd campaigns for safer, healthier food and is funded by public subscriptions and donations.

factors in soy protein which may be responsible for, or contribute to, claims for serum cholesterol reductions. While PTI's application considers isoflavones (phytoestrogens) to be the effective agent, the FDA has already concluded that is 'is not persuaded that the isoflavone component of soy protein is a relevant factor to the diet-disease relationship'.

Current scientific understanding of the components of soy protein - their absorption, distribution, metabolism and effects (both risks and benefits) - remains limited. It is known that levels of isoflavones can vary enormously within soy, dependent on many factors including the variety of soy crop, environmental conditions pre-harvest and methods of food preparation and processing post-harvest. There are likely to be similar variations in other potentially active components. Therefore, any measurement of soy protein, by whatever means, is unlikely to ensure a consistent amount of relevant 'active ingredients'. Hence there is great potential for such a claim to mislead consumers.

We therefore urge the FDA not to accept this health claim proposal on the grounds that there is no reliable method of testing soy protein in all circumstances. Furthermore, as we have previously stated, there are insufficient scientific grounds to support the claim, PTI have failed to address potential health risks and it would be highly inappropriate to advocate, through such a health claim that the general population increase its consumption of soy while such concerns remain outstanding.

The Food Commission understands that the FDA has a legal obligation to consider the issues raised by our responses before making its final decision about the wisdom of permitting this health claim.

Yours faithfully



Sue Dibb
Co-director

001028



AMERICAN ACADEMY OF PEDIATRICS

Committee on Nutrition

Soy Protein-based Formulas: Recommendations for Use in Infant Feeding

ABSTRACT. The American Academy of Pediatrics is committed to the use of maternal breast milk as the ideal source of nutrition for infant feeding. Even so, by 2 months of age, most infants in North America are formula-fed. Despite limited indications, the use of soy protein-based formula has nearly doubled during the past decade to achieve 25% of the market in the United States. Because an infant formula provides the largest, if not sole, source of nutrition for an extended interval, the nutritional adequacy of the formula must be confirmed and the indications for its use well understood. This statement updates the 1983 Committee on Nutrition review¹ and contains some important recommendations on the appropriate use of soy protein-based formulas.

ABBREVIATION. IgE, immunoglobulin E

BACKGROUND

Although soy protein-based nutrition has been used during infancy for centuries in the Orient, the first use of soy formula feeding in this country was in 1909.² In 1929, Hill and Stuart³ proposed soy protein-based feeding for infants with intolerance to cow milk-based feeding.

Before the 1960s, soy protein-based formulas used soy flour, which imparted a tan color and nutty odor to the formula, and infants consuming it often had diarrhea and excessive intestinal gas. These features and symptoms were attributed to residual indigestible carbohydrates in the soy.^{4,5} Since the mid-1960s, a soy protein isolate has been used, reducing these concerns and greatly increasing acceptance of the product.

COMPOSITION OF ISOLATED SOY PROTEIN-BASED FORMULAS

The isolated soy protein-based formulas currently on the market are all free of cow milk-protein and lactose, and prepared so they that provide 67 kcal/dL. All are iron-fortified and meet the vitamin, mineral, and electrolyte specifications addressed in the 1976 guidelines from the American Academy of Pediatrics for feeding full-term infants⁶ and established by the US Food and Drug Administration.⁷

The protein is a soy isolate supplemented with L-methionine, L-carnitine, and taurine to provide protein at 2.45 to 3.1 g/100 kcal or 1.65 to 2.1 g/dL. The harvested soybean is processed by removal of

the hull to yield a pulp that is then refined to soybean oil and soybean flake. The defatted flakes are processed into soy flour, soy protein isolate, or soy cotyledon fiber. Soy protein isolate is extracted in a slightly alkaline solution and precipitated at the isoelectric point of 4.5 to yield a purity of at least 90% soy protein on a dry basis.⁸

Supplementation with L-methionine began by the early 1970s. In 1979, Fomon et al⁹ demonstrated improved biological quality of the protein with the addition of sulfur-containing amino acids. Subsequent studies in 1986 demonstrated that at a protein intake of 1.8 g/100 kcal, methionine was required to improve nitrogen balance, whereas at intakes of 2.2 and 2.6 g/100 kcal, methionine supplementation improved weight gain, urea nitrogen excretion, and albumin synthesis.¹⁰ Before the routine supplementation of soy protein formulas with methionine, infants with undiagnosed, untreated cystic fibrosis were particularly at risk for severe hypoalbuminemia and edema when fed soy proteins, a risk that remains in soy, cow milk, and breastfed infants with cystic fibrosis until the initiation of pancreatic enzyme therapy.^{11,12}

Carnitine, which is required for the optimal mitochondrial oxidation of long-chain fatty acids, is deficient in foods of plant origin and is added to soy formula to the level in breast milk, as is taurine, an amino acid that is abundant in human milk. Taurine functions as an antioxidant and, along with glycine, is a major conjugate of bile acids in early infancy.

The fat content of soy protein-based formulas is derived primarily from vegetable oils. The quantity of specific fats varies by manufacturer and is usually similar to those in the corresponding cow milk-based formula. The fat content ranges from 5.3 to 5.5 g/100 kcal or 3.6 to 3.8 g/dL. The oils used include soy, palm, sunflower, olein, safflower, and coconut.

Carbohydrate is provided lactose free, as corn starch, corn starch hydrolysate, tapioca starch, or sucrose, with content ranging from 10.0 to 10.2 g/100 kcal or 6.7 to 6.9 g/dL. Polysaccharide, in the form of supplemented soy fiber, has been added to one soy protein-based formula.¹³

Until 1980, mineral absorption from soy formulas was erratic because of poor stability of the suspensions and the presence of excessive soy phytates in the formula.¹⁴ Not surprisingly, conflicting results of studies addressing the adequacy of bone mineralization were reported.¹⁵⁻¹⁷ With the present formulations, bone mineralization, serum levels of calcium and phosphorus, and alkaline phosphatase levels in full-term infants through 6 to 12 months of age are

The recommendations in this statement do not indicate an exclusive course of treatment or serve as a standard of medical care. Variations, taking into account individual circumstances, may be appropriate.
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equivalent to those seen with cow milk-based formulas.¹⁷⁻¹⁹ Because soy protein isolate formulas still contain 1.5% phytates and up to 30% of the total phosphorus is phytate-bound, the total phosphorus and calcium content of the formulas is ~20% higher than in cow milk-based formula, while still maintaining mandated calcium to available phosphorus ratio (1 to 2.0:1).

The soy phytates and fiber oligosaccharides also bind iron and zinc.²⁰ All soy-based formulas thus are iron-fortified and have proved as effective as iron-fortified (12 mg/L) cow milk-based formulas in the prevention of iron deficiency in infants.^{21,22} With radiolabeled zinc, the highest absorption of zinc is from human milk (41%) and the lowest is from soy formula (14%).²³ All soy protein-based formulas thus are zinc-fortified.^{20,23} In one infant, the phytates may have interfered with the uptake of exogenous thyroid hormone, binding the T4 within the lumen, increasing fecal loss, and reducing the efficacy of oral thyroid hormone.²⁴

Early studies revealed that the full nutritional value of soybean protein is achieved only after heat has been applied. Subsequent studies confirmed the presence of a number of heat-labile factors with biological activity in soybean-based products. The most prominent of these factors is a soybean protease inhibitor with the properties of an antitrypsin, antichymotrypsin, and antilelastin.²⁵ Soybean protein isolate, as heated for infant formulas, removes 80% to 90% of this protease inhibitor activity and renders it nutritionally irrelevant. There also are heat-stable factors that remain in the soy protein isolate, including the low-molecular-weight fibers, phytates, saponins, and phytoestrogens.

The phytoestrogens demonstrate physiologic activity in rodent models and, per unit of body weight, the infant's potential intake of phytoestrogen from isolated soy protein-based formula is higher than that demonstrated to influence the menstrual cycle of humans.²⁶ Very limited human data to date, however, suggest that soy phytoestrogens have a low affinity for human postnatal estrogen receptors and low potency in bioassays.²⁵ A number of studies are addressing this issue at this time.

In 1996, the American Academy of Pediatrics issued a statement on aluminum toxicity in infants and children and discussed the relatively high content of aluminum in soy-based formulas.²⁷ Although the aluminum content of human milk is 4 to 65 ng/mL, that of soy protein-based formula is 600 to 1300 ng/mL.²⁸⁻³¹ The source of the aluminum is the mineral salts used in formula production. Aluminum, which makes up 8% of the earth's crust as the third most common element, has no known biological function in humans.²⁸ The toxicity of aluminum is traced to increased deposition in bone and in the central nervous system, particularly in the presence of reduced renal function in preterm infants and children with renal failure. Additional potential sources of aluminum include total parenteral nutrition solutions, renal dialysis fluids, and aluminum-containing antacids. Because aluminum competes with calcium for absorption, increased amounts of

dietary aluminum from isolated soy protein-based formula may contribute to the reduced skeletal mineralization (osteopenia) observed in preterm infants and infants with intrauterine growth retardation.³² Term infants with normal renal function do not seem to be at substantial risk for aluminum toxicity from soy protein-based formulas.⁸

SOY PROTEIN-BASED FORMULAS IN TERM INFANTS

Numerous studies have documented normal growth and development in term neonates fed methionine-supplemented isolated soy protein-based formulas.^{9,33-37} Average energy intakes in infants receiving soy protein formulas also are equivalent to those achieved with cow milk formula.⁸ The serum albumin concentration, as a marker of nutritional adequacy, also is normal,^{8,10,34,38} and bone mineralization also is equivalent to that documented with cow milk-based formula.³⁵⁻³⁹ Additional studies confirm that soy protein formulas do not interfere with the normal immune responses to oral immunization with polio vaccine.^{39,40}

SOY PROTEIN-BASED FORMULAS IN PRETERM INFANTS

Preterm infants who weighed from 1500 to 1800 g and were fed methionine-supplemented soy protein-based formulas demonstrated significantly less weight gain, less length gain, and lower serum albumin levels than that achieved with cow milk-based formulas.⁴¹ With lower birth weights, ie, <1500 g, data conflict; one study demonstrated equivalent growth and plasma protein levels,⁴² whereas another demonstrated significant reductions in both.⁴³

All three studies of preterm infants agreed, however, that serum phosphorus levels were lower in the preterm infants fed soy protein-based formula and, when measured, the alkaline phosphatase levels were higher.^{41,42} As anticipated from these observations, the osteopenia of prematurity is reportedly increased in low birth weight infants receiving soy protein-based formulas.^{44,45} Even with supplemental calcium and vitamin D, radiographic evidence of increased osteopenia was present in 32% of 125 preterm infants fed soy protein-based formula.⁴⁵

When combined with concerns about aluminum toxicity, the failure to achieve equivalent growth rates or albumin levels consistently and the reduced bone mineralization lead to the conclusion that soy protein-based formulas should not be fed to low birth weight preterm infants. The newer cow milk protein-based formulas designed for preterm infants are clearly superior.

USE IN DISORDERS OF CARBOHYDRATE METABOLISM

When strict dietary lactose elimination is required in the management of infants with galactosemia or primary lactase deficiency, the soy protein formulas are safe and cost-effective. Soy protein-based formulas with sucrose as the carbohydrate are contraindicated in sucrase-isomaltase deficiency and in hereditary fructose intolerance.

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Results of studies in animal models using a diabetes-prone rat suggested an increased frequency of diabetes when ingesting a soybean meal diet. However, when soy protein isolate or hydrolyzed soy protein feedings were used, no significant increase in diabetes was noted. This suggests that the factor contributing to the increased frequency of diabetes in this animal model is not the soy protein present in infant formulas.⁴⁶

USE IN ACUTE DIARRHEA AND SECONDARY LACTASE DEFICIENCY

Because of the role of lactose-free soy protein-based formulas in the management of long-term lactose restriction, a number of studies have addressed the role of these formulas in the recovery from acute infantile diarrhea complicated by transient lactase deficiency. After immediate rehydration, most infants can be managed successfully with continued breastfeeding or standard cow milk or soy formula.^{47,48} In an extensive review, Brown⁴⁷ noted that the dietary failure rate of lactose-containing formulas was 22%, whereas that of lactose-free formulas was 12%. In a study comparing breast milk, cow milk-based formula, and soy protein-based formula, no difference was found in the rate of recovery from rotavirus or norovirus diarrhea based on nutritional therapy.⁴⁹ Although not significant from the perspective of nutritional compromise, the duration of diarrhea has been reported to be shorter in infants receiving soy protein-based formula.^{50,51} The duration of liquid stools may be reduced further by adding additional soy polysaccharide fiber⁵² or by resuming a mixed-staple diet.⁵³

ANTIGENICITY OF SOY PROTEIN-BASED FORMULAS

Any ingested large molecular weight protein is a potential antigen to the intestinal immune system. In soy protein isolate, 90% of the pulp-derived protein resides in two major heat-stable globulins: β -conglycin, with a molecular weight of 180 000, and glycinin, with a molecular weight of 320 000. The former has three subunits, and the latter has six.⁵⁴ After enteric digestion, the number of potential antigens generated at the mucosal surface is enormous.⁵⁵ As a result, the *in vitro* demonstration of antigen-specific antibody can be difficult. The antigenicity of soy protein, suspected since 1934,⁵⁶ was documented in low-risk infants by Eastham et al⁵⁷ in 1982. Intrauterine sensitization has been documented by demonstrating antigen-specific antibody in human amniotic fluid.⁵⁸

Severe gastrointestinal reactions to soy protein formula have been described for >30 years⁵⁹ and encompass the full gamut of disease seen with cow milk protein in infancy—enteropathy, enterocolitis, and proctitis. Small-bowel injury, a reversible celiac-like villus injury that produces an enteropathy with malabsorption, hypoalbuminemia, and failure to thrive, has been documented in at least four studies.⁶⁰⁻⁶³ To date, those afflicted have responded to the elimination of soy protein-based formulas and are no longer sensitive by 5 years of age. Severe enterocoli-

tis manifested by bloody diarrhea, ulcerations, and histologic features of acute and chronic inflammatory bowel disease also has been well described in infants receiving soy protein-based formulas.⁶⁴⁻⁶⁶ They respond quickly to elimination of the soy formula and introduction of a hydrolyzed protein formula. Their degree of sensitivity to soy protein during the first few years of age can remain dramatic; thus, casual use of soy-based formula is to be avoided.^{68,69} Most children, but not all, can resume soy protein consumption safely after 5 years of age. In addition, up to 60% of infants with cow milk protein-induced enterocolitis also will be equally sensitive to soy protein.⁶⁸⁻⁷⁰ It is theorized that the intestinal mucosa damaged by cow milk allows increased uptake and, therefore, increased immunologic response to the subsequent antigen soy. Eosinophilic proctocolitis, a more benign variant of enterocolitis, also has been reported in infants receiving soy protein-based formula.⁷⁰⁻⁷³

These dietary protein-induced syndromes of enteropathy and enterocolitis, although clearly immunologic in origin, are not immunoglobulin E-mediated, reflecting instead an age-dependent transient soy protein hypersensitivity.⁷⁴ Because of the reported high frequency of infants sensitive to both cow milk and soy antigens, soy protein-based formulas are not indicated in the management of documented cow milk protein-induced enteropathy or enterocolitis.

ALLERGENICITY OF SOY PROTEIN-BASED FORMULAS

Recognizing that soy protein is antigenic does not mean that soy protein is highly allergenic. To address immunoglobulin E (IgE)-mediated hypersensitivity to soy protein-based formula, three types of studies have been performed. The first addresses the frequency with which proven allergy develops in healthy infants fed cow milk- or soy protein-based formulas. The second addresses the same question in infants at high risk according to a family history of allergic responses to dietary protein. The symptom is usually eczema, and the high-risk history usually includes a family history of atopic disease (eg, asthma, allergic rhinitis, or eczema). The third type of study addresses the response of infants with proven cow milk allergy to subsequent ingestion of soy protein-based formula. The problem with these studies is with the definition of allergy, which included fussiness, colic, emesis, a positive RAST antibody, and/or a positive double-blind, placebo-controlled challenge.

In a prospective study of healthy infants fed breast milk, cow milk formula, or soy-based formula, Halpern et al⁷⁵ documented allergic responses to soy in 0.5% of infants and to cow milk in 1.8%. This frequency is consistent with the summary by Fomon⁷⁶ that in 3 decades of study of soy-based formulas, <1% of soy formula-fed infants had adverse reactions. In a national survey of pediatric allergists, the occurrence of allergy to cow milk was reported at 3.4%, whereas allergy to soy protein was reported to be 1.1%.⁷⁷ Two large studies of infants with atopic dermatitis addressed the frequency with which a

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double-blind, placebo-controlled challenge with soy protein was positive. Sampson⁷⁸ documented soy positivity in 5% of 204 patients, whereas Businco et al⁷⁹ implicated soy in 4% of 143 children.

Prospective studies of high-risk infants suggest that soy protein-based formula has no relative value over cow milk formula in the prophylaxis or prevention of allergic disease.⁸⁰⁻⁸⁴ Furthermore, the use of soy protein-based formula during the first 3 months of age does not reduce the frequency of positive antibody responses to cow milk formula introduced later in infancy.⁸⁵ When human milk feeding is supplemented with soy formula in high-risk infants, the anticipated frequency of eczema by 2 years of age is not significantly reduced.^{79,80,86,87} Interpretation of these data is obscured by multiple alterations in the maternal diet and by environmental stimuli. The issue of delay in allergic disease, as opposed to the prevention of allergic disease, awaits the result of long-term investigations. Fortunately, true anaphylaxis after soy protein exposure has been reported only once.⁸⁸ According to the data now available, isolated soy protein-based formula has no advantage over cow milk-based formula for supplementing the diet of a breastfed infant.

Two studies documented the frequency of tolerance to soy protein in a small number of children with documented allergy to cow milk protein as defined by a positive skin test and positive double-blind, placebo-controlled challenge. The rate of combined positivity to cow milk and soy approximated 10%.^{89,90}

TREATMENT OF COLIC WITH SOY PROTEIN-BASED FORMULA

Colicky discomfort, apparently abdominal in origin, is described by the parents of 10% to 20% of infants during the first 3 months of age.⁹¹ Although many factors have been implicated, parents frequently seek relief by changing infant formula. Although some calming benefit can be attributed to the sucrose^{92,93} and fiber content,⁹³ controlled trials of cow milk and soy protein-based formulas have not demonstrated a significant benefit from soy.^{94,95} The value of parental counseling as to the cause and duration of colic seems greater than the value of switching to soy formula.⁹⁶ Because most colicky behavior diminishes spontaneously between 4 and 6 months of age, any intervention at that time can be credited anecdotally.

CONCLUSIONS AND RECOMMENDATIONS

1. In term infants whose nutritional needs are not being met from maternal breast milk or cow milk-based formulas, isolated soy protein-based formulas are safe and effective alternatives to provide appropriate nutrition for normal growth and development. Isolated soy protein-based formula has no advantage over cow milk protein-based formula as a supplement for the breastfed infant.
2. Because soy protein-based formulas are lactose-free, they are appropriate for use in infants with galactosemia and hereditary lactase deficiency.

3. Parents seeking a vegetarian infant can be advised to use isolated soy protein-based formula.
4. Most previously well infants with acute gastroenteritis can be managed after rehydration with continued use of human breast milk or standard dilutions of cow milk-based formulas. Isolated soy protein-based formulas are indicated when lactose intolerance has been documented.
5. The routine use of isolated soy protein-based formula has no proven value in the prevention or management of infantile colic.
6. The routine use of isolated soy protein-based formula has no proven value in the prevention of atopic disease in healthy or high-risk infants.
7. Infants with documented cow milk protein-induced enteropathy or enterocolitis frequently are as sensitive to soy protein and should not be given isolated soy protein-based formula routinely. They should be provided formula derived from hydrolyzed protein or synthetic amino acid.
8. Most infants with documented IgE-mediated allergy to cow milk protein will do well on isolated soy protein-based formula.
9. Soy protein-based formulas are not designed or recommended for preterm infants who weigh <1800 g.

COMMITTEE ON NUTRITION, 1995 to 1997

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William J. Cochran, MD
Carlos A. Flores, MD
Michael K. Georgieff, MD
Marc S. Jacobson, MD
Alan M. Lake, MD

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National Institute of Diabetes & Digestive & Kidney Diseases
Ephraim Levin, MD
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American Dietetic Association
Elizabeth Yetley, PhD
Food and Drug Administration
Stanley Zlotkin, MD
Canadian Paediatric Society

AAP SECTION LIAISON

Ronald M. Lauer, MD
Section on Cardiology

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Topics covered include experimental and clinical nutrition research, dietetics, food science, nutrition in medicine, and food and nutrition legislation and policy as developed and implemented by national and international bodies.

Sections include in-depth reviews and brief critical reviews, editorials, nutrition grand rounds, point-of-view columns, nutrition policy perspectives, and an updated calendar of events. Readers are encouraged to comment on published articles through letters to the editor.

All submissions are subject to review.

