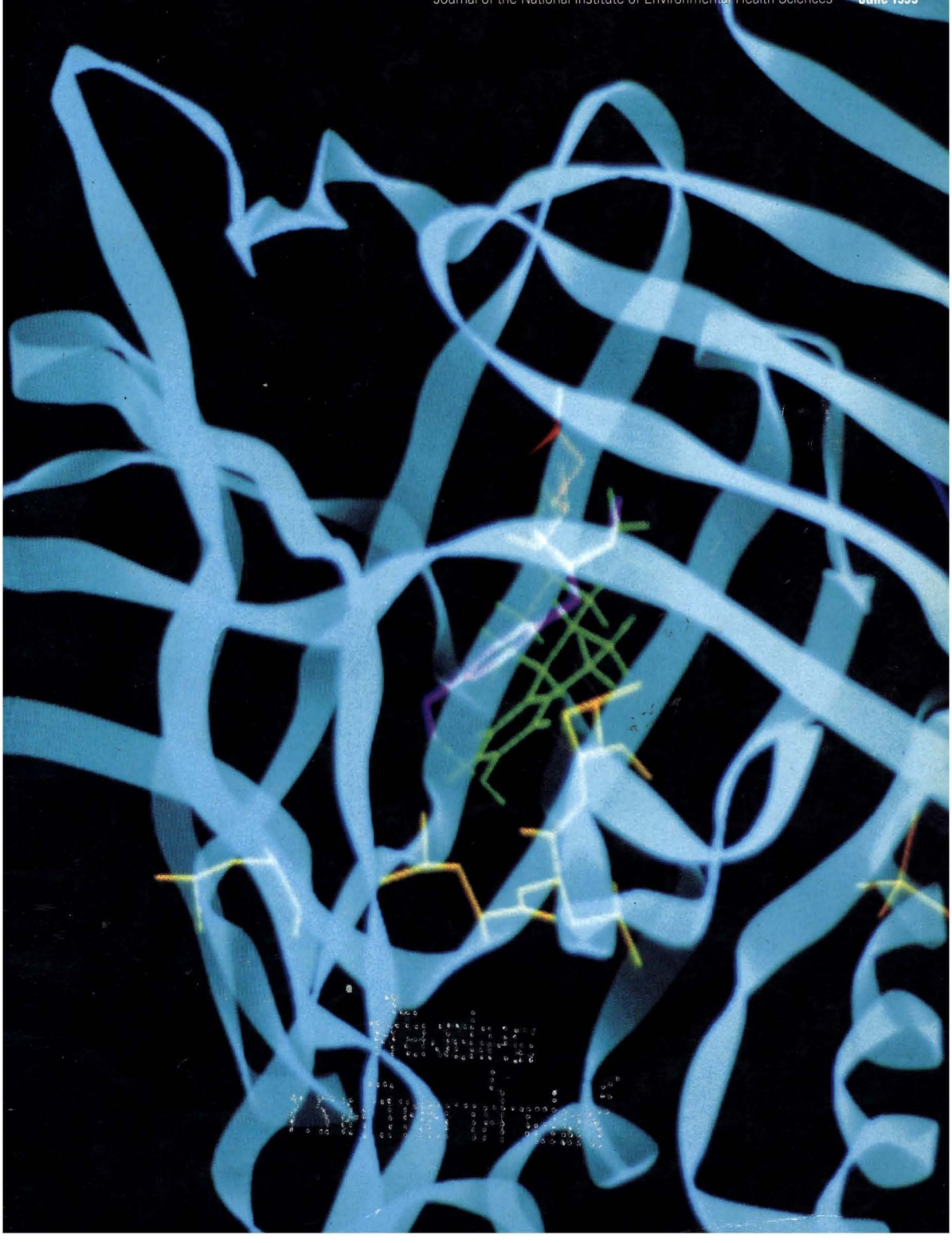
Environmental Health
perspectives
Volume 103
Number 6
Pages 525–632
Journal of the National Institute of Environmental Health Sciences
June 1995



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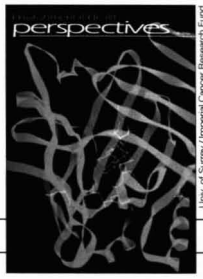
On The Cover:
The human estrogen receptor gene has assumed an important role in research on the effects of xenoestrogens (see Markaverich et al., p. 574, and Jobling et al., p. 582). PHOTO COURTESY OF DAVID F. V. LEWIS.