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Karen Oerter Klein, M D
For more than 60 years, soy-based infant formulas have been fed to millions of infants worldwide and studied in controlled clinical research. These products provide essential nutrients required for normal growth and development. The safety of isoflavones in soy-based products, including infant formulas, has been questioned recently owing to reports of possible endocrine effects in animals and in cultured cells. The literature offers no evidence of endocrine effects in humans from infant consumption of modern soy-based formulas. Growth is normal and no changes in the timing of puberty or in fertility rates have been reported in humans who consumed soy formulas as infants. Consequently, soy-based infant formulas continue to be a safe, nutritionally complete feeding option for most infants.
- Special Article*
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Julie A. Mennella, Ph D, and Gary K. Beauchamp, Ph D
Anyone who has observed infants for any period of time can testify to the intense activity occurring in and around their mouths—the primary site for learning in the first few months of life. Before they are even able to crawl, infants have learned much about their new sensory world. Through recent research we have begun to explore the impact of these early experiences on infants' acceptance of solid foods and how they explore objects in their environment. We have also begun to focus on the sensory experiences of the formula-fed infant, in particular, how their responses to particular formulas, which are extremely unpalatable yet to be done. It is clear, however, that infants are not passive receptacles for flavored foods. Parents who offer a variety of foods will provide both a nutritious, well-balanced diet, as well as an opportunity for their children's own personal preferences to develop.
- Nutrition Grand Rounds*
- 212 **Individual Variability in Homocysteine Response to Folate Depletion: An Unusual Case**
Robert A. Jacob, Ph D
Research within the past decade has shown that even moderately elevated plasma homocysteine concentrations are associated with increased risk of vascular disease. A variety of genetic and nutritional factors can affect homocysteine concentrations, with folate nutriture being one of the most influential. Plasma homocysteine responses of healthy adults to folate depletion and repletion vary substantially, even when many nutritional and lifestyle factors are normalized in a metabolic unit. The case of a woman with a highly exaggerated homocysteine response to moderate folate depletion is presented. A variety of possible factors relating to homocysteine metabolism are discussed, yet no convincing explanation for the unusual pattern is apparent. The case demonstrates that the homocysteine response to folate can be highly variable between individuals, and suggests that further research on the genetic determinants of the human folate requirement is warranted.
- Nutrition Science ↔ Policy*
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John T. Cook, Ph D
In 1996, the U.S. Congress passed the Personal Responsibility and Work Opportunity Reconciliation Act, which had as one of its effects the withdrawal of food stamp eligibility for many legal immigrants. This analysis from the Tufts University Center on Hunger, Poverty, and Nutrition Policy examines the nutritional impact of this legislation on legal immigrants and discusses the arguments for restoration of these nutritional benefits. At press time, the U.S. Congress had just passed legislation to restore food stamp benefits to many legal immigrants.

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Isoflavones, Soy-based Infant Formulas, and Relevance to Endocrine Function

Karen Oerter Klein, M.D.

For more than 60 years, soy-based infant formulas have been fed to millions of infants worldwide and studied in controlled clinical research. These products provide essential nutrients required for normal growth and development. The safety of isoflavones in soy-based products, including infant formulas, has been questioned recently owing to reports of possible endocrine effects in animals and in cultured cells. The literature offers no evidence of endocrine effects in humans from infant consumption of modern soy-based formulas. Growth is normal and no changes in the timing of puberty or in fertility rates have been reported in humans who consumed soy formulas as infants. Consequently, soy-based infant formulas continue to be a safe, nutritionally complete feeding option for most infants.

Introduction

For more than 60 years, soy-based infant formulas have been fed to millions of infants worldwide and studied in controlled clinical research. These products provide the nutrients necessary for normal growth and development.¹⁻⁷ Soy-based formulas were developed for infants who could not tolerate either the milk protein or the lactose (milk sugar) found in milk-based formulas. Early soy-based infant formulas used soy flour as the protein source and contained no lactose. The vast majority of soy formulas manufactured in the United States over the past 30 years ("modern infant formulas"), however, have relied on a more highly refined protein source, soy protein isolate,⁸ and contain no lactose. The isoflavone content of soy protein isolate can vary among regions/countries and with seasonal fluctuations. Soy protein isolate has reduced levels of isoflavones compared with soybeans and soy flour.⁹

When an infant has a family history of allergy or ex-

hibits signs of milk intolerance, the physician may recommend a soy-based formula to avert possible allergic reactions. There may also be a role for soy-based formula in recovery from diarrhea,¹⁰ kwashiorkor,¹¹ and colic.¹² Soy-based formulas currently constitute approximately 25% of the U.S. infant formula market¹³ and approximately 10% of some international markets.

The safety of isoflavones in soy-based products, including infant formulas, has been questioned recently because of reports of possible endocrine effects in animals and in cultured cells.^{9,68,77-79,82-86,91-92,98,118,126,127,139} Extrapolation of these observations to human infants, however, is not appropriate, because the biologic activity of isoflavones is species and organ specific as well as age dependent. More importantly, there are no reports to date of endocrine effects in human infants who consume modern soy-based infant formulas. A recent brief review of isoflavones and soy-based infant formulas concludes that any theoretical risks of isoflavones in soy-based infant formulas have not been recognized clinically, and thus remain only speculative.¹⁴ Additionally, no increased incidence of endocrine effects has been documented in infants in Asian populations, whose traditional diets include large amounts of soy products.

Based on a review of the literature on isoflavones from 1946 to 1997, this article focuses on three major areas of interest regarding the potential relevance of infant exposure to isoflavones in soy-based infant formulas. First, what are the levels of isoflavones to which infants consuming soy-based infant formulas are exposed? Second, what is known about the bioavailability of isoflavones to the infant? Third, what endocrine effects (e.g., growth and pubertal/reproductive development), if any, occur from the ingestion of isoflavones in the infant? Areas for possible future research also are identified.

Background

Phytoestrogens, dietary estrogens, and plant estrogens are interchangeable terms for compounds that are similar to naturally occurring steroidal estrogens in structure but are much weaker biologically. Phytoestrogens are present

Dr. Klein is with the Department of Clinical Science, A I duPont Hospital for Children, Wilmington, DE 19899, USA

naturally in many foods of plant origin (e.g., rice, pomegranates, apples, wheat, garlic, oats, coffee, fennel, licorice, barley, parsley, cherries, yeast, potatoes, soybeans, and soy-based products, including animal feeds)¹⁵⁻²² They also have been identified in alcoholic beverages²⁶⁻²⁹

Phytoestrogens are classified into three major categories: isoflavones, lignans, and coumestans. Isoflavones occur principally in legumes (e.g., soybeans) and related food products, the main isoflavones of interest from a dietary perspective are genistein, daidzein, biochanin A, and formononetin. This report uses the term isoflavone to refer to daidzein and genistein, the two principal isoflavones in soybeans; phytoestrogens not found in soy formulas are not discussed.

Isoflavones are chemically similar to endogenous estrogen (estrogen found in the human body), but are much weaker 10,000–140,000 times less potent in animal and in vitro models than estradiol, the major endogenous estrogen found in humans (Table 1)^{18,30-32}. The relative potency of these compounds in animal and in vitro models varies considerably and depends on many factors, including target tissue, functional state of the target tissue, species and age of the subject, route of delivery, dose, length of exposure, and metabolism.^{18,30-32} Furthermore, it is important to note that these wide ranges are derived from studies of cell-culture systems or animal models, rather than from direct effects in humans or high-dose studies in human infants. Potencies of isoflavones in human infants remain unknown. Therefore, caution must be taken in extrapolating this information to infants.

By utilizing methods such as high-performance liquid chromatography (HPLC) and gas chromatography-mass spectroscopy (GC-MS), investigators have measured isoflavones in many foods, including infant formulas, as well as human and animal tissues and fluids (e.g., saliva, blood, urine, and feces). (See the sections below on isoflavone levels.) The excretion of isoflavones has been correlated with diet; vegetarians excrete high concentrations of isoflavones.^{33,34}

Metabolism and Mechanism of Action

The metabolism and disposition of isoflavones is not completely defined in humans.³⁵⁻³⁸ Following ingestion of isoflavone-rich foods, isoflavones are hydrolyzed in the intestinal tract, absorbed in the small intestine and possibly the colon in deconjugated forms, and then undergo conjugation by hepatic enzymes, followed by biliary and urinary excretion. Isoflavones can be deconjugated again following biliary excretion into the intestinal tract, reabsorbed, and further metabolized.^{37,39,40} Thus, unconjugated (free) and conjugated forms of isoflavones circulate in the blood.

Isoflavone hydrolysis and deconjugation in the intestinal tract depends on the presence of intestinal en-

Table 1. Relative Potency of Principal Isoflavones in Soy as Compared with Estrogens^{18,30-32}

Main Endogenous Estrogens:	
Estradiol	1
Estrone	0.5
Principal Isoflavones in Soy	
Genistein	0.0001–0.0008
Daidzein	0.000007–0.00002

zymes as well as bacteria (e.g., lactobacilli, bacteroides, and bifidobacteria)^{36,37} Gut flora changes from infancy to adulthood.⁴¹ The gut is sterile at birth, but within the first week of life it begins to develop flora; lactobacilli predominate only during the first days of life but remain present in the gut of both breast-fed and formula-fed infants. By the end of the first week of life, bifidobacteria are predominant in the breast-fed infant. Although bifidobacteria are found in significant numbers in the formula-fed infant as well, bacteroides also are present. Owing to these individual variations, it is unknown when an infant acquires the flora necessary to metabolize isoflavones. A recent study indicates that 4-month-old human infants can absorb significant levels of isoflavones.⁴² The absolute efficiency of this absorption, however, is unknown, as is the bioactivity of the conjugated forms of isoflavones measured in these infants.⁴³ Even if an infant is able to metabolize isoflavones, other factors, such as transit time, which is decreased in infants, may result in less absorption.

Metabolic pathways for daidzein and genistein have been proposed, based on the isoflavoid metabolites found in human urine, and are shown in Figure 1.³⁸ The authors of the proposed pathways note the individual variability of metabolic response to daidzein that results in either O-demethylangolisin (O-DMA) or equol, a mammalian isoflavone formed by intestinal bacteria. The metabolic fate of daidzein may be of significance, since equol is known to be substantially more estrogenic than both daidzein and O-DMA.³⁵ There are several reports of urinary levels of equol in humans and animal species consuming phytoestrogens.⁴⁴⁻⁴⁶ Plasma equol levels were recently reported in human infants.⁴² (See the further discussion of these data in the section on isoflavone levels in infants.)

Although a detailed discussion of the mechanism of action of isoflavones is beyond the scope of this paper, a general discussion is relevant. Isoflavones can bind estrogen receptors,³⁰ stimulate the production of sex hormone-binding globulin, and inhibit enzymes such as tyrosine protein kinase and estrogen synthetase.⁵¹ Different mechanisms of action for a single phytoestrogen are possible in different species, in different target organs, and at different ages. For example, equol binds to estrogen receptors in the immature (3 weeks old) rat,⁵² and

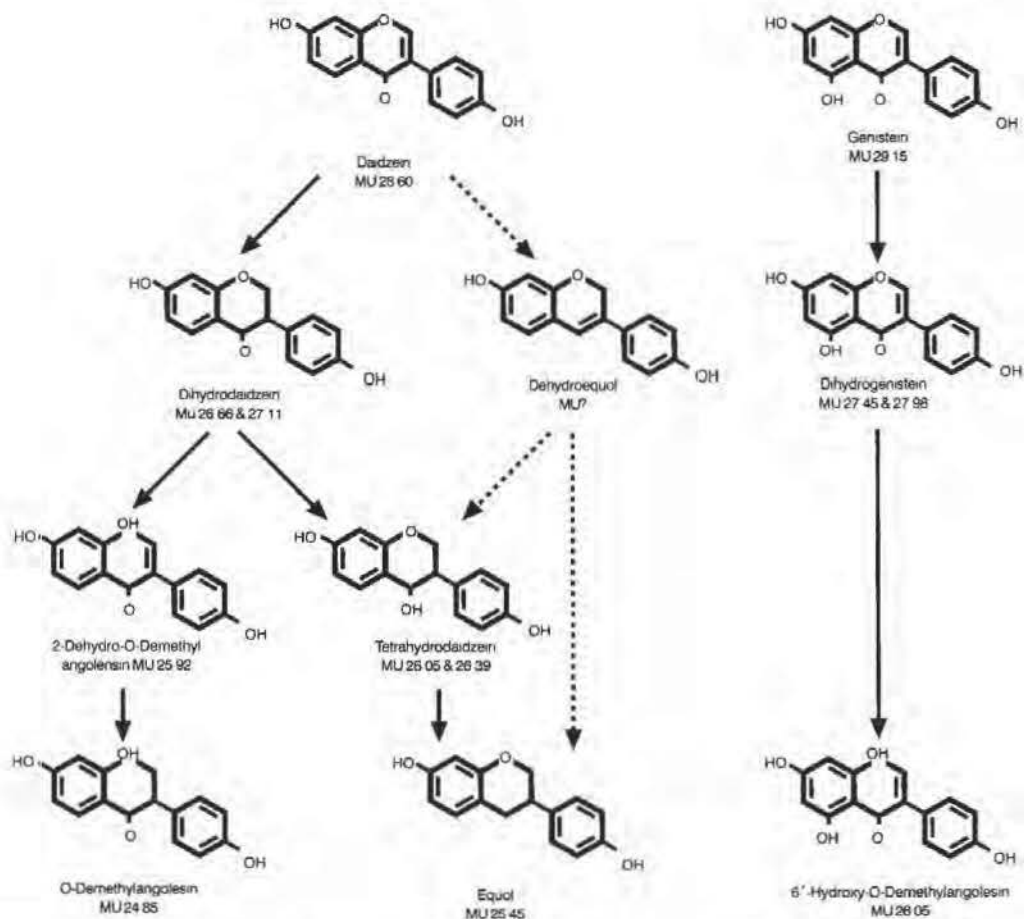


Figure 1 Proposed metabolic pathways for daidzein and genistein. Reprinted with permission of Elsevier Science from Joannou GE, Kelly GE, Reeder AY, et al. *J Steroid Biochem Molec Biol* 1995;54:167-84. ©1995, Elsevier Science, Oxford, England.

genistein and daidzein bind to mature sheep uterine estrogen receptors.⁵³

Through binding to estrogen receptors, isoflavones can either act as endogenous estrogens or antiestrogens, i.e., block the action of estrogens. Estrogenic and antiestrogenic activity is described in rat and mice uteri.^{54,55} Compounds that bind weakly to hormone receptors and serve as weak agonists, such as isoflavones, often have a U-shaped dose-response curve. At low doses of compound, the endogenous hormone is displaced from the receptor by the compound. Because there is less agonist activity, the net hormonal activity is negative, or antiestrogenic. At mid-concentrations of compound, enough hormone is displaced from the receptor, but higher compound levels compensate, yielding little biologic ac-

tivity. At very high doses, such compounds act like the hormone that was displaced. For isoflavones, the specific dose-response curve is unknown and may, in fact, vary among species, at different ages, and in different target tissues. According to this model, because infants have relatively high endogenous estradiol levels, isoflavones may have very little, if any, additional effect.

Isoflavone Levels in Soy-based Infant Formulas, Cow's Milk, and Human Milk

Reinli and Block's 1996 literature review⁵⁶ of the levels of isoflavones in a wide variety of foods cited U.S. infant formula data ranging from 34 to 42 µg/g wet weight (as consumed, equivalent to 34-42 µg/mL) total isoflavones. Also reporting in 1996, Knight et al.⁵⁷ found much lower

levels of isoflavones in Australian soy formulas, ranging from 4 to 20 µg/g dry weight, however, neither the methodology for isoflavone determination nor information about the soy formula (e.g., percentage of soy isolate and caloric content) was reported. By using methods that discriminated among the various conjugates, Setchell et al.⁴² reported in 1997 data for U.S. soy-based infant formulas ranging from 32 to 47 µg/mL total isoflavones (4.8–6.9 mg/100 kcal, assuming 20 kcal/fluid ounce of formula). Also in 1997, Murphy et al.³⁸ reported total isoflavone levels in U.S. infant formulas ranging from 25 to 30 µg/mL of reconstituted formula, the researchers adjusted total isoflavones for their molecular weight differences and expressed them as the free isoflavone forms (i.e., totals of genistein, daidzein, and glycitein), which they said may explain the lower values compared with other reported infant formula data.³⁸

Using his data on isoflavone levels in soy-based infant formulas, Setchell and his colleagues⁴² estimated infant exposure to isoflavones. Based on a 4-month-old infant's daily intake of approximately 900–1000 mL liquid (about 32 oz ready-to-feed or 120 g powdered soy infant formula) and containing 32–47 µg/mL isoflavones, the infant's estimated isoflavone exposure would be approximately 32–47 mg/day, or 5–8 mg/kg body weight/day. With Setchell's calculations and estimates of isoflavones' relative potency (10,000–140,000 times less than estradiol) based on model systems, an infant theoretically may be exposed to the equivalent of 0.2–4.7 µg estradiol/day from the ingestion of isoflavones.⁴² For comparison, this level of exposure would be similar to that received from ingestion of from one one-hundredth to one-fifth of an oral contraceptive pill, not several contraceptive pills, as has been suggested.³⁸ It is important to note that this type of comparison is highly speculative, and its appropriateness is questionable. It is provided only to correct inaccurate information reported in the lay press, no peer-reviewed

scientific articles have published such comparisons.

Furthermore, it is important to remember the inherent limitations in the above comparisons. Estimated estrogenic potencies for isoflavones have been derived from cell-culture systems or animal models and, thus, may not be relevant to human infants. Additionally, most of the isoflavones in soy formulas are in the conjugated (bound) form,⁴² which may be less biologically active than unconjugated (free) forms. Thus, biologic activity may not be assumed from the presence of isoflavones alone.

Isoflavone levels in soy-based infant formula have been compared with estradiol levels in human milk and cow's milk. Estrogen levels in human milk are high at birth and decrease rapidly during the first month.^{40–42} Human milk estrogen levels have been reported as high as 39 pg/mL for estradiol and 1177 pg/mL for the estrone glucosiduronate (one-half the potency of estradiol).⁵³ Another study reports much higher levels in human colostrum—0.5 ng/mL (500 pg/mL) for estradiol and 4–5 ng/mL (4000–5000 pg/mL) for estrone—and cow's milk, with estradiol and estrone concentrations at 4–14 pg/mL and 34–55 pg/mL, respectively. By the fifth postpartum day, however, levels of estrogen in human milk and cow's milk were similar.⁶⁴

Infant Isoflavone Levels Compared with Endogenous Estradiol Levels

Data on isoflavone levels in infants are limited, owing partially to ethical considerations that restrict the collection of body fluids/tissues (e.g., blood) and require invasive techniques. Available data vary considerably and are summarized in Table 2.^{42,65}

Genistein and Daidzein

Plasma concentrations of genistein and daidzein in infants fed soy-based infant formulas were recently reported to be significantly greater than in infants fed cow's milk—

Table 2. Isoflavone Levels in Infants Fed U.S. Soy-based Formula, U.S. Milk-based Formula, or Human Milk

Author	Fluid Measured	n	Age	Genistein (ng/mL)	Daidzein (ng/mL)	Equol (ng/mL)
Infants fed U.S. soy-based infant formula						
Setchell et al (1997) ⁴²	Plasma	7	4 mo	684 ± 443	295 ± 59.9	2.0
Venkataraman et al (1993) ⁶⁵	Urine	8	2 mo	26,451 ± 8559	25,399 ± 8081	6.4 ± 6.4
		7	4 mo	8758 ± 3808	17,577 ± 5452	1.5 ± 1.5
Infants fed U.S. milk-based infant formula						
Setchell et al (1997) ⁴²	Plasma	7	4 mo	3.2 ± 0.7	2.1 ± 0.3	4.1 ± 0.5
Venkataraman et al (1993) ⁶⁵	Urine	5	2 mo	205 ± 52	155 ± 64	42.6 ± 2.3
		6	4 mo	536 ± 393	706 ± 555	40.5 ± 3.3
Infants fed human milk						
Setchell et al (1997) ⁴²	Plasma	7	4 mo	2.8 ± 0.7	1.5 ± 0.1	0.5
Venkataraman et al. (1993) ⁶⁵	Urine	5	2 mo	1284 ± 1089	697 ± 653	2.9 ± 2.9
		5	4 mo	161 ± 108	179 ± 80	59 ± 42

based formulas or human milk.⁴² Similarly, significantly higher levels of urinary genistein and daidzein have been reported in a limited number of human infants consuming soy-based infant formulas than in infants consuming human milk or milk-based infant formulas. However, there is a wide range of overlap in the individual values.⁶⁵

Equol

Venkataraman et al.⁶⁵ reported similar urinary equol levels in 2- and 4-month-old infants fed cow's milk-based or soy-based formulas, but significantly different levels for 2-month-old versus 4-month-old breast-fed infants.⁶⁵

Setchell et al.⁴² reported higher plasma equol levels in infants consuming cow's milk-based formula versus infants consuming soy-based formula and human milk. He commented that the highest concentration of equol in infants fed cow's milk-based formula is explained by the presence of isoflavones in cow's milk. Because equol is a mammalian isoflavone formed from daidzein by intestinal bacteria, the lower urinary levels of equol in infants fed soy-based formulas and human milk may be due to lack of appropriate intestinal microflora or to inactivity of the metabolic enzymes.

Endogenous Estrogen Levels

Endogenous estrogen levels peak by 4 months of age in young infants.⁶⁶ Endogenous estradiol levels can be as high as approximately 8 ng/dL (80 pg/mL) in 2- to 4-month-old infant girls and approximately 4 ng/dL (40 pg/mL) in 2- to 4-month-old infant boys.

The statement by Setchell et al.⁴² that isoflavone levels in soy-fed infants are 13,000–22,000 times higher than

endogenous estrogen levels at the same age deserves further comment. Isoflavones are much less potent (14,000–100,000 times weaker than endogenous estrogens based on model systems), so the relative theoretical potency of isoflavone levels in the soy-fed infant is comparable to endogenous estrogen levels in all infants. Although data on infant isoflavone levels support the absorption of isoflavones by the infant, these data do not indicate any biologic or clinical effect of the isoflavones on the infant. In addition, endogenous estradiol levels are not reported for the same infants. Of particular relevance is that it is unknown whether isoflavones consumed by an infant fed soy formula contribute estrogenic activity (perhaps in addition to endogenous estrogen), suppress endogenous estrogen levels, or have no effect.

Endocrine Effects of Isoflavones

Animal Studies

The effects of isoflavones on reproductive organ functions depend on the species and age studied, the end-point measured, and the dose, route, and duration of administration.^{67,68} For example, selected nonhuman studies of sheep,^{69–75} mice,⁷⁶ rats,^{30,32,77–83} and cheetahs^{86,87} have demonstrated that both effects and noneffects on fertility and sexual behavior may be associated with isoflavone consumption. Table 3^{35,71,75–77,81,86,88–92} shows the variability of effects and further emphasizes the inappropriateness of extrapolating animal studies to humans.

Peripubertal monkeys, the animal model theoretically closest to the human, experienced no effect on uterine, prostatic, or testicular weight when given total isoflavones

Table 3. Variability in Isoflavone Sensitivity: Examples of Species- and Organ-specific Effects

Species	Age	Dose	Duration	Effect	Reference
Sheep	Adult	Variable	Several seasons	Infertility	Bennetts et al. (1946) ⁷⁵
Sheep	Adult	Variable	Several seasons	↑ Uterine wt	Bennet et al. (1967) ⁷¹
Mouse	Adult	0.5–1.0 mg G	Once	No ↑ estrogen activity	Shutt (1967) ⁸⁸
Mouse	3–4 weeks	800–5000 µg G	3 days	↑ Uterine wt and antiestrogenic	Folman & Pope (1966) ³⁵
Mouse		10 mg G	Once	↑ Uterine wt	Wong & Flux (1962) ⁷⁶
Mouse CD-1		12 mg G	Once	No ↑ uterine wt	Farnakidis & Murphy (1984) ⁸⁹
Rat	Neonatal	1000 µg G	Once	↓ Adult GnRH	Faber & Hughes (1991) ⁷⁷
Rat	Neonatal	100 µg G	Once	↑ Adult GnRH	Faber & Hughes (1991) ⁷⁷
Rat	20–30 days	800 µg G	Once	↑ Uterine wt.	Kitts et al. (1980) ⁸¹
Cheetah	Adult	50 mg D & G	Per day	Infertility	Setchell et al. (1987) ⁸⁶
Monkey	Peripubertal	9.41 mg/kg BW D & G	6 mo	No ↑ uterine wt, no hormone ▲	Anthony et al. (1996) ⁹⁰
Human	Premenopausal	45 mg/d I	1 mo	↓ LH	Cassidy et al. (1994) ⁹¹
Human	Postmenopausal	165 mg/d I		No ▲ SHBG, LH, FSH	Baird et al. (1995) ⁹²

Note: BW = bodyweight, D = daidzein, G = genistein, I = isoflavones, SHBG = sex hormone-binding globulin, LH = luteinizing hormone, FSH = follicle stimulating hormone, ▲ = change, ↑ = increase, ↓ = decrease.

in the amount of 9.41 mg/kg body weight daily for 6 months.⁹⁰ The monkeys also had no change in estradiol, testosterone, thyroxine, or sex hormone-binding globulin levels. It is important to note that the dose per body weight (9.41 mg/kg) received by the monkeys⁹⁰ is higher than the estimated maximum infant exposure from soy formula (5–8 mg/kg body weight).⁴⁷

Sheep need a prolonged exposure (two grazing seasons) before the hypothalamus is inhibited.⁷⁵ The classic report of “clover disease” in sheep⁷³ showed a relationship between clover consumption, increased uterine weight, and infertility. Further study of this report suggested that the increase in uterine weight was not directly related to the amount of genistein ingested, but that the formononetin content of the clover was more important.⁹¹ The ewes in this study were of fertile age when these changes occurred. In contrast, the human infant consumes soy-based infant formula many years before she is expected to be fertile. Another report suggests that changes in sexual behavior of ewes are too slight to account for the infertility.⁹¹ To further illustrate species variability, cattle are less sensitive to isoflavones than are sheep.⁹⁵

In mice, genistein has been reported to cause uterine hypertrophy after a single dose of 10 mg.⁷⁶ However, in another study, a higher dose of 12 mg genistein in CD-1 mice caused no change in uterine weight.⁸⁹ This difference may reflect a difference in strain, i.e., the CD-1 mouse strain is not affected at the same exposure level as is the B6D2F1 mouse strain. Uterine hypertrophy from estrone and estradiol was inhibited in 3–4-week-old mice receiving 800–5000 µg/day genistein for 3 days.⁵⁵ Various mouse strains respond differently to the estrogenic activity of genistein, as measured by uterine weight increase after exposure to isoflavones.^{89,96} Other changes from isoflavone administration in mice include altered vaginal cornification,⁹⁷ genital tract changes,^{98,99} and decreased ovulation and increased embryo degeneration.¹⁰⁰ There have been no descriptions of change in the timing of the onset of puberty.

Genistein administered subcutaneously at doses of 0.5–10 mg had no effect on a bioassay of uterus and vagina in mice.⁸⁸ The higher dose of 10 mg decreased the effects of estradiol by 54% in the same assay. This is consistent with a weak estrogen displacing estradiol and leading to a net negative effect.

Neonatal rats consuming 1000 µg genistein experienced a decrease in gonadotropin-releasing hormone (GnRH) response as adults, whereas rats receiving 100 µg genistein experienced an increase in luteinizing hormone (LH) response to GnRH as adults.⁷⁷ This illustrates that the direction of the effect is dose and age dependent. Rat uterine weight was increased in 20–30-day-old animals consuming 800 µg genistein.⁸¹ Neonatal exposure to genistein (100–1000 µg/day for 10 days) alters the sexually dimorphic nucleus of the preoptic area of the brain in

adult rats.⁷⁷

Infertility and liver disease were described in cheetahs after they received 50 mg/day daidzein and genistein.⁸⁶ This study concluded that dietary isoflavone may be one of several influences on the decreasing fertility of the cheetah. The cheetah is extraordinarily susceptible to isoflavone consumption, because cats in general poorly conjugate steroids in the liver, which means that more unconjugated (free and thus bioactive) isoflavones are circulating in the cheetah.⁸⁶

One report suggests that decreased reproduction/fertility in parrots is due to consumption of feed containing high levels of isoflavones.⁷⁹ However, the effects on the parrots have not been directly linked to isoflavones.

As with many toxicity studies, there can be effects seen in animals that are not seen in humans. However, many of the effects can be addressed indirectly. For example, if the human pubertal GnRH response were altered by infant ingestion of soy-based formula, there should be an alteration in the timing of puberty or a change in fertility. Neither of these effects has been reported in humans.

Human Studies

As previously noted, it is clear from the literature that different species and different tissues are affected by isoflavones in markedly different ways. It is difficult to know which tissues, if any, are affected in infants, and the variation among species makes extrapolation to infants inappropriate.

Endogenous estrogens in humans stimulate uterine cells to increase in size and number, affect breast development and lactation, stimulate linear growth and epiphyseal maturation and eventually epiphyseal fusion, and may alter lipoprotein metabolism. Synthetic estrogens (estrogen compounds made by man) can produce similar effects and cause gynecomastia in men and children.^{101–103} Gynecomastia has not been reported in infants who consume soy-based infant formulas.

Growth

Infants fed soy-based infant formulas grow and develop normally. There are many reports of large numbers of term infants consuming either soy-based infant formula, cow's milk-based infant formula, or human milk in which all three groups have equivalent rates of linear growth, growth velocity, weight gain, and head circumference growth.^{104–114} Bone mineral content is similar in infants who consume soy-based formula versus infants who consume human milk or cow's milk-based formula.^{5,115,116}

Pubertal/Reproductive Development

The hypothalamic-pituitary-gonadal axis is suppressed by high levels of estrogen. This axis is responsible for pubertal development in adolescents and for fertility in adults. Its role during infancy has not been completely elucidated.

Endogenous estradiol levels can be as high as 8 ng/dL (80 pg/mL) in 2–4-month-old infant girls and 4 ng/dL (40 pg/mL) in 2–4-month-old infant boys.⁶²

Known influences of hormones on sex organ development occur before birth. Because disturbed development of sex organs owing to hormonal influences occurs in utero, foods consumed during infancy are not relevant to sex organ development.

There have been no reports of infants fed soy-based formulas developing breast buds. Breast enlargement in an infant ingesting human milk from a mother on oral contraceptives has been reported.¹¹⁷ If isoflavones are absorbed in a significant amount and yet do not cause breast development or increased growth rate, both of which are accepted as early indicators of estrogenic effect in human children, it is very unlikely that they cause any other acute or delayed adverse endocrine effects.

It is unknown whether isoflavones consumed by an infant fed soy formula contribute estrogenic activity (perhaps in addition to endogenous estrogen), suppress endogenous estrogen levels, or have no effect. During infancy, there is no pubertal or reproductive development, so it would be unlikely that later puberty or fertility would be affected by infant exposure to isoflavones. Inhibition of sexual maturation has not been studied in humans who consumed soy-based formulas as infants, but there is no evidence that puberty does not follow the normal timing of onset and progression in these individuals.

Studies of adults have shown that age is an important determinant of the effects of isoflavone consumption in women. Studies of premenopausal women have reported influences on the menstrual cycle.^{91,118} In an uncontrolled study, GnRH-stimulated LH and follicle stimulating hormone (FSH) levels were suppressed and follicular phase length was increased in six premenopausal women who ingested a daily diet containing 60 g soy-based protein (45 mg isoflavones/day) for 1 month.⁹¹ Because luteal phase length was not altered, neither ovulation nor fertility should have been altered, although this was not directly measured in the study.

Ninety-one postmenopausal women were studied after ingesting a diet containing soy-based foods or a usual diet for 4 weeks. The soy diet provided a daily intake of 165 mg isoflavones. Although anticipated, no estrogenic effects were seen in the liver or pituitary as measured by no change in sex hormone-binding globulin or gonadotropin levels. The overall vaginal maturation index did not differ between the groups, but the percentage of vaginal superficial cells increased slightly (19% of those on the soy diet compared with 8% of the controls) ($p=0.06$).⁹² These doses are similar on a body weight basis to the doses consumed by infants fed soy formulas.

An increased incidence of male reproductive tract disorders, including reduced sperm counts over the last 50 years, has been alleged.¹¹⁹ Decreased sperm count is sug-

gested, but not scientifically proven, to possibly be the result of increased exposure to dietary estrogens.¹²⁰ Other recent studies, however, have shown no changes in sperm counts over the past 20–25 years and have demonstrated that there are significant regional differences in sperm counts.¹²¹ Furthermore, there are no epidemiologic data to suggest a higher incidence of decreased sperm count or other fertility problems in populations consuming traditional diets that include large amounts of soy products.

Thyroid Function

Goiter in infants who consumed soy formula was reported prior to the use of modern soy protein isolate formulas.^{122–125} One case report of an infant with congenital hypothyroidism and persistent elevation of thyroid-stimulating hormone (TSH) on soy formula has been reported.¹²⁶ Two infants with congenital hypothyroidism were described to have increased thyroxine levels after discontinuation of soy formula, and one infant was described to have an elevated TSH level until discontinuation of soy formula.¹²⁷ This increased TSH level was attributed to decreased absorption of thyroid hormone replacement by infants consuming soy-based formulas.

Other

It is important to note that the large body of literature supports a beneficial role for isoflavones, particularly as a protective agent against cancer. There are several reviews of the relationship between isoflavone intake and cancer risk.^{128–131} There are many reports of the protective role of isoflavones against breast cancer.^{91,132–137} Additionally, the higher levels of isoflavones in Japanese men relative to American and European men has been correlated to Japan's low mortality rate for prostate cancer.¹³⁸ Primate resistance to hormonal manipulations to induce mammary and genital carcinoma may be related to high isoflavone levels in the diet.¹³⁹ Tumor cell lines are responsive to isoflavones.^{140,141} For example, genistein stimulates sex hormone-binding globulin production and inhibits Hep-G2 cancer cell proliferation. Consequently, there is interest in using isoflavones (e.g., genistein) for cancer chemoprevention.¹⁴²

In addition to a protective role in cancer prevention, isoflavones have been shown to have other beneficial effects. Cholesterol levels are decreased in infants and adults with increased consumption of soy products.^{143–148} Prostatitis is decreased in rats on soy diets.¹⁴⁹ Hot flashes are reported to be decreased in postmenopausal women.¹⁵⁰ Isoflavones also inhibit angiogenesis, and thus may protect against certain chronic diseases.^{151,152}

Conclusions

Soy-based infant formulas contain isoflavones that are absorbed and metabolized by infants consuming these products, although absorption and metabolism varies

greatly among individuals. The presence of isoflavones in human infants does not imply biologic or clinical activity. Equivalent estrogen exposure from isoflavone ingestion, and biologic or clinical effects from this exposure, if any, remain unknown. Additionally, endogenous estrogen activity in all infants is high for the first few months of life. Based on model systems and pharmacologic principles, isoflavones consumed by infants fed soy formula could theoretically contribute estrogenic activity (perhaps in addition to endogenous estrogen), suppress endogenous estrogen levels, or have no effect. The lack of reported effects on millions of infants consuming isoflavones in soy formulas suggests there are no biologic or clinical effects.

Although large doses of isoflavones have estrogenic effects in some animals, no parallel effects in human infants have been reported. Furthermore, it is not appropriate to extrapolate animal observations to human infants, because it is well documented that effects of isoflavones are dependent on numerous factors including species, age, dose, duration of exposure, metabolism, and individual variability.

Any long-term effects on humans from infant consumption of isoflavones in soy-based infant formulas would be expected to be similar to known estrogenic effects. Therefore, the relevant areas of interest include growth and pubertal and reproductive development. It has been well documented that infants fed soy-based infant formula grow and develop normally. There have been no reports of abnormal pubertal development in adolescents who received soy-based formula as infants. Similarly, there have been no reports of infertility in adults who consumed soy-based formula as infants.

The literature offers no evidence of estrogenic effects from isoflavones consumed by millions of infants fed modern soy-based infant formulas. Growth is normal and no changes in the timing of puberty or in fertility rates have been reported in humans who consumed soy formulas as infants. Consequently, soy-based infant formulas continue to be a safe, nutritionally complete feeding option for most infants.

Future Research

Because no endocrine effects have been reported with millions of infants consuming modern soy-based infant formulas, the incidence of endocrine effects, if any, must be extremely low. Human studies investigating possible biologic effects of isoflavones are feasible. Infants consuming soy infant formulas could be examined for breast development, advanced bone maturation (bone age determination), and thyroid goiter, and could have blood sampled for estradiol, LH, FSH, thyroxine, thyroid antibodies, and TSH levels. Adults who consumed soy infant formula as infants could be studied retrospectively, possible end-

points are fertility rate, incidence of precocious puberty, adult height relative to parents' heights, and sperm counts.

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Early Flavor Experiences: Research Update

Julie A. Mennella, Ph.D., and Gary K. Beauchamp, Ph.D.

Anyone who has observed infants for any period of time can testify to the intense activity occurring in and around their mouths—the primary site for learning in the first few months of life. Before they are even able to crawl, infants have learned much about their new sensory world. Through recent research we have begun to explore the impact of these early experiences on infants' acceptance of solid foods and how they explore objects in their environment. We have also begun to focus on the sensory experiences of the formula-fed infant, in particular, how their responses to particular formulas, which are extremely unpalatable to older children and adults, change during infancy. This is a relatively new and exciting area of study, with much research yet to be done. It is clear, however, that infants are not passive receptacles for flavored foods. Parents who offer a variety of foods will provide both a nutritious, well-balanced diet, as well as an opportunity for their children's own personal preferences to develop.

Introduction

Anyone who has observed infants for any period of time can testify to the intense activity occurring in and around their mouths—the primary site for learning in the first few months of life. During feeding, or while mouthing objects such as their hands and toys, infants use the sense of touch to discriminate between textures, and the senses of taste and smell to discriminate flavors. Before they are even able to crawl, infants have learned much about their new sensory world.

When we first explored the topic of early flavor experiences in *Pediatric Basics* (No. 65, Summer 1993), we knew that the sensory world of the infant was different than that of older children and adults. We also knew infants had an ability to detect some tastes and not others,

Dr. Beauchamp is Member and Director, and Dr. Mennella is Associate Member, Monell Chemical Senses Center, Philadelphia, PA 19104-3308, USA. This article is reprinted with permission of *Pediatric Basics*, Vol. 82. © 1998 Gerber Products Company.

and that breast-fed infants experienced a variety of flavors in their mothers' milk.

Through recent research we have begun to explore the impact of these early experiences on infants' acceptance of solid foods and how they explore objects in their environment. We have also begun to focus on the sensory experiences of formula-fed infants, in particular, how their responses to particular formulas, which are extremely unpalatable to older children and adults, change. Before we discuss infants' early flavor experiences, we must establish a basic understanding of taste and smell, the differences between them, and how they interact and integrate to produce an overall impression of flavor.

What Is Flavor?

The "flavor" we experience while eating foods is a product of two frequently confused chemical senses: taste and smell. Taste refers to the sensation occurring when chemicals stimulate taste receptors on the tongue and other parts of the oropharynx (Figure 1). The taste stimuli that interact with these receptors are often separated into a small number of "primary" tastes: sweet, salty, bitter, sour, and perhaps savory, the taste of *umami* or monosodium glutamate.

Smell, on the other hand, occurs when chemicals stimulate olfactory receptors located on a relatively small

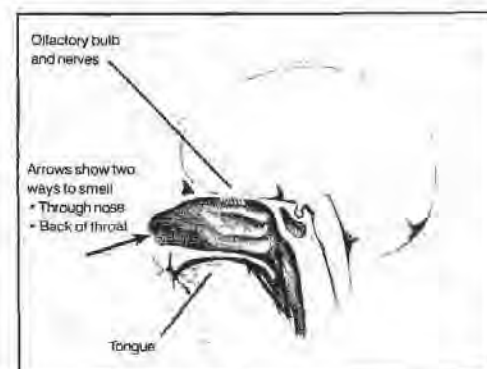


Figure 1. Orthonasal and retronasal routes of flavor perception.

patch of tissue in the nasal cavity. Unlike the sense of taste, there may be many different classes of odor stimuli, perhaps thousands. Odors can reach their receptors in two ways: they can enter the nostrils during inhalation (orthonasal route) or they can travel from the back of the nasopharynx toward the roof to the nasal cavity (retronasal route) during sucking in infants or chewing and swallowing in older children and adults (Figure 1)

Retronasal olfaction, the aroma of substances we put in our mouths, contributes significantly to the complexity of flavor. This is clearly noted by head cold sufferers who lose the ability to discriminate common foods when olfactory receptors are blocked. Similarly, foods often "taste" better after a person quits smoking perhaps because their sense of smell has improved,¹ allowing them to detect more subtleties of flavor. The role of smell in flavor is critical in distinguishing the flavor of strawberry from cherry, and in enjoying foods containing licorice, vanilla, and citrus.

It should be noted that other properties of food (e.g., texture, temperature, irritation) are also very important to its perceived flavor. However, little experimental work has been done in this area of infant flavor perception. Therefore, in this article we will focus on the infant's senses of taste and smell.

Developing Sensitivities and Preferences

The sensory world of the young infant differs from that of the adult in that the infant's sense of taste develops over time. Specifically, *sweet* responses are evident prenatally, and major changes are not known to occur postnatally. Similarly, the rejection of *sour* taste is evidenced from birth onwards, although it is clear that children often come to like very sour substances. How this happens is not known. *Salt* and *bitter* sensitivities appear to change postnatally, and the limited research on *umami* taste suggests that preferences are evident during infancy, but the context in which the tastant is experienced is critical.

Less is known about how olfactory perceptions and preferences change over time. Clearly, infants are able to detect and discriminate among a wide variety of odors shortly after birth. Although it is not yet known whether they hedonically respond to differences in odor quality, that is, which odors infants find pleasant, research has shown that newborns appear to be as sensitive to odors as adults, if not more so, and are capable of retaining complex olfactory memories.² When older, they will explore a scented toy differently than an unscented one and the way they respond to the scented toys is influenced by the amount of exposure they have had with that particular scent.³ These findings suggest that infants are able to detect and retain information about the chemical features of their environment.

Fetus and Premature Infants

Studies on Taste

Although amniotic fluid and embryonic membranes provide a series of barriers that protect the fetus from disturbances from the outside world, the fetus is nonetheless exposed to a variety of chemosensory stimuli in utero. The composition of amniotic fluid varies over the course of gestation, particularly as the fetus begins to urinate. By term, the fetus is actively swallowing almost a liter of amniotic fluid a day and has been exposed to a variety of substances including glucose, lactic acids, urea, amino acids, proteins, and salts.^{4,5} The apparatus needed to detect these stimuli, the taste buds, make their first appearance around the 7th or 8th week of gestation, and by 13 to 15 weeks, they begin to resemble those of the adult.⁶ Based on anatomical studies and studies on other animal species, they are presumably functional by the third trimester.

Studies on taste sensation in the preterm infant are rare, due in part to methodologic limitations. When preterm infants who had been fed exclusively via gastric tubes were presented with minute amounts of either glucose or water solutions intraorally, they exhibited more nonnutritive sucking in response to the glucose than the water.⁷

In another study using a methodology that embedded taste substances in a nipple-shaped gelatin medium,⁸ infants born preterm and tested between 33 and 40 weeks postconception produced more frequent, stronger sucking responses when offered a sucrose-sweetened nipple compared with a latex nipple. The results from these studies indicate that, prior to birth, the human infant possesses a sensory apparatus that can detect sweet tastes.

Studies on Smell

New research also confirms that the environment in which the fetus lives—the amnion—is indeed odorous. The odor can indicate certain disease states (such as maple syrup disease,⁹ phenylketonuria,¹⁰ and trimethylaminuria¹¹) or the types of foods eaten by the pregnant mother.¹² That the amniotic fluid and the newborn's body can acquire the odor of a spicy meal the mother ingested prior to giving birth suggests that odorous compounds in her diet can be transferred to the amniotic fluid.