Environmental Health Perspectives

Environmental Health Perspectives is published monthly by the Public Health Service, U.S. Department of Health and Human Services.
For subscription information, see page 625.

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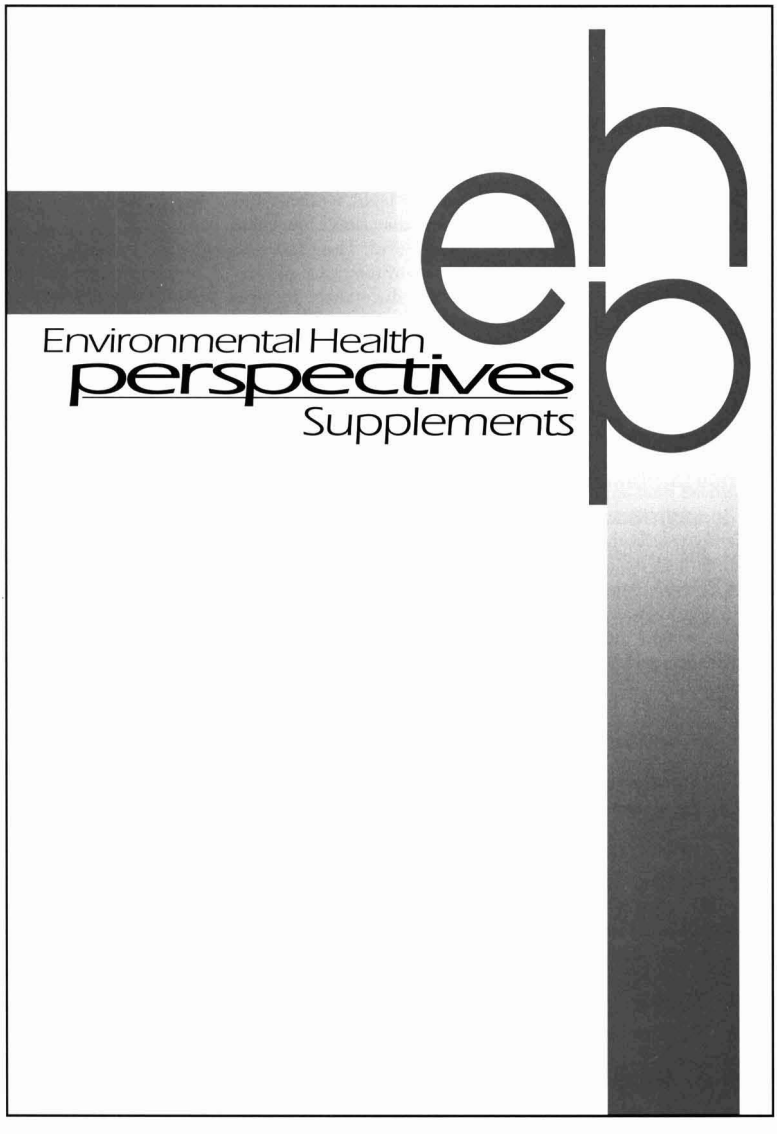

A Tradition of Progress

Environmental Health Perspectives Supplements continues its 20-year tradition of publishing the most important developments in the environmental health sciences arising from conferences, symposia, and workshops.

For subscription information, see p. 624

In This Issue

Household Pesticides

The first **Focus** article (p. 550) reports the use of 4 billion household pesticide applications per year. Although allergies to insect parts, toxins, and vector-borne diseases are valid reasons to reduce infestations, potential adverse health effects of household pesticides pose special risks for children and the elderly. More studies on the potential chronic toxicity of pesticides are needed, but in the meantime, simple improvements in warning labels could help to reduce accidental poisonings.