This has been experimentally demonstrated in a recent study in which amniotic fluid samples were obtained from pregnant women who were undergoing routine amniocentesis and who ingested either garlic or placebo capsules approximately 45 minutes before the procedure.¹³ As expected, the odor of amniotic fluid obtained from the women who ingested the garlic, as determined by adult human evaluators, was judged to smell stronger or more like garlic than the amniotic fluid from the women who did

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not consume the garlic

Because the normal fetus swallows significant amounts of amniotic fluid during the latter stages of gestation¹⁴ and has open airway passages that are bathed in amniotic fluid,¹⁵ the fetus may be exposed to a unique olfactory environment. Studies on other animals reveal that young and adult animals prefer certain odors that were experienced in utero.¹⁶ Whether similar mechanisms are operating in humans remains unknown. However, a recent study revealed that newborns can detect the odor of amniotic fluid and that they prefer the odor of their own amniotic fluids for at least the first few days of life.^{17,18}

Newborns

Studies on Taste

Facial expressions, which suggest contentment and liking or discomfort and rejection, have been used to assess the newborn's responsiveness to taste stimuli in some of the earliest investigations on human taste development (Figure 2). During the first few hours of life, infants display relatively consistent, quality-specific facial expressions when the sweet tastes of sucrose (facial relaxation, followed by positive mouth gaping), the sour taste of concentrated citric acid (lip pursing and facial grimace), and the bitter taste of concentrated quinine and urea (tongue protrusion and grimace) are presented into the oral cavity.¹⁹⁻²¹ No distinct facial response is evidenced with salt taste, however. Infants also display distinct positive facial expressions, similar to those observed with sweetness, when tasting soup to which monosodium glutamate (MSG) has been added when compared to the soup diluent alone.²² MSG alone does not appear to elicit those facial responses, however, raising the question of exactly what it is about the MSG-flavored soup that is preferred.

Intake studies, which compare how much an infant

consumes of a taste solution and a diluent solution during brief presentations, are the most frequent method used to evaluate taste preferences. Generally, intake studies use weaker concentrations of taste stimuli than studies on facial expressions. If an infant ingests more of the taste solution than a diluent, for example, one can infer that (a) the infant can detect the taste and, with less certainty, (b) the infant prefers or likes the tastant more than the diluent.

Consistent with the findings for premature infants, research has repeatedly demonstrated strong acceptance of sweet-tasting sugars by newborn infants. Within days after birth, infants are quite sophisticated sweet connoisseurs. They can detect even dilute sweet solutions and can differentiate varying degrees of sweetness and different kinds of sugars.²³ In addition, as was the case for premature infants, newborns will suck more in response to sweet stimuli.^{8,24} It is of interest to note that one of the most predominant taste qualities of the first food of all mammals, mother's milk, is its sweetness.

In addition to a preference for sweet tastes, infants also show physiologic responses to them. A small amount of a sweet-tasting liquid placed on the tongue of a crying newborn exerts a rapid, calming effect which persists for several minutes.^{25,26} The rapid onset of analgesia, which has been observed during such painful procedures as blood sampling and circumcision, suggests that afferent signals from the mouth, rather than gastric or metabolic changes, are responsible for such effects.

In contrast to the innate preference for sweet tastes, newborns reject the sour taste of citric acid.²⁷ Because only a handful of studies on sour tastes have been conducted with newborn infants, it is not known whether there are developmental changes in sensitivity or preference for sour-tasting fluids.

Conclusions regarding the neonate's response to bitter and salty tastes are more problematic and further re-

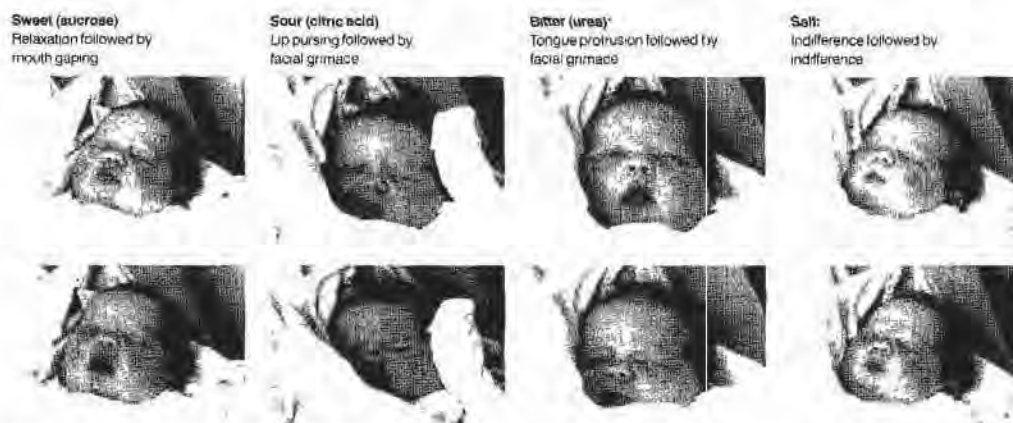


Figure 2. Sequence of facial expressions elicited from 3-day-old infant

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search is needed. Newborns respond with highly negative facial expressions to concentrated quinine and urea (Figure 2), but they do not reject moderate concentrations of urea.²¹ The reason for this difference remains unclear. Perhaps the newborn can detect bitter substances, but the ability to reject a substance or modulate intake will come as the infant matures. With regard to salt taste, studies measuring intake and facial expressions suggest that the newborn infant is indifferent to and may not detect salt. However, salt does appear to suppress some parameters of sucking in newborns. No studies suggest that the taste of salt is attractive to the newborn infant, however.

Although each measure has its limitations, the convergence of research findings supports the conclusion that the ability to detect sweets is evident very early in human development and that its hedonic tone—that is, its pleasantness—is also well developed at birth. It is likely that the innate preference for sweets and rejection of bitter tastes in humans is a consequence of selection, favoring animals who consumed high-energy, vitamin-rich fruit and vegetable diets, while avoiding bitter, poisonous fruits and plants. Although the preference for sweet tastes appears to be innate and persists throughout childhood, experience may also play an interacting role in development. However, contrary to popular beliefs, there is no scientific evidence in humans that variations in early exposure to sweets permanently alter the preference for sweet-tasting foods.²⁸

Studies on Smell

Odor preferences in newborns are more difficult to assess. However, we do know that, shortly after birth, human infants are able to detect a wide variety of odors, with perhaps the most salient of these odors originating from the mother. Within hours after birth, mothers and infants can recognize each other through the sense of smell alone. Day-old breast-fed infants spend more time orienting toward a breast pad previously worn by their lactating mothers than one worn by an unfamiliar lactating woman.²⁹⁻³¹ They move their head and arms less, suck more, and cry less when they are exposed to their mother's odors.^{32,33}

This ability of breast-fed infants to discriminate the odors of their mothers from those of other lactating women is not limited to odors emanating from the breast region, since they can also discriminate odors originating from their mother's underarms and neck. Interestingly, newborns preferred their mother's breast unwashed as compared to when it had been thoroughly washed and thereby less odorous.³⁴ Before the sense of sight is well developed, the recognition and preference for mother's odors may play an early role in guiding the infant to the nipple area and facilitating early nipple attachment and breast feeding.

Because bottle-fed infants do not discriminate their mothers' odors from those of an unfamiliar bottle-feeding mother,^{31,32} it has been suggested that breast-fed infants

are able to discriminate these odors because they, unlike bottle-fed infants, have prolonged periods of skin contact with their mothers and their nostrils are in close proximity to their mother's breasts and underarms during feeding. Recent studies, however, suggest that bottle-fed infants also prefer the breast odors of unfamiliar, lactating women.³⁵ Therefore, breast odors, or the volatile components of breast milk, may be particularly attractive to all newborns.

Older Infants

Studies on Taste

Babies beyond the neonatal period (one to 24 months) have been most neglected in studies on taste. Nonetheless, a few notable findings suggest that changes in taste responses occur during this time in development.

While newborns rejected concentrated bitter-tasting solutions (urea), more recent studies revealed that relatively low concentrations of urea were not rejected in newborn infants, but rejection was evident among infants who were 14 to 180 days of age.³⁶ This is consistent with the idea that there is an early developmental change in bitter perception or the ability to regulate the intake of bitter solutions. As a practical matter, it could explain why older infants reject bitter-tasting foods, like green vegetables. Parents can expect a "learning period" when introducing these foods, and anticipate a need to introduce them slowly, but consistently. With exposure, eventually these foods may be tolerated and even enjoyed.

Developmental shifts in salt acceptability have also been demonstrated in several research studies. While newborn infants are indifferent to or reject salt relative to plain water, the preference for salt water relative to plain water first emerges at approximately 4 months of age.³⁷ Experience with salty tastes does not appear to play a major role in this shift from indifference or rejection of salt at birth to acceptance in later infancy.³⁷ Rather, this change in response may reflect postnatal maturation of central and/or peripheral mechanisms underlying salt taste perception, as has been demonstrated in animal model studies.³⁸ Thus, the preference that emerges at 4 months appears to be largely unlearned.

Research has also revealed that young children undergo another developmental shift in their preference for salt taste. By 18 months of age, children begin rejecting salted water and become more adult-like in their preferences, they begin exhibiting robust preferences for salt in soup and other foods such as carrots or pretzels.³⁹ In other words, the same level of saltiness may elicit either a positive or negative response depending on the medium in which salt is presented to the child. These studies underline the importance of sensory context in perceived pleasantness and preference.

Early Experience and Preference for Salt Taste

Although there is no evidence that high salt intake in infancy influences later preferences, there are data suggesting that the opposite is true. Several human studies have been conducted and were stimulated by a series of animal model studies that demonstrated that early alterations in sodium balance alter long-term salt preference behavior.^{38,40} Rat pups whose mothers were severely salt-restricted during an early period of gestation have altered sensitivity, both behaviorally and electrophysiologically, when tested at various times after birth.⁴⁰

In the human studies, the adult offspring (college students) of mothers who experienced considerable morning sickness during their pregnancies had greater salt preferences compared with students whose mothers suffered little or no morning sickness.⁴¹ The authors suggest that morning sickness leads to transient fluid and sodium depletion in a manner analogous to sodium depletion reported in the animal model studies. Consistent with these findings, 12- to 14-year-old children who had been erroneously fed a chloride-deficient formula during infancy had heightened preference for salty (but not sweet) food relative to their unexposed siblings.⁴² Because chloride deficiencies mimic sodium deficiencies in some ways (e.g., altered hormonal profile), this finding is consistent with the hypothesis that early sodium depletion leads to heightened preferences many years later.

Studies on Smell

During the past decade, research at Monell has focused on the early olfactory experiences of the human infant, using mother's milk as the medium for these experiences. Our research revealed that human milk, like the milk of other animals, is indeed rich in flavors⁴³ which directly reflect the foods and spices (e.g., garlic, mint, vanilla, carrot) eaten by the mother. The breast-feeding infant's ability to detect the sensory changes in the mother's milk is suggested by the infant's altered suckling behavior when the milk is flavored, that is, the infant feeds longer and sucks more overall when the milk is flavored with either garlic or vanilla.^{44,45} The mouth movements made during suckling may facilitate the retronasal perception of the volatiles in the milk, enhancing the infant's ability to "taste" the change. Moreover, experience with a flavor in mother's milk modifies how that infant responds to that flavor during subsequent feedings.⁴⁶ Interestingly, formula-fed infants responded in a similar manner when we added the flavor of vanilla to their formula, they sucked more during their initial exposure to the flavor, but this response diminished after repeated exposures.⁴⁵

The flavor world of breast-fed infants is potentially much richer than previously thought. Because the chemical senses are not only functioning during infancy, but change during development, breast-fed infants may be

afforded an opportunity to learn about the flavor of the foods of their people long before solids are introduced.

Alcohol and Breast Feeding

The flavor of human milk is also altered when nursing women drink alcohol, a beverage that has been recommended for centuries to nursing mothers as an aid to lactation. Folklore relates that drinking small quantities of alcohol shortly before nursing increases milk yield, facilitates milk let-down, and relaxes both the mother and her baby. Contrary to this lore, research has demonstrated that breast-fed infants consume significantly less milk during the 3 to 4 hours after their mothers drink an alcoholic beverage.^{46,47} This rejection was not due to infants responding to the altered flavored of the milk, however.⁴⁸ Whether the alcohol was having a pharmacological effect on the nursing mother, the infant, or both is the subject of present investigations. Whatever the case, it would seem that the recommendation for a nursing mother to drink a glass of beer or wine before nursing may actually be counterproductive. While the mother may be more relaxed after a drink, her baby will ingest less milk. Moreover, infants appear to be learning about the flavor of alcohol as evidenced by changes in their suckling behavior.⁴⁹

Conclusions

As a relatively new and exciting area of study, many questions remain unanswered about the infant's sense of taste and smell. The long-term goals of research at Monell are to uncover whether early exposure to flavors, most often experienced in amniotic fluid, mother's milk, or formula, affects later preferences, the development of food habits, and the willingness to accept new foods at weaning or thereafter. Although much research is still needed to fully understand the impact of early flavor experiences of the human infant, it is clear that they are not passive receptacles for flavored foods. Rather, they will avidly accept some flavors, while decidedly rejecting others.

We are particularly intrigued with the notion that there may be sensitive periods during early development when experiences with flavors produce particularly enduring preferences. The concept of sensitive periods, first introduced into the field of behavior from embryological studies by the ethologist Konrad Lorenz,⁵⁰ implies that there is a period during early development when the organism is primed to receive and perhaps permanently encode important environmental information. An early knowledge of what is safe, appropriate, and nutritious foods would intuitively be important information for the fetus, infant, and young child. This is not to say that later learning is not important, but it highlights the possible significance of these very early experiences. Studies on breast-fed infants and infants fed casein hydrolysate formulas provide possible model systems to further study this issue.

Because every baby is an individual, with distinct likes and dislikes, parents should expect that their child will need time to learn to like some foods while never liking others. Parents who offer their babies and growing children a variety of foods will provide both a nutritious, well-balanced diet as well as an opportunity for their child's own personal preferences to develop.

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Individual Variability in Homocysteine Response to Folate Depletion: An Unusual Case

Robert A. Jacob, Ph.D.

Research within the past decade has shown that even moderately elevated plasma homocysteine concentrations are associated with increased risk of vascular disease. A variety of genetic and nutritional factors can affect homocysteine concentrations, with folate nutrition being one of the most influential. Plasma homocysteine responses of healthy adults to folate depletion and repletion vary substantially, even when many nutritional and lifestyle factors are normalized in a metabolic unit. The case of a woman with a highly exaggerated homocysteine response to moderate folate depletion is presented. A variety of possible factors relating to homocysteine metabolism are discussed, yet no convincing explanation for the unusual pattern is apparent. The case demonstrates that the homocysteine response to folate can be highly variable between individuals, and suggests that further research on the genetic determinants of the human folate requirement is warranted.

Introduction

Although clinical homocystinuria has been known for some 30 years to result in vascular disease at an early age, a profusion of research within the past decade has shown that even moderately elevated plasma homocysteine (Hcy) concentrations are associated with increased risk of vascular diseases.^{1,2} A review of some 38 studies relating plasma Hcy levels to vascular disease, and folate nutrition to Hcy, concluded that plasma Hcy is a strong and independent risk factor for vascular disease.¹ A 5 $\mu\text{mol/L}$ increase in plasma Hcy was estimated to increase heart disease risk by about 70% (risk equivalent to +0.5 mmol/L serum cholesterol), and an increased folate intake of 200 $\mu\text{g/d}$ was estimated to reduce Hcy levels by about 4 $\mu\text{mol/L}$.¹

Like plasma lipids, a variety of factors have been identified as contributing to an individual's predisposition to

elevated Hcy levels.³⁻⁵ These can be broadly classified into genetic and environmental influences. The former include rare cystathionine β -synthase (CBS) and methionine synthase defects and the more common genetic variant of the methylenetetrahydrofolate reductase (MTHFR) enzyme, which occurs in approximately 10% of the population and predisposes these individuals to high Hcy levels.⁶⁻⁸ Some physiologic factors include age, gender, renal function, and hormonal status. Environmental influences include smoking and, more importantly, nutrition. As shown in Figure 1, methionine, folate, choline (as betaine), and vitamins B₁₂, B₆, and B₂ are intimately involved in Hcy metabolism and can affect plasma Hcy levels substantially.^{3,4} Of the B vitamins, folate nutrition generally seems to exert the greatest influence on Hcy concentrations, although vitamin B₁₂ may be more important for elderly who suffer from cobalamin malabsorption.^{9,10}

Owing to the variety of genetic and environmental factors that can affect Hcy metabolism, the specific cause of elevated plasma Hcy levels in an individual might not be clear. We determined the Hcy response to folate depletion and repletion in two controlled metabolic unit studies

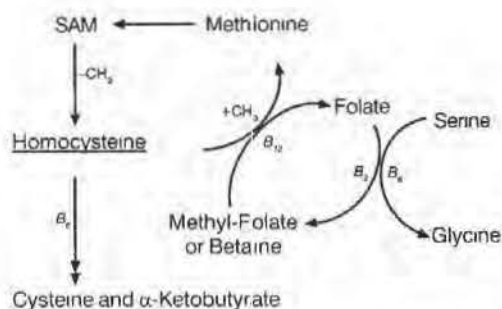


Figure 1 B vitamins involved in homocysteine metabolism. Vitamin B₆ (pyridoxal-5-phosphate)-dependent enzymes are cystathionine β -synthase and serine hydroxymethylase, the vitamin B₁₂ (cobalamin)-dependent enzyme is N-5-methyltetrahydrofolate homocysteine methyltransferase (methionine synthase), and the vitamin B₂ (FAD)-dependent enzyme is 5,10-methylenetetrahydrofolate reductase. The folate form is tetrahydrofolate.

Dr Jacob is a Research Chemist, Western Human Nutrition Research Center, Agricultural Research Service, U.S. Department of Agriculture, Presidio Box 29997, Building 1110, San Francisco, CA 94129.

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of adult men and women carried out in 1990 and 1995, respectively.^{11,12} This format allowed for the study of the plasma Hcy response to folate nutriture while other factors of diet and lifestyle that might also have affected Hcy levels were held constant.

Individual Variability in Homocysteine Response to Folate Depletion

In the 1990 study of folate depletion in men, plasma Hcy changed inversely with dietary folate intake and plasma levels.¹¹ However, the time course and magnitude of Hcy changes differed substantially among the 10 individuals. All subjects had normal baseline Hcy levels, below 12 $\mu\text{mol/L}$.¹² During 4 weeks of folate depletion at 25 μg folate/day (one-eighth of the recommended dietary allowance [RDA]), four subjects attained moderately elevated Hcy (above 16 $\mu\text{mol/L}$), five subjects rose slightly but remained in the normal range, and a tenth subject showed a delayed rise to 17 $\mu\text{mol/L}$. Although it was tempting to explain this latter abnormal pattern as an artifact, it was found to be reproducible in the second half of the study, which was essentially a repeat of the first half. Pearson correlation

coefficients (R) for linear regressions of plasma Hcy against folate gave negative coefficients for individuals and ranged from an R^2 of 0.02 to 0.94. Because diet and lifestyle were essentially the same for all, the observed individual differences in the Hcy response to changes in folate intake were attributed to differing degrees of folate body pool depletion and/or differing genetic dispositions that affected folate/Hcy pathways.¹¹

An Unusual Case

In 1995, we assessed plasma Hcy response to folate depletion in a second study of 10 postmenopausal women who received 56–516 $\mu\text{g/day}$ over 13 weeks (the 1989 folate RDA for women is 180 $\mu\text{g/day}$).¹³ The study design can be seen in Figure 2, which shows folate intake and plasma levels. Like the earlier study of men, each subject served as her own control. The decreasing intersubject variance in plasma folate seen in Figure 2 (group standard deviation [SD] was 5.9 nmol/L at day 6 [baseline] and 1.6 nmol/L at day 49) shows the normalizing effect of the controlled folate intake. The decreasing intersubject variance of plasma folate levels as the study progressed, however,

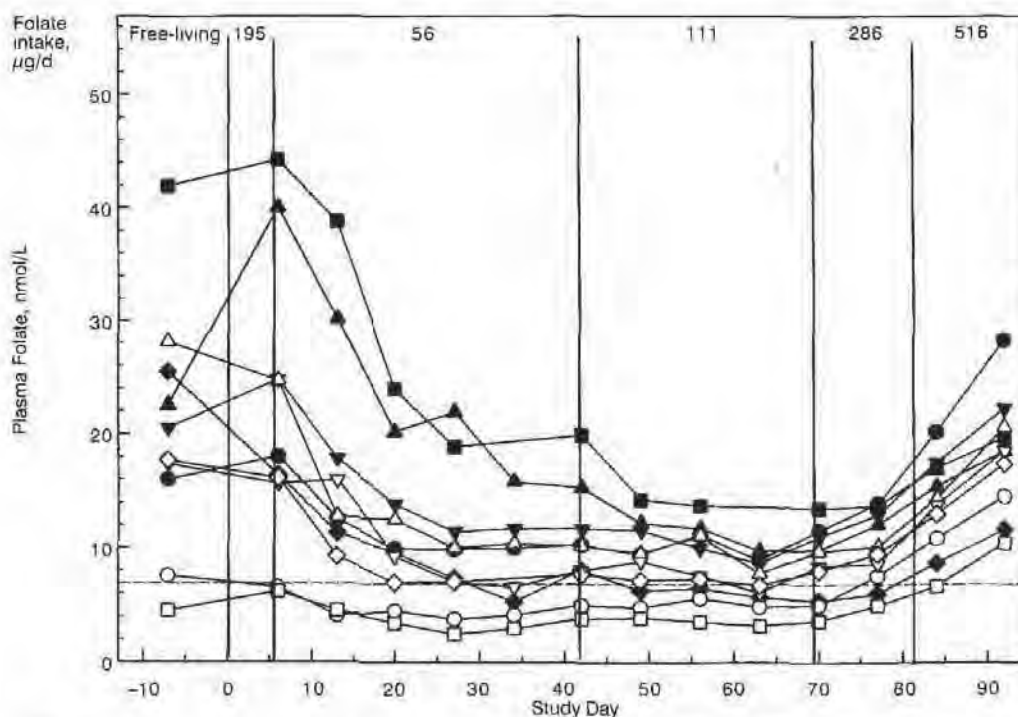


Figure 2 Plasma folate concentrations of 10 postmenopausal women receiving various dietary intakes of folate (shown at top in $\mu\text{g/day}$). Lower limit of normal range for plasma folate is shown as horizontal dashed line at 6.8 nmol/L. Subject SW is shown by solid triangles.

did not reflect similar changes in body or tissue folate pools, as the latter change much more slowly than plasma folate and Hcy¹⁴

As in the men's study, there was an inverse relation between folate intake and plasma Hcy levels in women, and plasma Hcy responded differently among individuals, as seen in Figure 3. Although all subjects, except HM (open circles), followed the general inverse relation, the magnitude of Hcy response to folate depletion was different. The Hcy values for three subjects changed only within the normal range, five subjects showed mild elevations, and one subject, SW (solid triangles), showed an extraordinary increase to 30.9 $\mu\text{mol/L}$. The high baseline Hcy value of subject HM may have been due to environmental factors that were altered favorably upon entry into the study, resulting in a decline in Hcy despite a low folate intake. For example, this subject had the lowest vitamin B₁₂ values throughout the study, 185–235 pmol/L; however, these values do not represent cobalamin deficiency. As seen in Figure 2, this subject also had low plasma folate until the last repletion period.

Subject SW showed an unusually large Hcy increase upon folate depletion. Because plasma Hcy is an independent risk factor for vascular disease in the population at large, it is useful to determine the possible causes of the exaggerated Hcy response in this case.

Causes for Exaggerated Homocysteine Response

Several possible causes for the unusually large Hcy response of subject SW to folate depletion can be considered. The obvious explanations involve deficiencies of nutrients involved in Hcy or methylation pathways, or genetic faults in Hcy metabolism. Other likely causes include the presence of a temporary iron deficiency anemia and the cessation of hormone replacement therapy (HRT).

Nutrient Deficiencies of Methylation Pathways

The subject may have had unusually severe deficiencies of nutrients involved in the Hcy or methylation pathways, such as folate, choline, or other B vitamin cofactors. Low folate status could have been a factor if SW began the study with low body stores of folate and she, therefore,

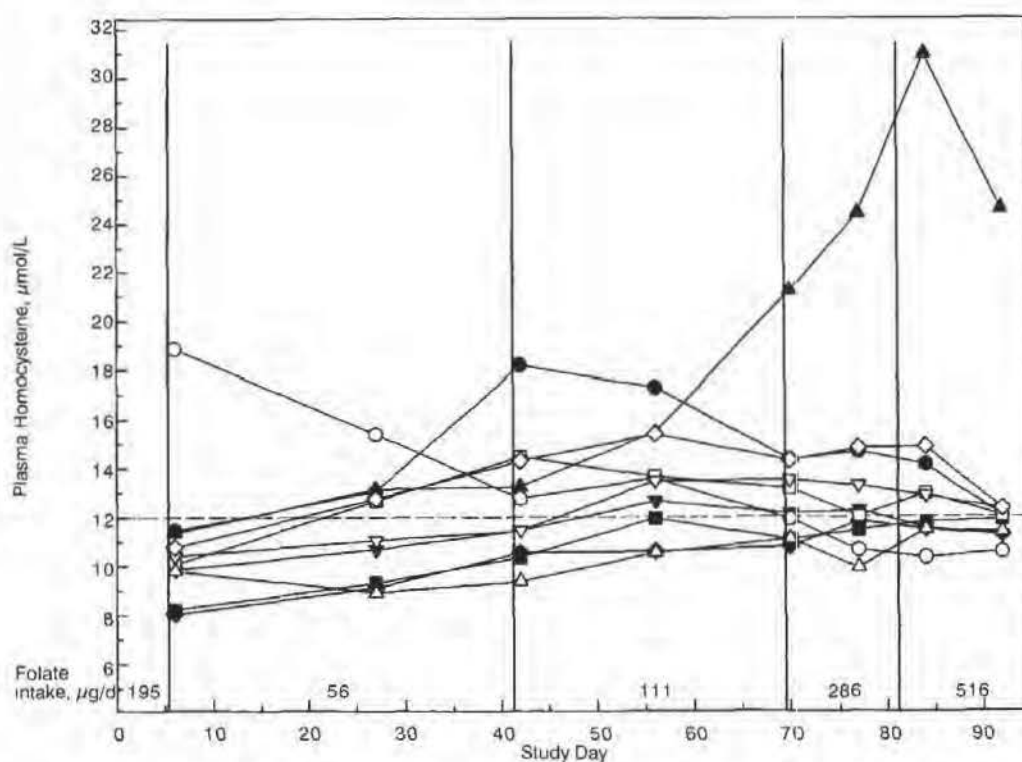


Figure 3. Plasma total homocysteine (Hcy) concentrations of 10 postmenopausal women receiving various dietary intakes of folate (shown at bottom in $\mu\text{g/day}$). Upper limit of normal range for plasma Hcy is shown as horizontal dashed line at 12 $\mu\text{mol/L}$. Subject SW is shown by solid triangles.

suffered greater tissue folate depletion during the 9 weeks of low folate intake. This does not appear to be the case, as her plasma folate started high and remained above the group average throughout the study (Figure 2). Moreover, her red cell folate, a better measure of overall body folate status than plasma folate, started and remained highest of all subjects, at 1341–1821 nmol/L (normal range is 317–1422 nmol/L¹⁵). Additionally, a clinical measure of folate deficiency, mean corpuscular volume (MCV), was normal throughout (91–85 fL from beginning to end), and other observed measures of folate depletion, lymphocyte DNA hypomethylation, and urinary malondialdehyde as thiobarbituric acid reactive substances (a measure of lipid peroxidation)¹² were similar for SW compared with the group as a whole.

The experimental diet was also low in the exogenous methyl donor choline, providing an average of 147 mg/day total choline, compared with usual adult intakes of 600–1000 mg/day in Western diets.¹⁶ The low choline content of the diet may have predisposed subjects to higher Hcy levels, because choline can contribute to remethylation of Hcy via betaine.¹⁶ However, the choline status of SW was not unusual compared with the other subjects, her plasma choline was 5.6 and 4.7 μ mol/L at days 42 and 70, compared with group means (range) of 5.6 (3.8–7.4) μ mol/L and 5.1 (3.6–6.5) μ mol/L, respectively. Intake of the other major dietary methyl donor, methionine, was adequate, with the 4-day rotating diet providing a daily average intake of 780 mg methionine and 420 mg cysteine, 132% of the estimated adult requirement of 910 mg/day for methionine plus cysteine.¹⁷

Likewise, deficiency of the other B vitamin cofactors required for Hcy metabolism did not appear to be involved, because the diet plus supplements provided the following amounts as a percentage of the current RDA: 234% of B₆, 124% of B₁₂, and 200% of B₂.¹² The plasma vitamin B₁₂ concentration of subject SW decreased from 509 pmol/L at baseline to 322 pmol/L at day 49 and then gradually increased to 374 pmol/L at the end of the study. However, these levels were second highest of all subjects throughout the study and were well within the normal range of 148–616 pmol/L throughout.¹³

Genotype

Genetic variations of enzymes involved in the Hcy/methionine pathways might affect the Hcy response to folate depletion. The most likely genetic variant involved would be the relatively common MTHFR 677 cytosine-to-thymine mutation, which occurs in about 10% and 40% of the general population in homozygous and heterozygous forms, respectively.^{4,6,18} This mutation produces an alanine-to-valine substitution in the enzyme protein, resulting in reduced enzyme activity and impaired formation of 5-methyltetrahydrofolate. Homozygosity for this variant

has been associated with elevated plasma Hcy levels and exaggerated Hcy response to folate depletion,^{4,6} but, generally, not increased coronary artery disease.^{7,18} SW was found to be heterozygous for the mutant allele, as determined by polymerase chain reaction (PCR) amplification of the lymphocyte-derived MTHFR gene, restriction enzyme digestion with *Hinf*I, and agarose gel electrophoresis.¹⁹ This is a common MTHFR genotype, which is not associated with elevated Hcy levels⁸ and, therefore, does not explain SW's exaggerated Hcy response to folate depletion. It is possible that less common genetic mutations of Hcy pathway enzymes, i.e., CBS or methionine synthase, may be involved. Boddie et al.²⁰ recently reported that heterozygotes for CBS deficiency have increased ratios of plasma Hcy:folate and Hcy cysteine compared with controls.

Iron Deficiency and Hormone Replacement

Upon examining the reasons for SW's abnormal Hcy response to folate depletion, it should be noted that she developed a mild anemia during the low folate intake period, as shown by declining hemoglobin levels in the first half of the study (Figure 4). This occurred despite a total iron intake of 31 mg/day, 313% of the current RDA¹⁷ (6 mg from the diet and 25 mg from mineral supplements consumed at each meal). The anemia was not of a macrocytic nature, since the red cell MCV did not increase. At day 57 she began taking an additional daily iron supplement of 325 mg ferrous sulfate for the remainder of the study, after which the anemia promptly resolved.

Iron and folate deficiencies frequently occur together, and megaloblastic folate deficiency secondary to iron deficiency has been reported in both rats and man.^{21,22} Suggested mechanisms included a decrease in activity of the folate pathway enzyme glutamate formimino transferase or increased erythrocyte turnover owing to iron deficiency.²¹ Further studies, however, were not confirmatory, and the question of the effects of iron deficiency on folate metabolism is currently unresolved.^{23,24}

It is tempting to speculate that the exaggerated Hcy response of SW may have been due to a secondary effect of iron deficiency, however, the absence of megaloblastosis and the relatively late changes in Hcy relative to hemoglobin levels argue against this (plasma Hcy levels continued to increase after day 70, when hemoglobin was repleted to normal). Again, except for Hcy response, SW appeared to have no more severe induced folate deficiency than the other subjects. There is no clear precedent for hypothesizing that iron deficiency predisposes to elevations of plasma Hcy, but the many reported relations between iron and folate, some noted above, certainly leave open this possibility.

Finally, an analysis of the effect of HRT is warranted, because this may have affected plasma Hcy levels in the

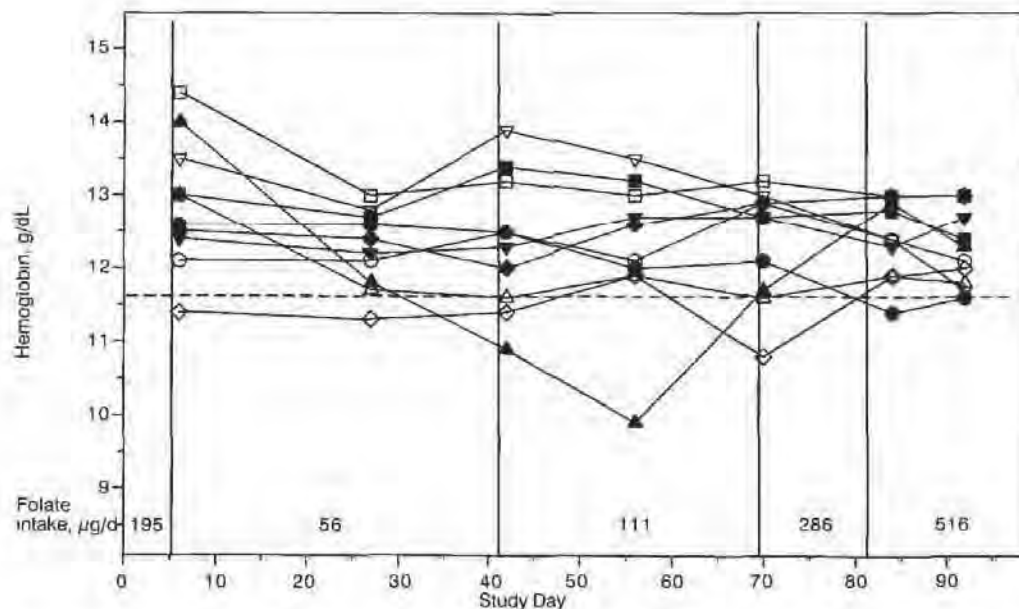


Figure 4. Blood hemoglobin concentrations of 10 postmenopausal women receiving various dietary intakes of folate. Lower limit of normal range is shown as horizontal dashed line at 11.6 g/dL. Subject SW is shown by solid triangles.

women studied. A prospective study of 21 healthy postmenopausal women showed that initiation of HRT lowered serum Hcy levels an average of 11% over the first 6 months, with the decrease being 17% for women with high initial Hcy levels.²⁵ Of the 10 postmenopausal women in the present report, six were not on HRT, three continued their prestudy HRT throughout the study, and SW stopped her HRT upon entry into the study. She remained off HRT for the remainder of the study. Hence, cessation of SW's HRT regimen at the beginning of the study may have affected her Hcy response to folate depletion. Plasma folate and Hcy concentrations were not significantly different throughout the study in the three women taking HRT compared with those who were not. Given this observation and the reported moderate effects of HRT on serum Hcy, it seems unlikely that the exaggerated Hcy response of SW was due primarily to changes in hormonal status.

Conclusion

Examination of the responses of plasma Hcy to folate depletion of 10 postmenopausal women support the conclusion that substantial individual differences exist, even when many environmental and lifestyle factors are normalized. The exaggerated Hcy response of subject SW does not appear to be related to differences in nutrient commonly related to methylation pathways or to the relatively common variant of the MTHFR gene. Changes in

iron or hormone status unique to SW may have played a role in her exaggerated Hcy response, but little is known about the effect of these factors on Hcy metabolism. Further research on the relationships among iron deficiency, folate utilization, and Hcy are necessary, because earlier studies did not measure Hcy levels.

Because the above factors do not provide a convincing explanation for SW's unusual Hcy response, it is likely that the explanation lies in a genetic variant of a Hcy pathway enzyme that is less common, or less well known, than the MTHFR variant. If so, the results for subject SW indicate that the putative gene-based impairment is overcome by a higher folate intake, and important only at a low folate intake. This leads to the hypothesis, stated recently by Rosenberg and Rosenberg,⁸ that genetic disposition is a significant factor that determines the human folate requirement and that further research of this question is needed.

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The Food Stamp Program and Low-Income Legal Immigrants

John T. Cook, Ph.D.

Editors' note: In 1996, the U.S. Congress passed the Personal Responsibility and Work Opportunity Reconciliation Act, which had as one of its effects the withdrawal of food stamp eligibility for many legal immigrants. The following analysis from the Tufts University Center on Hunger, Poverty, and Nutrition Policy examines the nutritional impact of this legislation on legal immigrants and discusses the arguments for restoration of these nutritional benefits. At press time, the U.S. Congress had just passed legislation to restore food stamp benefits to many legal immigrants

Introduction

In January 1998, 20.3 million Americans received food stamp benefits.¹ This represents a reduction of more than 26.2% from the high of 27.5 million in 1994.² This decrease in participation in the Food Stamp Program is largely due to improvements in the national economy during this period. Part of the decrease, however, is a result of changes brought about by the Personal Responsibility and Work Opportunity Reconciliation Act (PRWORA) of 1996.

One of the changes implemented by PRWORA was to deny food stamp eligibility for many legal immigrants. Although illegal immigrants were already prohibited from receiving food stamps before PRWORA, legal immigrants who were otherwise eligible could not be denied food stamps solely on the basis of their immigration status. This brief review provides information about the impact of the changes in food stamp eligibility brought about by PRWORA and the nutritional benefits of food stamps for low-income legal immigrants.

Who Are Legal Immigrants?

The Census Bureau reports that in 1996 there were 24.6 million foreign-born persons (9.3% of the total population) living in the United States.³ That year, 32.2% of the

foreign-born population in the United States (about 8 million persons) were naturalized citizens.³ Most of the remainder were legal permanent residents, or legal immigrants.

Legal immigrants are persons born in a foreign country (also sometimes referred to as "aliens") who are given legal admission into the United States by the federal government. The U.S. Immigration and Naturalization Service (INS) and the Census Bureau monitor and classify legal immigrants by the number admitted into the United States during a given year. For example, in 1995, a total of 720,461 immigrants were admitted and a little more than half (52.8%) were classified as "new arrivals" by the INS, whereas a little less than half were "adjustments," or persons whose immigration status was changed administratively from another category to immigrant status. During this same year, 22.6 million nonimmigrants were admitted into the United States for temporary periods (many of whom may have become future "adjustment" legal immigrants).⁴

The 720,461 legal immigrants who entered the United States in 1995 were classified in several different categories by the INS upon entry. Most were categorized as so-called preference immigrants, meaning they were either family-sponsored immigrants (mostly spouses, sons, and daughters of naturalized American citizens or other permanent legal residents) or employment-based immigrants (persons entering either at the request of American employers or independently for employment). In 1995, preference immigrants constituted 45% of all immigrants admitted into the United States. Seventy-four percent of the preference immigrants were family sponsored, whereas about 26% were employment based. Employment-based legal immigrants constituted just under 12% of all immigrants admitted in 1995.

The second largest category, constituting 30% of all immigrants in 1995, was "immediate relatives." These are parents, spouses, or children of U.S.-born citizens, or orphans adopted by U.S. citizens. Refugees and asylum seekers, or "asylees," constituted 16% of all immigrants in 1995, with the remaining 9% classified as "other immigrants." This latter group consists primarily of "diversity program" immigrants admitted under laws intended to diversify immigration.

Dr. Cook is Director for Research, Center on Hunger and Poverty, Tufts University, Medford, MA 02155, USA.

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Many eligible immigrants are allowed to change their status from temporary resident to legal permanent resident by making application to the INS. Some immigrants also apply for and have their status changed to naturalized citizenship after a period of 3–5 years as legal permanent residents. Over longer periods of time (e.g., several years or decades) legal permanent residents and naturalized citizens become part of the U.S. “foreign-born” population.

Legal Immigrants and the Food Stamp Program Under PRWORA

Under PRWORA, legal immigrants are considered either “newly arrived immigrants” (i.e., they arrived in the United States after August 22, 1996) or “current immigrants” (i.e., they arrived before August 22, 1996). Although each of these two categories of immigrants is treated differently for Temporary Assistance for Needy Families (TANF) cash assistance under PRWORA, both current and newly arrived legal immigrants, with few exceptions, are denied federal food stamp benefits. The exceptions include certain refugees, asylees, and persons whose deportation has been withheld (for the first 5 years in the country), persons on active military duty or veterans and their spouses and children, and persons who have worked in the United States for at least 40 calendar quarters. For other poor legal immigrants, the Food Stamp Program—the nation’s primary nutrition safety-net program that provides protection from food insecurity, hunger, and malnutrition—is no longer available.

The U.S. Department of Agriculture (USDA) reports that during the fiscal year 1995, approximately 1.8 million legal immigrants received food stamps. Of these, 1.4 million (77%) were legal permanent residents. Nearly two-thirds of food stamp recipient households containing legal permanent residents also contained a U.S. citizen. Most of these households consisted of legal permanent resident parents living with U.S.-born children. Nearly one-third of all households receiving food stamps with a legal permanent resident in fiscal year 1995 contained an employed worker, versus only 20% of all food stamp households.

A December 1997 General Accounting Office (GAO) study reports that the USDA estimated that 935,000 legal immigrants lost their federal food stamp benefits as of December 1997 because of PRWORA.²⁵ However, as of that time, USDA also estimated that about 241,000 of the legal immigrants who had lost their food stamps were receiving food stamps funded by states. The study also reports that as of December 1997, 20 states were providing some kind of assistance to individuals who had lost federal food stamp benefits. Ten states were purchasing federal food stamps with their own funds for certain legal immigrants, primarily children and elderly. In the remain-

ing 10 states, food assistance was being supported with state funds through existing public or private emergency food assistance programs. In the 10 states purchasing and distributing federal food stamps with state funds, an estimated 241,000 legal immigrants were receiving state-funded food stamps. As of December 1997, therefore, 694,000 legal immigrants who had previously received food stamps were no longer receiving them. Among the 10 states providing state-funded food stamps for certain legal immigrants in December 1997 were California, Florida, New York, and Texas, where about 70% of all legal immigrants receiving food stamps that year resided.

Reports released by the Center on Budget and Policy Priorities in January and February 1998 indicate that as of that time period, 10 states (California, Florida, Illinois, Massachusetts, Maryland, Nebraska, New Jersey, New York, Rhode Island, and Washington) were providing state-funded food stamps to an estimated 250,000 immigrants, mostly children, elderly, and disabled, by purchasing them from the federal government. Two states (Minnesota and Texas) were providing cash assistance to legal immigrants in lieu of lost food stamp benefits. Thus, of the 935,000 legal immigrants who had lost food stamp benefits as of December 1997, 685,000 (73%) were still not receiving food stamps.^{6,7}

Nutrition Benefits of the Food Stamp Program

The Food Stamp Program is the nation’s most important and effective nutrition safety-net program. The program provides low-income families with resources to ensure them access to a healthful diet. According to a description on the USDA’s Internet website, the “Food Stamp Program represents the pledge that hunger will not be tolerated in America. It is the tangible expression of the principle that everyone has a right to food for themselves and their families.”

A large body of empirical research evidence indicates that the Food Stamp Program provides important nutrition benefits to low-income families who would otherwise be at high risk of hunger-related malnutrition and poor health.

A 1990 study⁸ conducted by Mathematica Policy Research, Inc., for USDA found that, overall, households participating in the Food Stamp Program have a higher average money value of food used at home per person than low-income nonrecipients, and that participants receive more nutrients for each dollar’s worth of food used at home.

A 1992 study by Korenman and Miller⁹ (conducted under contract for USDA) on the effects of Food Stamp Program participation on child and maternal health found that food stamp use during pregnancy is associated with a reduction in the incidence of low-birth-weight infants born to women with incomes below 50% of poverty.