Diet and Cancer

Scientists and the public share a gut feeling that proper diets may reduce cancer. Although the second **Focus** article (p. 556) supports this hypothesis, proof is wanting. The best information is derived from animal studies, which have repeatedly shown that reduction in caloric intake reduces the incidence of tumors. Multimillion-dollar studies are underway in an attempt to explain the mechanisms involved between certain foods and cancer, with hopes that dietary regimens can some day be used to reduce the incidence of cancer.

Sugar for Benzene

Techniques for replacing benzene with glucose for production of commercial chemicals has been accomplished using genetic engineering with microbes. The **Innovations** article (p. 564) offers promise that instead of using an non-renewable resource like petroleum to synthesize benzene, fields of trees or plains of grasses could supply glucose as raw material and eliminate dangerous by-products from benzene chemistry in the bargain.

Fungicides and Human Health

Occupational exposure to the common class of fungicides known as the dithiocarbamates occurs in various industries and in farming. Parent ethylenebisdithiocarbamate compounds are toxic, as is the metabolite ethylenethiourea. Chronic human exposure leads to risks for carcinogenesis, as proven in animal studies. Houeto et al. (p. 568) urge that further human studies are necessary to quantitate these risks, but that regular surveillance of humans exposed to dithiocarbamates be initiated now, with particular attention to thyroid and liver toxicity.

Estrogen Receptors

Phytoestrogens are plant-derived compounds like the isoflavonoid coumestrol which bind to estrogen receptors and exhibit estrogenic activity *in vivo*. Markaverich et al. (p. 574) showed that coumestrol was an atypical phytoestrogen because even though it increased the mass of the uterus, it failed to stimulate cellular DNA synthesis and proliferation in immature female rats whose ovaries had been removed. These data raise the possibility that environmental phytoestrogens may act differently in premenopausal and post-menopausal women, and also indicate an incomplete understanding for the proposed procarcinogenic or anticarcinogenic effects of compounds like coumesterol.

Estogenicity of Phthalates

Jobling et al. (p. 582) found that 10 of 20 chemicals present in sewage were weakly estrogenic, as judged by estrogen receptor binding, proliferation of breast cancer cells, or stimulation of an estrogen-responsive reporter gene. The data suggest that contaminants such as phthalate esters could exert cumulative effects with other natural estrogenic compounds and enhance potential adverse effects on fertility or estrogen-related carcinogenicity.

Prenatal BaP + Lead Affects Fertility

The effects of potential chemical synergism on fertility were examined in an animal model. Kristensen et al. (p. 588) exposed pregnant mice to subtoxic lead concentrations or to lead plus benzo[*a*]pyrene to determine the degree of interaction between these chemicals. Data collected from six-month continuous breeding trials of their offspring demonstrated that the combination of chemical exposure reduced almost all indicators of ovarian development and fertility compared to those observed in mice exposed to BaP alone. Lead concentrations ranged between 2.44 and 5.26 µM/L in the exposed groups. There are no equivalent human data to gauge the possible detrimental effects of multiple chemical exposure.

Pregnancy and Trihalomethanes

Savitz et al. (p. 592) evaluated the reproductive health consequences of human exposure to chlorinated chemicals in the drinking water. Records from North Carolina on miscarriages, preterm deliveries, and low birth weights were used to assess risks associated with water source, amount, and trihalomethane concentration. There was no clear association between chlorination byproducts and adverse pregnancy outcome.

Mortality in the Butadiene Industry

Mortality among 374 men working in rubber plants exposed to 1,3-butadiene revealed significantly elevated standardized mortality ratios for lymphosarcoma and reticulosarcoma based on 4 observed cases. Ward et al. (p. 598) examined records from plants where butadiene was a primary product and neither benzene nor ethylene oxide was present. An excess of these tumor types was also observed in the only other cohort of butadiene production workers previously studied. These data add to the weight of evidence for carcinogenicity of 1,3-butadiene in humans.