The Tufts University Center on Hunger and Poverty, in a 1995 study,¹⁰ showed that poor children in families receiving food stamps are significantly better nourished than poor children in families that do not receive food stamps. This study also found that, compared with poor nonrecipients, nutrient intakes of young children (ages 1–5 years) who receive food stamps are better for 15 of 16 nutrients examined. For 10 of these nutrients, the proportions of children receiving food stamps with intakes below 70% of the recommended dietary allowance (RDA) were statistically significantly lower than for poor nonrecipients. For protein, riboflavin, and vitamin B₁₂, the proportions of children with inadequate intakes were 72–100% lower among Food Stamp Program participants. For folate and magnesium, the proportions of children receiving food stamps with substandard intakes were 50% lower than those not receiving food stamps. For calories, calcium, and vitamin B₆, the proportions of food stamp recipient children with inadequate intakes were more than 30% lower.

A recent study examined the prevalence of hunger and food insecurity in adult patients of an urban county hospital in Minnesota.¹¹ The patients whose food stamps had been eliminated or reduced within the previous year were significantly more likely to report not having enough food, not eating for a whole day, going hungry but not eating, and cutting the size of meals or skipping meals. The study also found that loss of food stamp benefits was a significant predictor of both food insecurity and hunger.

Are Legal Immigrants Protected by Charitable Emergency Food Assistance?

Although many legal immigrants receive food from private emergency food assistance programs, many of these programs do not have the resources to cover the large additional need caused by loss of food stamp benefits. Moreover, many programs anticipate even greater demand for their services as even more poor people lose cash and food assistance benefits in the coming years as a result of PRWORA.

The Tufts Center on Hunger and Poverty estimates that reductions in Food Stamp Program expenditures resulting from PRWORA (\$27 billion over the first 6 years after the law was enacted) will lead to an average annual reduction in food available to poor households of more than 4 billion pounds.¹² The center also projects that Second Harvest, the nation's largest distribution network for donated and recovered food, with 186 food banks serving nearly 50,000 local charitable agencies, will be able to provide less than one-fourth of this additional food if it continues to grow at the same annual rate as it did from 1991 to 1994. If the Second Harvest network is able to double its annual rate of growth, it will be able to provide less

than one-third of the additional food needed.

The aforementioned GAO report indicates that in December 1997, 13 states were using other state-funded food assistance programs to provide emergency food for legal immigrants. These state-funded food assistance programs are in addition to state-funded food stamp programs, which purchase food stamps from the federal government for distribution to legal immigrants and others who lost their food stamps as a result of PRWORA. The study reports that in the five localities surveyed, most nonprofit agencies contacted said they anticipate an increase in the need for their services as a result of welfare reform but are concerned that the supplemental services they can provide with their limited resources will not compensate for the basic food assistance lost from the federal Food Stamp Program.⁵

Conclusion

The U.S. population is very ethnically diverse, with nearly one in 11 Americans being foreign born. Historically, immigrants have been a source of cultural and economic richness in the American "melting pot." In most ways, legal immigrants are indistinguishable from any other of the 22.6 million foreign-born Americans. However, nearly a million poor legal immigrants, by virtue of their immigration status alone, were denied food stamp benefits, one of the most important forms of assistance in America's social safety net.

The empirical evidence, summarized above, indicates that participation in the Food Stamp Program provides important food and nutrient safeguards for poor legal immigrant families. However, even though some 250,000 legal immigrants who lost food stamps as a result of PRWORA are receiving food stamps paid for by states, approximately 685,000 are still not receiving them. Although some legal immigrants may receive supplemental food from charitable emergency food assistance providers, there is strong evidence that these programs will not be able to adequately address the food needs of all legal immigrants who have lost food stamps.

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Human and Clinical Nutrition

Dietary Soybean Protein Prevents Bone Loss in an Ovariectomized Rat Model of Osteoporosis^{1,2}

BAHRAM H. ARJMANDI,³ LEE ALEKEL, BRUCE W. HOLLIS,* DAXA AMIN,
MARIA STACEWICZ-SAPUNTZAKIS, PEILIN GUO AND SUBHASH C. KUKREJA[†]

Department of Human Nutrition and Dietetics, University of Illinois at Chicago, Chicago, IL 60612;

*Department of Pediatrics, Medical University of South Carolina, Charleston, SC 29425; and

[†]Department of Medicine, West Side Veterans Administration Medical Center, Chicago, IL 60612

ABSTRACT The purpose of this study was to examine whether soybean protein isolate prevents bone loss induced by ovarian hormone deficiency. Thirty-two 95-d-old Sprague-Dawley rats were randomly assigned to four treatment groups (sham-operated (sham); ovariectomized (ovx); ovx + soybean; ovx + 17 β -estradiol (E₂)) and killed after 30 d. Rats in the sham, ovx and ovx + 17 β -estradiol groups were fed a casein-based diet, and the soybean group was fed soybean protein isolate instead of casein; the diets were otherwise comparable. Rats in the ovx group had significantly lower densities of the right femur ($P < 0.001$) and the fourth lumbar vertebra ($P < 0.05$) than rats in the sham group. These lower bone densities were not observed in animals receiving 17 β -estradiol or fed soybean. The ovx group also had significantly ($P < 0.01$) greater serum concentrations of 1,25-dihydroxycholecalciferol than the other three groups. Our findings suggest that dietary soybean protein is effective in preventing bone loss due to ovarian hormone deficiency. Because serum activities of both alkaline phosphatase and tartrate-resistant acid phosphatase were significantly greater in the ovx group and in the ovx + soybean group but not in the group receiving 17 β -estradiol, compared with sham animals, this confirms that ovariectomy enhances and 17 β -estradiol suppresses the rate of bone turnover. Despite the higher rate of bone turnover in the soybean-fed animals, the vertebral and femoral bone densities of these rats were significantly greater than those of rats in the ovx group, suggesting that formation exceeded resorption. Further studies are needed to clarify whether this protective effect on bone is due to the protein itself or to the presence of isoflavones in soybean protein. *J. Nutr.* 126: 161-167, 1996.

INDEXING KEY WORDS:

- soybean protein isolate • estradiol
- ovariectomy • bone loss • rats

pausal osteoporosis) is by far the most common cause of age-related bone loss. A sharp decrease in ovarian estrogen production is the predominant cause of rapid hormone-related bone loss during the first decade after menopause (Gruber et al. 1984). Traditional therapies for postmenopausal osteoporosis have emphasized agents that inhibit bone resorption such as estrogen (Centrella and Canalis 1985), calcitonin (Canalis et al. 1988, Rudman et al. 1981) and bisphosphonates (Ben-net et al. 1984). Although the most effective method to reduce the rate of postmenopausal bone loss is estrogen replacement therapy, it may be accompanied by side effects (Genant et al. 1989) and is thus recommended only for women who are at high risk of osteoporosis and who have no contraindications. Recent advances in bone biology have led researchers to suggest using a combination of anti-resorptive agents, such as estrogen, and formation-stimulating agents, such as growth hormone, toward a cure for osteoporosis (Turner 1991). However, the potential bone-forming agents available today either may have serious side effects (e.g., growth hormone, anabolic steroids), may not improve bone quality, or may not decrease susceptibility to fracture (e.g., sodium fluoride). Therefore, it would be most helpful to discover a naturally occurring substance that minimizes bone loss in postmenopausal women, thus decreasing the necessity for drug therapy.

Some reports have indicated that ipriflavone, a synthetic flavonoid derivative (Agnusdei et al. 1989), is effective in preserving bone mass in several models of

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³ To whom correspondence and reprint requests should be addressed.

Osteoporosis that is associated with ovarian hormone deficiency following menopause (postmeno-

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experimental osteoporosis (Benvenuti et al. 1991, Yamazaki 1986, Yamazaki and Kinoshita 1986). These promising reports regarding the beneficial effects of isoflavone have led us to hypothesize that natural food sources with high concentrations of isoflavonoids might be equally effective in modulating bone mass due to ovarian hormone deficiency. To test this hypothesis we used rats and replaced the casein in their diets with soybean protein. The isoflavones found predominately in soybeans and soybean products are pharmacologically and structurally similar to the synthetic phytoestrogens (e.g., tamoxifen, ipriflavone) that have been shown to be effective in preventing or reducing bone loss. The potential effect of soybean protein on bone health has immense implications should this dietary source of isoflavones be demonstrated effective in the prevention or treatment of osteoporosis.

MATERIALS AND METHODS

Animals and diets. Thirty-two female Sprague-Dawley rats, aged 90 d, were purchased from Harlan Sprague Dawley (Indianapolis, IN) and used for this study when they were 95 d old. Upon arrival at our institution, the rats were housed in an environmentally controlled animal laboratory. Rats were acclimated to a standard laboratory nonpurified diet for 5 d. After acclimation, rats were divided by initial body weight into four blocks of eight rats each, using a randomized complete block design. Two animals from each of the four treatment groups were included in every block: 1) sham-operated (sham group); 2) ovariectomized (ovx group); 3) ovx + soybean group; 4) ovx + 17 β -estradiol (10 μ g/kg body wt per day) (ovx + E₂ group). All rats except those in the ovx + E₂ group received solvent vehicle. 17 β -Estradiol was dissolved in a small volume of absolute ethanol and the concentration was adjusted with sesame oil. The solvent vehicle contained similar volumes of ethanol and sesame oil. 17 β -Estradiol and solvent vehicle injections were given subcutaneously daily from the date of surgery. Rats were fed isonitrogenous and isocaloric experimental powdered diets. Rats in the sham, ovx, and ovx + E₂ groups were fed a powdered casein-based diet (Teklad, Madison, WI) that contained 0.4% calcium, 0.3% phosphorus and 0.195 nmol vitamin D₃/g diet. Rats in the ovx + soybean group were fed a similar powdered diet in which casein was replaced with soybean protein isolate (22.7 g/100 g diet; Protein Technologies International, St. Louis, MO) with the calcium and phosphorus levels adjusted to the levels in the casein-based diet (Table 1). All rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride and xylazine at doses of 100 mg/kg body wt and 5 mg/kg body wt, respectively. Bilateral ovariectomies were performed in the ovx, ovx + soy, and ovx + E₂ groups, and animals

TABLE 1

Composition of casein and soybean protein diets

Ingredient	Casein diet ¹	Soy protein diet
	g/kg diet	
Protein		
Casein	227.0	—
Soybean protein isolate ²	—	227.0
Carbohydrate		
Sucrose	417.6	417.6
Cornstarch	200.0	200.0
Fiber source (cellulose ³)	56.0	56.0
Fat (corn oil)	57.0	57.0
Vitamin mixture ⁴	10.0	10.0
Total cholecalciferol	0.0016	0.0016
Mineral mixture (Ca-P deficient) ⁵	13.4	13.4
Calcium carbonate	9.88	9.88
Sodium phosphate monobasic	3.88	3.88
Potassium phosphate monobasic	2.38	2.38
Potassium citrate monohydrate	0.90	0.90

¹ Teklad diet #88190 (Harlan Teklad, Madison, WI)

² Soy protein isolate obtained from Protein Technologies International (St. Louis, MO).

³ Alphacel obtained from ICN Biochemicals (Costa Mesa, CA).

⁴ Vitamin mixture (g/kg diet; TD 40060) obtained from Harlan Teklad (Madison, WI): p-aminobenzoic acid, 0.1101; ascorbic acid, 1.0166; biotin, 0.00044; vitamin B-12 (0.1% trituration), 0.0297; calcium pantothenate, 0.0661; choline dihydrogen citrate, 3.4969; folic acid, 0.00198; inositol, 0.1101; menadione, 0.0495; niacin, 0.0991; pyridoxine (HCl), 0.0220; riboflavin, 0.0220; thiamin HCl, 0.0220; dry retinyl palmitate, 0.0044; dry d,l- α -tocopheryl acetate, 0.2423; corn starch (diluent), 4.6669.

⁵ Mineral mixture (TD 79055) obtained from Harlan Teklad (Madison, WI). This mineral mixture is a modification of AIN 76 lacking calcium, phosphorus, and sucrose as diluent.

in the sham group were subjected to sham operation (Waynforth 1980). Rats that received 17 β -estradiol consumed food with ad libitum access, and their precise food intakes were measured every 3 d. Before each feeding, the food remaining was weighed, and the amount ingested was calculated. Rats in the other three groups were pair-fed (plus an additional 1 g/d for growth) to the mean intake of the ovx + E₂ group animals for 30 d from the date of surgery. The rats had free access to deionized water. We killed the animals 30 d after surgery, right femurs and fourth lumbar bones were dissected out for bone analyses. Blood was collected, and serum was separated, divided into small samples and stored at -20°C until required for analyses. Guidelines for the ethical care and treatment of animals from the Animal Care Committee of the University of Illinois at Chicago were strictly followed.

Bone length and density. The right femurs and the fourth lumbar vertebra were freed of soft tissue using small scissors, tweezers and cotton gauze. The length of each femur was measured with a vernier caliper. Before measurement of the bone density, the fe-

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mur was cut at the mid-diaphysis and the marrow washed out. Bone volume and density were measured by Archimedes' principle [Kalu et al. 1991]. Briefly, each bone was put in an unstoppered vial filled with deionized water, and the vial was placed under a vacuum for 90 min to ensure that all the trapped air diffused out of the bone. Each bone was removed from its vial, blotted with gauze sponge, weighed, and returned to the vial containing deionized water. The bone was reweighed in water and the density was calculated (g/cm^3 bone volume).

Bone calcium and phosphorus. The bones were hydrolyzed with 6 mol/L HCl at 110°C for 20 h. To measure calcium, aliquots of hydrolysate were properly diluted using 3.1 mmol/L lanthanum solution. Calcium was determined by atomic absorption spectrophotometry (model 503, Perkin-Elmer, Norwalk, CT), and the values were expressed as milligrams of calcium per cubic centimeter of bone volume. Aliquots of bone hydrolysate were also used for the colorimetric analysis of phosphorus with the technique of Tausky and Shorr (1953).

Serum total calcium and phosphate. Serum was appropriately diluted with 3.1 mmol/L lanthanum solution, and calcium was measured using atomic absorption spectrophotometry. Serum phosphate was assessed by the method of Tausky and Shorr (1953).

Serum tartrate-resistant acid phosphatase and alkaline phosphatase. Serum tartrate-resistant acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) were measured spectrophotometrically using kits (procedures no. 435 and 245, respectively) from Sigma Diagnostics (St. Louis, MO). The manufacturer's protocols were followed for each assay. The values for tartrate-resistant acid phosphatase and alkaline phosphatase were expressed in nanokatals per liter and microkatals per liter, respectively.

Vitamin D metabolites. The techniques used to measure the serum vitamin D metabolites were previously reported [Hollis 1986, Hollis et al. 1986]. Briefly, to determine 1,25-dihydroxycholecalciferol [$1,25(\text{OH})_2\text{D}_3$],⁴ serum samples were extracted with acetonitrile. After centrifugation the supernatant was decanted into a tube containing 0.4 mol/L K_2HPO_4 , pH 10.6, and vortexed. The extract was applied to a C_{18} -OH cartridge (Varian Corporation, Harbor City, CA) to isolate and purify $1,25(\text{OH})_2\text{D}_3$ prior to radio-receptor assay. Standards and samples were added to $1,25(\text{OH})_2\text{D}_3$ receptor solution prepared from calf thymus (Incstar Corporation, Stillwater, MN). Incubation was performed at 20°C for 45 min, after which tritium-labeled $1,25(\text{OH})_2\text{D}_3$ (Amersham, Arlington

Heights, IL) was added, and the incubation was continued for 45 min. After treatment with dextran-coated charcoal, bound and free hormones were separated, and serum concentrations of $1,25(\text{OH})_2\text{D}_3$ were estimated. The detection limit of the method is 3.90 fmol per tube. To determine serum 25-hydroxycholecalciferol [$25(\text{OH})\text{D}_3$], serum samples were extracted in acetonitrile. After centrifugation, a portion of the supernatant was subjected to direct radioimmunoassay using an antibody generated against the 23,24,25,26,27-pentol-22-carboxylic acid of vitamin D using a radioiodinated tracer. The detection limit of the assay is 7.49 nmol/L serum.

Statistical analysis. Data analysis (GraphPad InStat Software version 2.00, 1993, San Diego, CA) involved estimation of means and SD for each of the groups (Snedecor and Cochran 1967). Analysis of variance was performed to determine whether there were significant ($P < 0.05$) differences among the groups. When an ANOVA indicated any significant difference among the means, the Newman-Keuls multiple comparison test was used to determine which means were significantly different [Chew 1977].

RESULTS

Body weights and food intake. The four treatment groups started with similar mean body weights, but at the end of the study, the ovx group had a significantly higher and the ovx + E_2 group a significantly lower mean body weight than the sham group or the ovx + soybean group (Table 2). This lower mean final body weight of 17β -estradiol-treated rats was not a result of significantly less food intake, because all the treatment groups were pair-fed and hence their food intakes were similar.

Organ weights. The organs that were weighed are listed in Table 2 and are presented relative to body weight. Ovariectomy caused atrophy of the uterus. This was prevented by the 17β -estradiol treatment but not by dietary soybean protein. The abdominal fat mass was significantly lower in animals fed the soybean diet compared with rats in the sham group or the ovx + E_2 group. The left and right kidneys of the 17β -estradiol-treated rats were significantly heavier than those of rats in the ovx group or the ovx + soybean groups but similar to those of the sham group. The other relative organ weights were not significantly different among groups.

Bone density, bone calcium and phosphorus. Animals in the ovx group had significantly lower densities of the right femurs and the fourth lumbar vertebra compared with the sham group (Fig. 1). The animals treated with 17β -estradiol had similar or significantly higher bone densities than rats in the sham group or the ovx + soybean group. The rats fed the

⁴ Abbreviations used: $25(\text{OH})\text{D}_3$, 25-hydroxycholecalciferol; $1,25(\text{OH})_2\text{D}_3$, 1,25-dihydroxycholecalciferol. Group abbreviations: sham group, sham-operated rats; ovx group, ovariectomized rats; ovx + E_2 group, ovariectomized rats receiving daily injections of 17β -estradiol; ovx + soybean group, ovariectomized rats fed soybean protein isolate.

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TABLE 2

Effects of ovariectomy (ovx), soybean protein, and 17 β -estradiol (E₂) on food intake, body weights, and relative organ weights in rats^{1,2}

Measure	Experimental group			
	Sham	Ovx	Ovx + soy	Ovx + E ₂
Food intake, g/d	12.3 \pm 1.5	12.2 \pm 1.4	12.1 \pm 1.3	11.2 \pm 4.6
Initial body weight, g	249 \pm 16.0	251 \pm 10.1	249 \pm 8.2	250 \pm 9.3
Final body weight, g	274 \pm 14.3 ^b	295 \pm 15.3 ^c	274 \pm 13.8 ^b	253 \pm 6.6 ^a
Organ weights, g/100 g body wt				
Spleen	0.24 \pm 0.015	0.23 \pm 0.034	0.22 \pm 0.029	0.25 \pm 0.024
Uterus	0.160 \pm 0.044 ^b	0.051 \pm 0.003 ^a	0.062 \pm 0.018 ^a	0.182 \pm 0.020 ^b
Liver	3.03 \pm 0.47	3.13 \pm 0.61	3.08 \pm 0.66	3.27 \pm 0.24
Heart	0.35 \pm 0.022	0.36 \pm 0.034	0.35 \pm 0.018	0.39 \pm 0.059
Abdominal fat	3.56 \pm 1.39 ^b	2.36 \pm 0.78 ^{a,b}	1.97 \pm 0.99 ^a	3.30 \pm 0.79 ^b
Left kidney	0.34 \pm 0.039 ^{a,b}	0.30 \pm 0.027 ^a	0.31 \pm 0.036 ^a	0.36 \pm 0.04 ^b
Right kidney	0.35 \pm 0.029 ^b	0.31 \pm 0.031 ^a	0.31 \pm 0.036 ^a	0.37 \pm 0.032 ^b

¹ Values are means \pm SD, n = 8 except for spleen relative weights, n = 6.

² Within a row, values with different superscripts are significantly different (P < 0.05)

soybean diet had significantly higher mean bone densities of the right femur and fourth lumbar vertebra than rats in the ovx group.

The right femur calcium content (mmol calcium/cm³ bone) was not significantly different among any of the treatment groups compared with the value for

intact animals (sham 7.3 \pm 0.77, ovx 7.3 \pm 0.57, ovx + soybean 7.5 \pm 0.62, and ovx + E₂ 8.0 \pm 0.60). Estrogen-treated animals had a significantly greater fourth lumbar vertebra calcium content than the ovx group or sham group but not the ovx + soybean group (Fig.

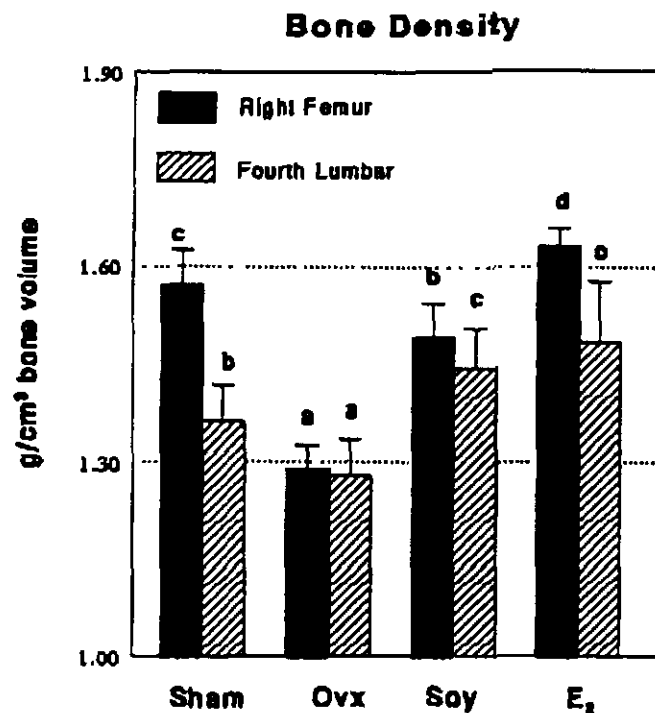


FIGURE 1 Right femur and fourth lumbar vertebra bone densities of sham-operated (sham), ovariectomized (ovx), ovx + soybean (soy) and ovx + 17 β -estradiol (E₂) rats. Values are means \pm SD, n = 8 per group. For each bone, densities with different letters are significantly different (P < 0.05).

Calcium and Phosphorus 4th Lumbar Vertebra

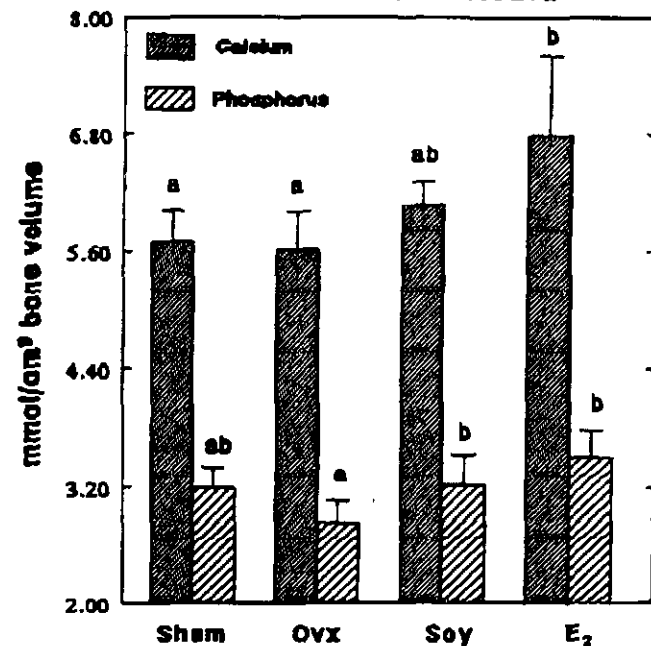


FIGURE 2 Fourth lumbar vertebra calcium and phosphorus contents of sham-operated (sham), ovariectomized (ovx), ovx + soybean (soy), and ovx + 17 β -estradiol (E₂) rats. Values are means \pm SD, n = 8 per group. Calcium or phosphorus values with different letters are significantly different (P < 0.05).

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2). The ovx + E₂ group and ovx + soybean group had significantly greater bone phosphorus contents of the fourth lumbar vertebra (Fig. 2) than the ovx group but not the sham group.

Serum vitamin D metabolites, alkaline phosphatase, tartrate-resistant acid phosphatase, total calcium and phosphate. The serum concentrations of vitamin D metabolites, alkaline phosphatase, tartrate-resistant acid phosphatase, total calcium and phosphate are presented in Table 3. The serum concentrations of 25(OH)D₃ were similar among the treatment groups. However, the serum concentrations of 1,25(OH)₂D₃ were significantly higher in the ovx group than in any other group. Serum alkaline phosphatase and tartrate-resistant acid phosphatase activities were similar in the ovx group and ovx + soybean group and were significantly higher than in both the sham group and the ovx + E₂ group. Serum concentrations of total calcium and phosphorus were not significantly influenced by any of the dietary treatments.

DISCUSSION

The main purpose of this study was to evaluate whether soybean protein isolate is effective in preventing bone loss due to ovariectomy and, if so, whether it functions in a manner similar to estrogen. One of the treatment groups in this study received estrogen. This group served as a positive control group, because the bone-conserving effects of estrogen are well established in an ovariectomized rat model of osteoporosis (Kalu et al. 1991). Our data on bone density and bone calcium support the observations of other investigators that bone loss due to ovarian hormone deficiency is prevented by estrogen administration (Kalu et al. 1991). Additionally, in line with findings of numerous studies, estrogen suppressed the ovariectomy-induced rise in concentrations of biochemical

markers of bone turnover, alkaline phosphatase and tartrate-resistant acid phosphatase (Raisz 1988).

In accord with previous evidence (Kalu et al. 1991), rats in the ovx group had lower densities of the right femur and the fourth lumbar vertebra, the loss of which was completely prevented in animals receiving 17β-estradiol. Compared with the casein diet, the soybean protein diet was also effective in preventing bone loss in the fourth lumbar vertebra and somewhat effective in the right femur. Thus, soybean protein isolate seems to have more of an effect on trabecular than on cortical bone. Because trabecular bone is readily lost due to ovariectomy in this animal model (Wronski et al. 1989), it is reasonable that this type of bone may be more responsive to treatment than is cortical bone.

These observations are supported by recent observations of Omi et al. (1994), who reported a positive effect of a soybean milk-based diet on the bone density of six week-old female rats. They suggested that this positive effect of a soybean milk-based diet may be due to enhanced intestinal calcium absorption. Although we did not assess intestinal calcium absorption in this study, the enhanced intestinal absorption of calcium along with modulation of parathyroid hormone and renal function may provide a partial explanation for the beneficial effects of soybean protein on bone health, as has been suggested by Kalu et al. (1988). Additionally, high protein diets have been linked to increased urinary calcium excretion (Hegsted and Linkswiler 1981), possibly due to the oxidation of sulfur-containing amino acids. Hypercalciuria is thought to be minimized by plant-based diets, because animal-based diets are higher in sulfur-containing amino acids. A recent examination of the cross-cultural variations in animal protein consumption and hip fracture incidence in women over 50 y of age from 34 surveys in 16 countries found a strong positive association when female fracture rates were regressed against estimates of dietary animal protein intakes (Abelow et al. 1992). The association could not be fully explained

TABLE 3

Effects of ovariectomy (ovx), soybean protein, and 17β-estradiol (E₂) on serum concentrations of vitamin D metabolites, calcium and phosphorus and alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activities in rats^{1,2}

Serum measure ³	Experimental group			
	Sham	Ovx	Ovx + Soy	Ovx + E ₂
25(OH)D ₃ , nmol/L	70.9 ± 17.72	69.9 ± 15.47	57.7 ± 11.48	57.4 ± 8.49
1,25(OH) ₂ D ₃ , pmol/L	85.5 ± 30.67 ^a	141.6 ± 56.92 ^b	81.3 ± 20.53 ^a	56.9 ± 12.73 ^a
Calcium, mmol/L	3.39 ± 0.29	3.45 ± 0.28	3.69 ± 0.2	3.55 ± 0.22
Phosphorus, mmol/L	1.96 ± 0.44	2.17 ± 0.53	2.40 ± 0.69	2.15 ± 0.37
ALP, μkat/L	1.20 ± 0.21 ^a	1.84 ± 0.41 ^b	1.97 ± 0.87 ^b	1.13 ± 0.19 ^a
TRAP, nkat/L	48.3 ± 10.5 ^b	64.3 ± 6.8 ^c	70.3 ± 11.7 ^c	29.3 ± 9.8 ^a

¹ Values are means ± SD, n = 8.

² Within a row, values with different superscripts are significantly different (P < 0.05).

³ 25(OH)D₃ = 25-hydroxycholecalciferol; 1,25(OH)₂D₃ = 1,25-dihydroxycholecalciferol.

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by either dietary calcium or total energy intake. The implication was that plant-based (e.g., soybean) diets should be recommended for positive calcium balance in postmenopausal women.

Contrary to the general expectation, ovariectomy significantly increased serum concentrations of $1,25-(\text{OH})_2\text{D}_3$ compared with concentrations in sham animals, whereas 17β -estradiol administration and soybean protein consumption prevented the rise in $1,25(\text{OH})_2\text{D}_3$ due to ovariectomy. As we have previously reported, 17β -estradiol administration results in lower serum concentrations of vitamin D metabolites, while stimulating calcium absorption in intact animals (Arjmandi et al. 1994a). Thus, with respect to the vitamin D metabolites, the phytoestrogens in soybean may be functioning in a manner similar to 17β -estradiol. The vitamin D data suggest that although soybean does not enhance calcium absorption by increasing circulating concentrations of vitamin D metabolites, it is nevertheless effective in preventing bone loss due to ovarian hormone deficiency.

Because serum concentrations of both alkaline phosphatase and tartrate-resistant acid phosphatase were significantly greater in the ovx group and the ovx + soybean group than in the sham and ovx + E_2 groups, this may suggest that soybean protein does not inhibit bone turnover as does 17β -estradiol. Histomorphometric and molecular biology studies are warranted to explain the mechanism of this altered bone turnover and apparent increase in bone formation. Further studies are needed to clarify whether this protective effect on bone is due to the protein itself or to the presence of isoflavones, such as genistein and daidzein, in soybeans (Anderson et al. 1995).

With respect to food intake and body weight, animals in the ovx group had significantly greater final body weights than rats in the sham group. This greater body weight gain due to ovariectomy, despite similar food consumption, has been well documented (Kalu et al. 1991). Both estrogen administration and the soybean diet prevented this ovariectomy-induced body weight gain. Although estrogen treatment is expected to prevent body weight gain due to ovariectomy, to our knowledge this is the first time that such an observation has been made for soybean consumption. Although we have no definitive explanation as to why this occurs, we speculate that soybean protein, which contains isoflavones (such as genistein and daidzein), might serve as a source of proestrogenic compounds. The soybean diet also significantly reduced the abdominal fat in comparison with the sham animals, in spite of equal final body weights, thus suggesting a tissue-specific effect. This finding is of particular interest because it raises the question of whether soybean protein isolate stimulates the synthesis of growth hormone, which is known to decrease adipose tissue mass (Rudman et al. 1990) and increase bone mass (Arjmandi et al. 1994b, Kalu et al. 1993).

As expected, animals in the ovx group had uterine atrophy, which was prevented by 17β -estradiol administration but not by the soybean diet. Isoflavones are readily converted by intestinal bacteria to equol, which is rapidly absorbed in the gut, conjugated in the liver and excreted in the urine (Axelso et al. 1984). Elevated urinary isoflavone concentrations (as much as 1000-fold) have been reported in humans following soybean consumption (Yamazaki and Kinoshita 1986). Both in vitro and in vivo studies have shown that genistein exerts a weak estrogenic effect, approximately 1×10^{-3} to 1×10^{-5} that of estradiol (Farmakalidis et al. 1985). Although some isoflavonoids have been reported to have estrogenic activity (Lerner et al. 1963) and to enhance the uterotrophic activity of estrogens, this was not supported by our data. Thus, isoflavonoids in soybean protein seem to differ from estrogen with respect to uterotrophic activity.

Further studies are needed to examine the effects of soybean protein on body composition, including bone, and calcium metabolism. Animal models of osteoporosis are necessary to elucidate the mechanisms, whereas assessing whether soybean protein has a bone-conserving effect should be addressed in a clinical trial.

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Dietary Soybean Protein Prevents Bone Loss in an Ovariectomized Rat Model of Osteoporosis¹⁹²

BAHRAM H. ARJMANDI,³ LEE ALEKEL, BRUCE W. HOLLIS,* DAXA AMIN, MARIA STACEWICZ-SAPUNTZAKIS, PEILIN GUO AND SUBHASH C. KUKREJA†

Department of Human Nutrition and Dietetics, University of Illinois at Chicago, Chicago, IL 60612;

*Department of Pediatrics, Medical University of South Carolina, Charleston, SC 29425; and

†Department of Medicine, West Side Veterans Administration Medical Center, Chicago, IL 60612

ABSTRACT The purpose of this study was to examine whether soybean protein isolate prevents bone loss induced by ovarian hormone deficiency. Thirty-two 95-d-old Sprague-Dawley rats were randomly assigned to four treatment groups [shamoperated (sham); ovariectomized (ovx); ovx + soybean; ovx + 17 β -estradiol (E₂)] and killed after 30 d. Rats in the sham, ovx and ovx + 17 β -estradiol groups were fed a casein-based diet, and the soybean group was fed soybean protein isolate instead of casein; the diets were otherwise comparable. Rats in the ovx group had significantly lower densities of the right femur ($P < 0.001$) and the fourth lumbar vertebra ($P < 0.05$) than rats in the sham group. These lower bone densities were not observed in animals receiving 17 β -estradiol or fed soybean. The ovx group also had significantly ($P < 0.01$) greater serum concentrations of 1,25-dihydroxycholecalciferol than the other three groups. Our findings suggest that dietary soybean protein is effective in preventing bone loss due to ovarian hormone deficiency. Because serum activities of both alkaline phosphatase and tartrate-resistant acid phosphatase were significantly greater in the ovx group and in the ovx + soybean group but not in the group receiving 17 β -estradiol, compared with sham animals, this confirms that ovariectomy enhances and 17 β -estradiol suppresses the rate of bone turnover. Despite the higher rate of bone turnover in the soybean-fed animals, the vertebral and femoral bone densities of these rats were significantly greater than those of rats in the ovx group, suggesting that formation exceeded resorption. Further studies are needed to clarify whether this protective effect on bone is due to the protein itself or to the presence of isoflavones in soybean protein. *J. Nutr.* 126: 161–167, 1996.

INDEXING KEY WORDS:

- soybean protein isolate • estradiol
- ovariectomy • bone loss • rats

Osteoporosis that is associated with ovarian hormone deficiency following menopause (postmeno-

pausal osteoporosis) is by far the most common cause of age-related bone loss. A sharp decrease in ovarian estrogen production is the predominant cause of rapid hormone-related bone loss during the first decade after menopause (Gruber et al. 1984). Traditional therapies for postmenopausal osteoporosis have emphasized agents that inhibit bone resorption such as estrogen (Centrella and Canalis 1985), calcitonin (Canalis et al. 1988, Rudman et al. 1981) and bisphosphonates (Bennet et al. 1984). Although the most effective method to reduce the rate of postmenopausal bone loss is estrogen replacement therapy, it may be accompanied by side effects (Genant et al. 1989) and is thus recommended **only** for women who are at high risk of osteoporosis and who have no contraindications. Recent advances in bone biology have led researchers to suggest using a combination of anti-resorptive agents, such as estrogen, and formation-stimulating agents, such as growth hormone, toward a cure for osteoporosis (Turner 1991). However, the potential bone-forming agents available today either **may** have serious side effects (e.g., growth hormone, anabolic steroids), may not improve bone quality, or may not decrease susceptibility to fracture (e.g., sodium fluoride). Therefore, it would be most helpful to discover a naturally occurring substance that minimizes bone loss in postmenopausal women, thus decreasing the necessity for drug therapy.

Some reports have indicated that ipriflavone, a synthetic flavonoid derivative (Agnusdei et al. 1989), is effective in preserving bone mass in several models of

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³ To whom correspondence and reprint requests should be addressed.

experimental osteoporosis (Benvenuti et al. 1991, Yamazaki 1986, Yamazaki and Kinoshita 1986). These promising reports regarding the beneficial effects of isoflavones have led us to hypothesize that natural food sources with high concentrations of isoflavonoids might be equally effective in modulating bone mass due to ovarian hormone deficiency. To test this hypothesis we used rats and replaced the casein in their diets with soybean protein. The isoflavones found predominately in soybeans and soybean products are pharmacologically and structurally similar to the synthetic phytoestrogens (e.g., tamoxifen, ipriflavone) that have been shown to be effective in preventing or reducing bone loss. The potential effect of soybean protein on bone health has immense implications should this dietary source of isoflavones be demonstrated effective in the prevention or treatment of osteoporosis.

MATERIALS AND METHODS

Animals and diets. Thirty-two female Sprague-Dawley rats, aged 90 d, were purchased from Harlan Sprague Dawley (Indianapolis, IN) and used for this study when they were 95 d old. Upon arrival at our institution, the rats were housed in an environmentally controlled animal laboratory. Rats were acclimated to a standard laboratory nonpurified diet for 5 d. After acclimation, rats were divided by initial body weight into four blocks of eight rats each, using a randomized complete block design. Two animals from each of the four treatment groups were included in every block: 1) sham-operated (sham group); 2) ovariectomized (ovx group); 3) ovx + soybean group; 4) ovx + 17β -estradiol ($10\mu\text{g/kg}$ body wt per day) (ovx + E_2 group). All rats except those in the ovx + E_2 group received solvent vehicle. 17β -Estradiol was dissolved in a small volume of absolute ethanol and the concentration was adjusted with sesame oil. The solvent vehicle contained similar volumes of ethanol and sesame oil. 17β -Estradiol and solvent vehicle injections were given subcutaneously daily from the date of surgery. Rats were fed isonitrogenous and isocaloric experimental powdered diets. Rats in the sham, ovx, and ovx + E_2 groups were fed a powdered casein-based diet (Teklad, Madison, WI) that contained 0.4% calcium, 0.3% phosphorus and 0.195 nmol vitamin D_3/g diet. Rats in the ovx + soybean group were fed a similar powdered diet in which casein was replaced with soybean protein isolate (22.7 g/100 g diet, Protein Technologies International, St. Louis, MO) with the calcium and phosphorus levels adjusted to the levels in the casein-based diet (Table 1). All rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride and xylazine at doses of 100 mg/kg body wt and 5 mg/kg body wt, respectively. Bilateral ovariectomies were performed in the ovx, ovx + soy, and ovx + E_2 groups, and animals

TABLE 1
Composition of casein and soybean protein diets

Ingredient	Casein diet ¹	Soy protein diet
	g/kg diet	
Protein		
Casein	227.0	—
Soybean protein isolate ²	—	227.0
Carbohydrate		
Sucrose	417.6	417.6
Cornstarch	200.0	200.0
Fiber source (cellulose ³)	56.0	56.0
Fat (corn oil)	57.0	57.0
Vitamin mixture ⁴	10.0	10.0
Total cholecalciferol	0.0016	0.0016
Mineral mixture (Ca-P deficient) ⁵	13.4	13.4
Calcium carbonate	9.88	9.88
Sodium phosphate monobasic	3.88	3.88
Potassium phosphate monobasic	2.38	2.38
Potassium citrate monohydrate	0.90	0.90

¹ Teklad diet #88190 (Harlan Teklad, Madison, WI)

² Soy protein isolate obtained from Protein Technologies International (St. Louis, MO).

³ Alphacel obtained from ICN Biochemicals [Costa Mesa, CA].

⁴ Vitamin mixture (g/kg diet; TD 40060) obtained from Harlan Teklad (Madison, WI): p-aminobenzoic acid, 0.1101; ascorbic acid, 1.0166; biotin, 0.00044; vitamin B-12 [0.1% (trituration), 0.0297; calcium pantothenate, 0.0661; choline dihydrogen citrate, 3.4969; folic acid, 0.00198; inositol, 0.1101; menadione, 0.0495; niacin, 0.0991; pyridoxine [ICN], 0.0220; riboflavin, 0.0220; thiamin HCl, 0.0220; dry retinyl palmitate, 0.0044; dry d, l- α -tocopheryl acetate, 0.2423; corn starch (diluent), 4.6669.

⁵ Mineral mixture (TD 79055) obtained from Harlan Teklad (Madison, WI). This mineral mixture is a modification of AIN 76 lacking calcium, phosphorus, and sucrose as diluent.

in the sham group were subjected to sham operation (Waynforth 1980). Rats that received 17β -estradiol consumed food with ad libitum access, and their precise food intakes were measured every 3 d. Before each feeding, the food remaining was weighed, and the amount ingested was calculated. Rats in the other three groups were pair-fed (plus an additional 1 g/d for growth) to the mean intake of the ovx + E_2 group animals for 30 d from the date of surgery. The rats had free access to deionized water. We killed the animals 30 d after surgery; right femurs and fourth lumbar bones were dissected out for bone analyses. Blood was collected, and serum was separated, divided into small samples and stored at -20°C until required for analyses. Guidelines for the ethical care and treatment of animals from the Animal Care Committee of the University of Illinois at Chicago were strictly followed.

Bone length and density. The right femurs and the fourth lumbar vertebra were freed of soft tissue using small scissors, tweezers and cotton gauze. The length of each femur was measured with a vernier caliper. Before measurement of the bone density, the fe-

mur was cut at the mid-diaphysis and the marrow washed out. Bone volume and density were measured by Archimedes' principle [Kalu et al. 1991]. Briefly, each bone was put in an unstoppered vial filled with deionized water, and the vial was placed under a vacuum for 90 min to ensure that all the trapped air diffused out of the bone. Each bone was removed from its vial, blotted with gauze sponge, weighed, and returned to the vial containing deionized water. The bone was reweighed in water and the density was calculated (g/cm^3 bone volume).

Bone calcium and phosphorus. The bones were hydrolyzed with 6 mol/L HCl at 110°C for 20 h. To measure calcium, aliquots of hydrolysate were properly diluted using 3.1 mmol/L lanthanum solution. Calcium was determined by atomic absorption spectrophotometry (model 503, Perkin-Elmer, Norwalk, CT), and the values were expressed as milligrams of calcium per cubic centimeter of bone volume. Aliquots of bone hydrolysate were also used for the colorimetric analysis of phosphorus with the technique of Taussky and Shorr (1953).

Serum total calcium and phosphate. Serum was appropriately diluted with 3.1 mmol/L lanthanum solution, and calcium was measured using atomic absorption spectrophotometry. Serum phosphate was assessed by the method of Taussky and Shorr (1953).

Serum tartrate-resistant acid phosphatase and alkaline phosphatase. Serum tartrate-resistant acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) were measured spectrophotometrically using kits (procedures no. 435 and 245, respectively) from Sigma Diagnostics (St. Louis, MO). The manufacturer's protocols were followed for each assay. The values for tartrate-resistant acid phosphatase and alkaline phosphatase were expressed in nanokatal per liter and mikrokatal per liter, respectively.