Mercury Exposure from Eating Seabass

Knobeloch et al. (p. 604) present a case study of a Wisconsin family who exhibited blood mercury levels 6- to 10-fold above normal. No sources of exposure were present; mercury came from a constant diet of fish. A variety of fish were eaten, but only seabass contained 0.5–0.7 mg/kg mercury; consumption estimates suggested the family consumed an average daily mercury intake of 0.5–0.8 µg/kg per week. Avoiding seabass consumption for 6 months resulted in blood mercury concentrations dropping to normal in the parents (3–5 µg/L), and sequential blood samples confirmed that half of the mercury was eliminated within 60 days.

Xenoestrogens in Food Cans

Brotons et al. (p. 608) report that extracts from canned foods or packing water exhibited some estrogenic activity in an MCF7 proliferative cell E-screen test. The lacquer-coated food cans were purchased in supermarkets from Spain and the United States. Some of the cans were packed in Brazil, France, and Turkey. Bisphenol A leached from the lacquer coating was identified by mass spectrometry as the contaminant with estrogenic activity in the canned food and water.

ANNOUNCING

DIOXIN'95

15TH INTERNATIONAL SYMPOSIUM ON CHLORINATED DIOXINS AND RELATED COMPOUNDS

AUGUST 21-25, 1995
EDMONTON, ALBERTA, CANADA

The 15th International Symposium on Chlorinated Dioxins and Related Compounds (DIOXIN'95) will define the state of knowledge and future trends in formation, sources, incineration, transport and fate, emission controls, remediation, human and ecosystem toxicology, epidemiology, mechanism of action, risk, regulatory approaches, and analytical methods for these chemicals. Although the chlorinated dioxins and furans are the focus of the meeting, significant coverage will be given to other organochlorine chemicals such as PCBs, pesticides, herbicides, terphenyls, naphthalenes, toxaphene, chlorophenols, chlorobenzenes, and short-chain aliphatics. A plenary session is being organized to address the current knowledge on the human risk of Dioxins/Furans (speakers: Professor Stephen Safe, Dr. Ellen Silbergeld, additional speakers to be announced). In addition, pre-Symposium workshops are being organized for "Critical Factors in Environmental Sampling and Analysis"; "Immunoassay Analysis of Hazardous Wastes"; and "Accelerated Solvent Extraction".

Interested persons should contact the DIOXIN'95 Secretariat for registration forms and additional details. Bound volumes of four-page short papers of all presentations will be provided to delegates at the time of registration. Symposium co-chairs: Dr. S. Ramamoorthy, Alberta Environmental Protection; Dr. R.E. Clement, Ontario Ministry of Environment and Energy.

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Ecosystem Services: An Essential Component of Sustainable Use

Ecosystem services are those functions of natural systems perceived as beneficial to human society. Examples of ecosystem services are maintenance of atmospheric gas balance and water quality and preserving and providing genetic material for pest-resistant plants that will grow under conditions that the more commonly used agricultural plants will not [for a discussion, see Westman (*1*) and Cairns and Niederlehner (*2*)]. At the planet's present population density of approximately 5.6 billion and the probability that it will reach 10 billion before the middle of the next century, human society is dependent on both a technological and ecological life support system. Since huge numbers of people now live in urban areas with little contact with natural systems, society is often unaware of its dependency on these systems. Making a commitment to ensuring that future generations have the same amenities that we enjoy involves protecting both the technological and ecological components of our life support system. Because the development of technological services may impair the delivery of ecosystem services, attention must be given to balancing the delivery of both technological and ecosystem services (*3*).

One of the serious flaws in the development of sustainable use of the planet for the future is the failure to anticipate episodic events that are either beyond human control or are not predicted by present models. For example, crop failures due to pests, plant diseases, drought, exceptionally heavy rainfall, or depletion of the soils causes poor production. In the reports of extremely heavy flooding in northern California during March 1995, it was not stressed that the flooding might have been markedly reduced had California not lost 91% of its wetlands in the last 200 years (*4*) or had it not devegetated large areas while simultaneously increasing the percentage of impervious surfaces such as roads, parking lots, and buildings. Each of these decisions (to fill in a wetland here, to build a parking lot there, to put a housing development somewhere else, and log yet another area) undoubtedly made sense when the decision was made in isolation from all the other decisions, but, when taken in the aggregate, all of these small decisions usually have environmental and other impacts far beyond those contemplated. This effect has been called the "tyranny of small decisions" by the economist A. E. Kahn (*5*) and subsequently by the ecologist W. E. Odum (*6*). Given the present level of information and the processing and storage capabilities of today's computers, there is little excuse for not becoming aware of the aggregate effects of a series of seemingly unrelated decisions. In addition to cumulative impacts, we should also determine if the planet has a time-dependent carrying capacity. ("Carrying capacity" is defined as that degree of environmental use beyond which no major human population increase will occur.) Can we continue present practices forever? Can we assume that long periods of equilibrium will exist in the next century? Some safety factors must be built in for the protection of ecosystem services.

To protect ecosystem services, we must determine which functions of ecosystems are essential to the survival of human society. As a corollary, we must determine which of the ecosystem services will not continue if other ecological functions, not perceived as beneficial to human society, are degraded. In this context, five assertions follow:

1) *Ecosystem services are as important to the survival of present human society as technological services.* For most of human existence, our life support system has been entirely ecological. Following the agricultural and industrial revolutions, the population exploded, per capita affluence increased dramatically, and, instead of being thinly distributed across the planet, large numbers of people were concentrated in urban and suburban areas. Without the technological life support system, which delivers food, energy, and transportation, the present population density, level of affluence, and distribution would not be possible. However, the operation of the technological life support system is threatening the ecological life support system, without which continued use of the planet at present population densities and levels of affluence will not be possible.

2) *Replacing the services provided by natural systems with comparable services provided by technological systems will be at least an order of magnitude more expensive.* Probably the best evidence on cost is from the space effort and Biosphere 2. Avise (*7*) notes that the estimated cost of supplying ecosystem services to seven people in Biosphere 2 was $9 million per person per year. Arguably, if these services were provided to larger numbers of people, there would be an economy of scale, but technology to do so at a reasonable cost does not appear probable for at least five decades or perhaps never.

3) *Sustainable use of the planet is impossible without ecosystem services.* Effective sustainable use will depend on robust estimates of the planet's carrying capacity for people at particular levels of affluence and dependence on technology and energy. This level of use must not threaten the integrity of the ecological life support systems.

4) *The quantity of ecosystem services per capita can be increased through ecological restoration of damaged ecosystems.* Obviously, damaged ecosystems are unlikely to provide the same level of services as healthy ecosystems. Therefore, without increasing the area involved, the level of services can be improved through restoration, repair, and healing.

5) *Ecosystem services can also be improved with existing undamaged ecosystems by focusing on their health rather than merely protecting them.* This assertion is merely an extension of the previous one. Once the systems are restored, repaired, or healed, they must be kept healthy to get optimal levels of services.

If we are indeed dependent on an ecological life support system, then attention must be given to this system lest it fail through ever-increasing pressure of population, expectations of affluence, and technological impacts. Because not much attention has been given to ecosystems as life support systems, this information must be generated quickly. Otherwise, we may lose components that are irreplaceable. The use of the word "services" has its drawbacks because we might be conditioned to think of ecosystems only in terms of their service functions. However, aesthetic appreciation, compassion for other species, and our responsibility as stewards of the planet cannot be ignored. On the other hand, for those who do not accept these views, the fact that our survival may depend on ecosystems services may change their behavior if the evidence is persuasive.

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XIVth World Congress on Occupational Safety and Health
April 22–26, 1996
Madrid, Spain

The XIVth World Congress on Occupational Safety and Health will be held in Madrid from April 22 to April 26, 1996. The organizers are the Spanish Ministry of Labour and Social Security, through the National Institute for Occupational Safety and Health (INSHT), the International Labour Office (ILO), Geneva, and the International Social Security Association (ISSA), Geneva.

These World Congresses, of which the first was held in Rome in 1955 and the last in New Delhi in 1993, have had such venues as Brussels, Paris, London, Zagreb, Vienna, Dublin, Bucharest, Amsterdam, Ottawa, Stockholm and Hamburg.