Vitamin D metabolites. The techniques used to measure the serum vitamin D metabolites were previously reported (Hollis 1986, Hollis et al. 1986). Briefly, to determine 1,25-dihydroxycholecalciferol [$1,25(\text{OH})_2\text{D}_3$],⁴ serum samples were extracted with acetonitrile. After centrifugation the supernatant was decanted into a tube containing 0.4 mol/L K_2HPO_4 , pH 10.6, and vortexed. The extract was applied to a $\text{C}_{18}\text{-OH}$ cartridge (Varian Corporation, Harbor City, CA) to isolate and purify $1,25(\text{OH})_2\text{D}_3$ prior to radio-receptor assay. Standards and samples were added to $1,25(\text{OH})_2\text{D}_3$ receptor solution prepared from calf thymus (Instar Corporation, Stillwater, MN). Incubation was performed at 20°C for 45 min, after which tritium-labeled $1,25(\text{OH})_2\text{D}_3$ (Amersham, Arlington

Heights, IL) was added, and the incubation was continued for 45 min. After treatment with dextran-coated charcoal, bound and free hormones were separated, and serum concentrations of $1,25(\text{OH})_2\text{D}_3$ were estimated. The detection limit of the method is 3.90 fmol per tube. To determine serum 25-hydroxycholecalciferol [$25(\text{OH})\text{D}_3$], serum samples were extracted in acetonitrile. After centrifugation, a portion of the supernatant was subjected to direct radioimmunoassay using an antibody generated against the 23,24,25,26,27-pentol-22-carboxylic acid of vitamin D using a radioiodinated tracer. The detection limit of the assay is 7.49 nmol/L serum.

Statistical analysis. Data analysis (GraphPad InStat Software version 2.00, 1993, San Diego, CA) involved estimation of means and SD for each of the groups (Snedecor and Cochran 1967). Analysis of variance was performed to determine whether there were significant ($P < 0.05$) differences among the groups. When an ANOVA indicated any significant difference among the means, the Newman-Keuls multiple comparison test was used to determine which means were significantly different (Chew 1977).

RESULTS

Body weights and food intake. The four treatment groups started with similar mean body weights, but at the end of the study, the ovx group had a significantly higher and the ovx + E_2 group a significantly lower mean body weight than the sham group or the ovx + soybean group (Table 2). This lower mean final body weight of 17β -estradiol-treated rats was not a result of significantly less food intake, because all the treatment groups were pair-fed and hence their food intakes were similar.

Organ weights. The organs that were weighed are listed in Table 2 and are presented relative to body weight. Ovariectomy caused atrophy of the uterus. This was prevented by the 17β -estradiol treatment but not by dietary soybean protein. The abdominal fat mass was significantly lower in animals fed the soybean diet compared with rats in the sham group or the ovx + E_2 group. The left and right kidneys of the 17β -estradiol-treated rats were significantly heavier than those of rats in the ovx group or the ovx + soybean groups but similar to those of the sham group. The other relative organ weights were not significantly different among groups.

Bone density, bone calcium and phosphorus. Animals in the ovx group had significantly lower densities of the right femurs and the fourth lumbar vertebra compared with the sham group (Fig. 1). The animals treated with 17β -estradiol had similar or significantly higher bone densities than rats in the sham group or the ovx + soybean group. The rats fed the

⁴ Abbreviations used. $25(\text{OH})\text{D}_3$, 25-hydroxycholecalciferol; $1,25(\text{OH})_2\text{D}_3$, 1,25-dihydroxycholecalciferol. Group abbreviations: sham group, sham-operated rats; ovx group, ovariectomized rats; ovx + E_2 group, ovariectomized rats receiving daily injections of 17β -estradiol; ovx + soybean group, ovariectomized rats fed soybean protein isolate.

TABLE 2

Effects of ovariectomy (ovx), soybean protein, and 17 β -estradiol (E₂) on food intake, body weights, and relative organ weights in rats^{1,2}

Measure	Experimental group			
	Sham	Ovx	Ovx + soy	Ovx + E ₂
Food intake, g/d	12.3 \pm 1.5	12.2 \pm 1.4	12.1 \pm 1.3	11.2 \pm 4.6
Initial body weight, g	249 \pm 16.0	251 \pm 10.1	249 \pm 8.2	250 \pm 9.3
Final body weight, g	274 \pm 14.3 ^b	295 \pm 15.3 ^c	274 \pm 13.8 ^b	253 \pm 6.6 ^a
Organ weights, g/100 g body wt				
Spleen	0.24 \pm 0.015	0.23 \pm 0.034	0.22 \pm 0.029	0.25 \pm 0.024
Uterus	0.160 \pm 0.044 ^b	0.051 \pm 0.003 ⁱ	0.062 \pm 0.018 ^a	0.182 \pm 0.020 ^b
Liver	3.03 \pm 0.47	3.13 \pm 0.61	3.08 \pm 0.66	3.27 \pm 0.24
Heart	0.35 \pm 0.022	0.36 \pm 0.034	0.35 \pm 0.018	0.39 \pm 0.059
Abdominal fat	3.56 \pm 1.39 ^b	2.36 \pm 0.78 ^{a,b}	1.97 \pm 0.99 ^a	3.30 \pm 0.79 ^b
Left kidney	0.34 \pm 0.033 ^{a,b}	0.30 \pm 0.027 ⁱ	0.31 \pm 0.036 ⁱ	0.36 \pm 0.04 ^b
Right kidney	0.35 \pm 0.029 ^b	0.31 \pm 0.031 ⁱ	0.31 \pm 0.036 ⁱ	0.37 \pm 0.032 ^b

¹ Values are means \pm SD, n = 8 except for spleen relative weights, n = 6.

² Within a row, values with different superscripts are significantly different (P < 0.05)

soybean diet had significantly higher mean bone densities of the right femur and fourth lumbar vertebra than rats in the ovx group.

The right femur calcium content (mmol calcium/cm³ bone) was not significantly different among any of the treatment groups compared with the value for

intact animals (sham 7.3 \pm 0.77, ovx 7.3 \pm 0.57, ovx + soybean 7.5 \pm 0.62, and ovx + E₂ 8.0 \pm 0.60). Estrogen-treated animals had a significantly greater fourth lumbar vertebra calcium content than the ovx group or sham group but not the ovx + soybean group (Fig.

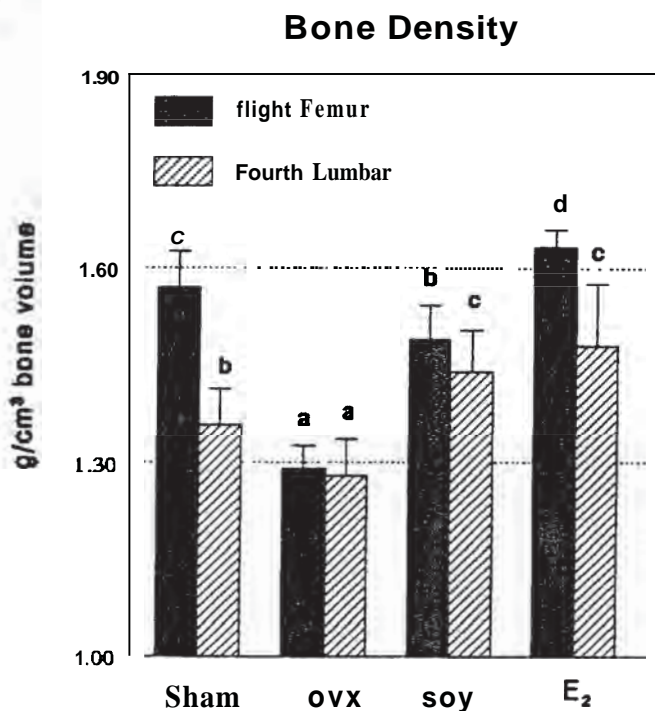


FIGURE 1 Right femur and fourth lumbar vertebra bone densities of sham-operated (sham), ovariectomized (ovx), ovx + soybean (soy) and ovx + 17 β -estradiol (E₂) rats. Values are means \pm SD, n = 8 per group. For each bone, densities with different letters are significantly different (P < 0.05).

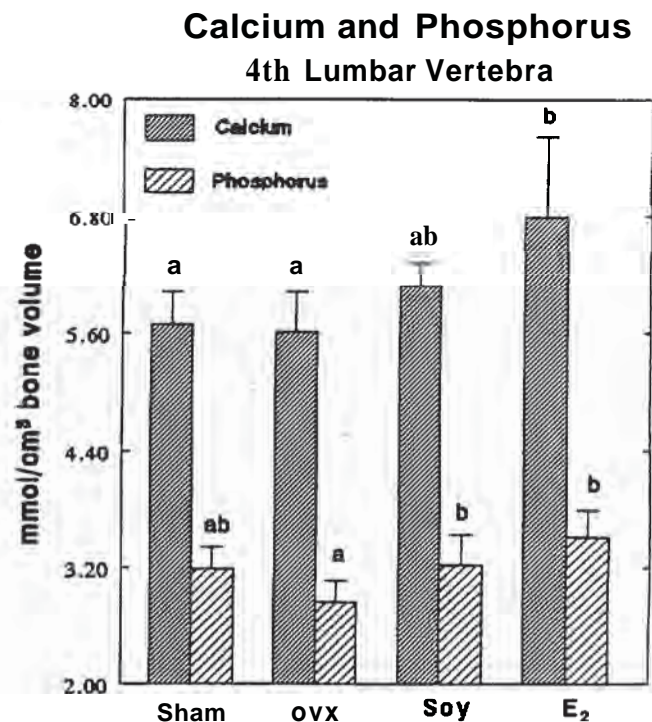


FIGURE 2 Fourth lumbar vertebra calcium and phosphorus contents of sham-operated (sham), ovariectomized (ovx), ovx + soybean (soy), and ovx + 17 β -estradiol (E₂) rats. Values are means \pm SD, n = 8 per group. Calcium or phosphorus values with different letters are significantly different (P < 0.05).

2). The ovx + E₂ group and ovx + soybean group had significantly greater bone phosphorus contents of the fourth lumbar vertebra (Fig. 2) than the ovx group but not the sham group.

Serum vitamin D metabolites, alkaline phosphatase, tartrate-resistant acid phosphatase, total calcium and phosphate. The serum concentrations of vitamin D metabolites, alkaline phosphatase, tartrate-resistant acid phosphatase, total calcium and phosphate are presented in Table 3. The serum concentrations of 25(OH)D₃ were similar among the treatment groups. However, the serum concentrations of 1,25(OH)₂D₃ were significantly higher in the ovx group than in any other group. Serum alkaline phosphatase and tartrate-resistant acid phosphatase activities were similar in the ovx group and ovx + soybean group and were significantly higher than in both the sham group and the ovx + E₂ group. Serum concentrations of total calcium and phosphorus were not significantly influenced by any of the dietary treatments.

DISCUSSION

The main purpose of this study was to evaluate whether soybean protein isolate is effective in preventing bone loss due to ovariectomy and, if so, whether it functions in a manner similar to estrogen. One of the treatment groups in this study received estrogen. This group served as a positive control group, because the bone-conserving effects of estrogen are well established in an ovariectomized rat model of osteoporosis (Kalu et al. 1991). Our data on bone density and bone calcium support the observations of other investigators that bone loss due to ovarian hormone deficiency is prevented by estrogen administration (Kalu et al. 1991). Additionally, in line with findings of numerous studies, estrogen suppressed the ovariectomy-induced rise in concentrations of biochemical

markers of bone turnover, alkaline phosphatase and tartrate-resistant acid phosphatase (Raisz 1988).

In accord with previous evidence (Kalu et al. 1991), rats in the ovx group had lower densities of the right femur and the fourth lumbar vertebra, the loss of which was completely prevented in animals receiving 17 β -estradiol. Compared with the casein diet, the soybean protein diet was also effective in preventing bone loss in the fourth lumbar vertebra and somewhat effective in the right femur. Thus, soybean protein isolate seems to have more of an effect on trabecular than on cortical bone. Because trabecular bone is readily lost due to ovariectomy in this animal model (Wronski et al. 1989), it is reasonable that this type of bone may be more responsive to treatment than is cortical bone.

These observations are supported by recent observations of Omi et al. (1994), who reported a positive effect of a soybean milk-based diet on the bone density of six week-old female rats. They suggested that this positive effect of a soybean milk-based diet may be due to enhanced intestinal calcium absorption. Although we did not assess intestinal calcium absorption in this study, the enhanced intestinal absorption of calcium along with modulation of parathyroid hormone and renal function may provide a partial explanation for the beneficial effects of soybean protein on bone health, as has been suggested by Kalu et al. (1988). Additionally, high protein diets have been linked to increased urinary calcium excretion (Hegsted and Linkswiler 1981), possibly due to the oxidation of sulfur-containing amino acids. Hypercalciuria is thought to be minimized by plant-based diets, because animal-based diets are higher in sulfur-containing amino acids. A recent examination of the cross-cultural variations in animal protein consumption and hip fracture incidence in women over 50 y of age from 34 surveys in 16 countries found a strong positive association when female fracture rates were regressed against estimates of dietary animal protein intakes (Abelow et al. 1992). The association could not be fully explained

TABLE 3

Effects of ovariectomy (ovx), soybean protein, and 17 β -estradiol (E₂) on serum concentrations of vitamin D metabolites, calcium and phosphorus and alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activities in rats^{1,2}

Serum measure ³	Experimental group			
	Sham	Ovx	Ovx + soy	Ovx + E ₂
25(OH)D ₃ , nmol/L	70.9 \pm 17.72	69.9 \pm 15.47	57.7 \pm 11.48	57.4 \pm 8.49
1,25(OH) ₂ D ₃ , pmol/L	85.5 \pm 30.67 ^a	141.6 \pm 56.92 ^b	81.3 \pm 20.53 ^a	56.9 \pm 12.73 ^a
Calcium, mmol/L	3.39 \pm 0.29	3.45 \pm 0.28	3.69 \pm 0.2	3.55 \pm 0.22
Phosphorus, mmol/L	1.96 \pm 0.44	2.17 \pm 0.53	2.40 \pm 0.69	2.15 \pm 0.37
ALP, μ kat/L	1.20 \pm 0.21 ^a	1.84 \pm 0.41 ^b	1.97 \pm 0.87 ^b	1.13 \pm 0.19 ^a
TRAP, nkat/L	48.3 \pm 10.5 ^b	64.3 \pm 6.8 ^c	70.3 \pm 11.7 ^c	29.3 \pm 9.8 ^a

¹ Values are means \pm SD, n = 8.

² Within a row, values with different superscripts are significantly different (P < 0.05).

³ 25(OH)D₃ = 25-hydroxycholecalciferol; 1,25(OH)₂D₃ = 1,25-dihydroxycholecalciferol.

by either dietary calcium or total energy intake. The implication was that plant-based (e.g., soybean) diets should be recommended for positive calcium balance in postmenopausal women.

Contrary to the general expectation, ovariectomy significantly increased serum concentrations of $1,25\text{-(OH)}_2\text{D}_3$ compared with concentrations in sham animals, whereas 17β -estradiol administration and soybean protein consumption prevented the rise in $1,25\text{-(OH)}_2\text{D}_3$ due to ovariectomy. As we have previously reported, 17β -estradiol administration results in lower serum concentrations of vitamin D metabolites, while stimulating calcium absorption in intact animals (Arjmandi et al. 1994a). Thus, with respect to the vitamin D metabolites, the phytoestrogens in soybean may be functioning in a manner similar to 17β -estradiol. The vitamin D data suggest that although soybean does not enhance calcium absorption by increasing circulating concentrations of vitamin D metabolites, it is nevertheless effective in preventing bone loss due to ovarian hormone deficiency.

Because serum concentrations of both alkaline phosphatase and tartrate-resistant acid phosphatase were significantly greater in the ovx group and the ovx + soybean group than in the sham and ovx + E_2 groups, this may suggest that soybean protein does not inhibit bone turnover as does 17β -estradiol. Histomorphometric and molecular biology studies are warranted to explain the mechanism of this altered bone turnover and apparent increase in bone formation. Further studies are needed to clarify whether this protective effect on bone is due to the protein itself or to the presence of isoflavones, such as genistein and daidzein, in soybeans (Anderson et al. 1995).

With respect to food intake and body weight, animals in the ovx group had significantly greater final body weights than rats in the sham group. This greater body weight gain due to ovariectomy, despite similar food consumption, has been well documented (Kalu et al. 1991). Both estrogen administration and the soybean diet prevented this ovariectomy-induced body weight gain. Although estrogen treatment is expected to prevent body weight gain due to ovariectomy, to our knowledge this is the first time that such an observation has been made for soybean consumption. Although we have no definitive explanation as to why this occurs, we speculate that soybean protein, which contains isoflavones (such as genistein and daidzein), might serve as a source of proestrogenic compounds. The soybean diet also significantly reduced the abdominal fat in comparison with the sham animals, in spite of equal final body weights, thus suggesting a tissue-specific effect. This finding is of particular interest because it raises the question of whether soybean protein isolate stimulates the synthesis of growth hormone, which is known to decrease adipose tissue mass (Rudman et al. 1990) and increase bone mass (Arjmandi et al. 1994b, Kalu et al. 1993).

As expected, animals in the ovx group had uterine atrophy, which was prevented by 17β -estradiol administration but not by the soybean diet. Isoflavones are readily converted by intestinal bacteria to equol, which is rapidly absorbed in the gut, conjugated in the liver and excreted in the urine (Axelso et al. 1984). Elevated urinary isoflavone concentrations (as much as 1000-fold) have been reported in humans following soybean consumption (Yamazaki and Kinoshita 1986). Both in vitro and in vivo studies have shown that genistein exerts a weak estrogenic effect, approximately 1×10^{-3} to 1×10^{-5} that of estradiol (Farmakalidis et al. 1985). Although some isoflavonoids have been reported to have estrogenic activity (Lerner et al. 1963) and to enhance the uterotrophic activity of estrogens, this was not supported by our data. Thus, isoflavonoids in soybean protein seem to differ from estrogen with respect to uterotrophic activity.

Further studies are needed to examine the effects of soybean protein on body composition, including bone, and calcium metabolism. Animal models of osteoporosis are necessary to elucidate the mechanisms, whereas assessing whether soybean protein has a bone-conserving effect should be addressed in a clinical trial.

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**McKenna & Cuneo, LLP**

Attorneys at Law

Los Angeles

San Francisco

San Diego

1900 K Street, N.W. ■ Washington, D.C. 20006-1108
202-496-7500 ■ Fax: 202-496-7756
www.mckennacuneo.com

Denver

Dallas

Brussels

July 14, 1999

Gary L. Yingling

202-496-7645

gary_yingling@mckennacuneo.com

Alan M. Rulis, Ph.D.
Director, Office of Premarket Approval (HFS-206)
Center for Food Safety & Applied Nutrition
U.S. Food and Drug Administration
200 C Street, S.W.
Washington, D.C. 20204

Re: Pre-Meeting on Isoflavones

Dear Dr. Rulis:

This is to confirm that my client, Archer Daniels Midland Company (ADM), has requested a pre-meeting for the purpose of discussing with the FDA Office of Premarket Approval (OPA) the current information that ADM has concerning the safe use of isoflavones and how that information may or should be provided to FDA.

Based on a recent conversation with Dr. Kahl of OPA, we understand that a meeting has been scheduled for July 21, 1999 at 2:30 p.m. at 1110 Vermont Avenue, N.W., in the 7th floor conference room. We will come to the 12th floor to sign in.

Attending the meeting will be: Dr. Water Glinsmann of Glinsmann Inc.; Dr. Joseph Borzelleca of Toxicology and Pharmacology Inc.; Dr. Mark Empie and Dr. Gary Miller of ADM; and myself.

We will be providing to the agency in the next day or two a copy of the final draft expert GRAS panel report which will include an executive summary and the

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McKenna & Cuneo, LLP

Attorneys at Law

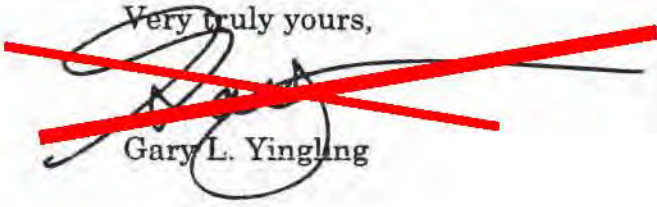
Alan M. Rulis, Ph.D.

July 14, 1999

Page 2

complete report. We will not be providing copies of the articles referenced in the report. If you have any questions, please do not hesitate to contact me.

Very truly yours,


Gary L. Yingling

GLY/lh

cc: ✓ Dr. Linda Kahl, FDA OPA
Dr. George Pauli, FDA OPA
Dr. Laura Tarantino, FDA OPA

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Historical Time Line

- ◆ Pre-meeting
- ◆ GRN 000001 filed March 5, 1998 notifying FDA of ADM self-affirmation of soy isoflavone extract
- ◆ Dr. Sheehan from NCTR submits letter objecting to GRAS status
- ◆ ADM meets with FDA to present information addressing endocrine issues (Sept. 9, 1998)
- ◆ ADM conducts additional literature search and seeks review

Historical Time Line

- ◆ File is closed by FDA on (Nov. 3, 1998) while information is updated to reflect new research results
- ◆ Additional information submitted to independent Expert Panel for review
- ◆ Expert Panel completes report
- ◆ Panel concludes ADM soy isoflavone extract is GRAS

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Conclusions of the Expert Panel

● **Estrogenic/Women's Health**

- There are no hormonal changes or they are of such modest effect as to be of little concern
- There is no support for suggested effects on fertility

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Conclusions of the Expert Panel

◆ Endocrine and Development

- Significant species differences in physiological response to isoflavones
- Animal studies do not indicate consistent compound and dose-dependent adverse effects
- Experience in Far Eastern diets and infant formulas provide no support for suggested effects on growth and development or endocrine function

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Conclusions of the Expert Panel

◆ **Thyroid**

- Thyroid impairment hypothesis is unsupported by existing data in animal and human studies

◆ **Vascular dementia**

- No linkage between soy isoflavones and vascular dementia or neuroendocrine disorders

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Conclusions of the Expert Panel

◆ Health Benefits

- Data is suggestive of positive health benefits for soy isoflavones toward breast and prostate cancer risk reduction, maintenance of healthy bone calcium and plasma cholesterol levels, and supportive of cardiovascular health.

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Conclusions of the Expert Panel

◆ Conclusion

- ADM's soy product, Novasoy™ isoflavones, is GRAS for the proposed uses.

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