The XIVth World Congress, to be held in Madrid, aims to be an open forum for all persons involved in risk prevention at work, safety and health safety specialists, occupational health physicians, labour inspectors, persons directly concerned with safety and health at work, including entrepreneurs and managers in enterprises, trade union representatives, manufacturers and importers, as well as heads of public administration and social security administrators.

The main focus of this Congress will be on the consequences for occupational safety and health of processes of international and regional integration (e.g. EU, NAFTA) and of the globalization of economic relations, on an in-depth analysis of chemical risks and on new proposals for cooperation and participation within enterprises. Other specific issues will also be dealt with, such as training and information, control of working conditions or new responsibilities. Special emphasis will be placed on small and medium-sized enterprises and sectors facing specific problems with regard to safety and health at work, such as the construction sector and agriculture.

In addition, as part of this Congress, the International Section "Electricity" of the ISSA will be organizing the 3rd International Film and Video Festival on Occupational Safety and Health.
Should you require any further information, please contact:

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COMPACT Predictions: Is There a Catch?

Lewis et al. reported a retrospective evaluation of COMPACT predictions of rodent carcinogenicity for 44 chemicals evaluated in long-term bioassays by the National Toxicology Program (*EHP* 103:178–184). They concluded that COMPACT performed quite well. I was surprised to read this, because I had seen the published COMPACT carcinogenicity predictions regarding these chemicals (*1*) and knew that the method had not performed particularly well. Thus, I was interested in learning how the method's predictive performance had been enhanced. After examining the paper by Lewis et al. (*1*) in more detail, it quickly became clear that the authors had employed several questionable data manipulations in their retrospective analysis to improve the performance of their method.

Publishing predictions of carcinogenicity before the study outcomes are known firmly establishes the predictions and permits an easy assessment of their accuracy. Prospective predictions are important because a predictive methodology that truly works should be able to predict the carcinogenic potential of untested chemicals. Unfortunately, COMPACT had little success in this regard. Based on Lewis et al.'s predictions (*1*), COMPACT was able to correctly predict the carcinogenicity outcome for only 56% (20/36) of the NTP chemicals, a success rate not significantly different from flipping a coin. The 16 chemicals for which these COMPACT predictions were inaccurate are given in Table 1.

Table 1. Chemicals for which COMPACT incorrectly predicted carcinogenicity

NTP noncarcinogens predicted to be carcinogens	NTP carcinogens predicted to be noncarcinogens
Promethazine	*o*-Benzyl-*p*-chlorophenol
Resorcinol	Methylphenidate hydrochloride
p-Nitrophenol	Diphenylhydantoin
Tricresyl phosphate	Tris(2-chloroethyl)phosphate
Chloramine	2,3-Dibromo-1-propanol
4,4'-Diamino 2,2'-stilbenedisulfonic acid	1,2,3-Trichloropropane
CI Pigment Red 23	
4-Hydroxyacetanilide (acetaminophen)	
HC Yellow 4	
p-Nitroaniline	

Table 2. COMPACT and Hazardexpert predictions for six NTP equivocal carcinogens

Chemical	COMPACT prediction	Hazardexpert prediction	Carcinogenicity outcome[a]
γ-Butyrolactone	-	-	-
Chloramine	+	-	-
CI Pigment Red 23	+	+	+
4-Hydroxyacetanilide	+	-	+
HC Yellow 4	-	+	-
p-Nitroaniline	+	+	+

[a]These carcinogenicity outcomes reflect the views of Lewis et al., not the conclusions of the NTP. Lewis et al. concluded that the carcinogenicity outcomes for all six chemicals were predicted correctly by COMPACT/Hazardexpert.

Given these results, it would have been appropriate for the authors to attempt to understand the reasons behind COMPACT's failures and to make the necessary modifications so that the method might be more successful when applied prospectively to a new set of chemicals. Instead, the authors reevaluated the predictions made for the 44 NTP chemicals and manipulated the data in various ways to show that COMPACT (when used in combination with Hazardexpert) really had only 5 discordant carcinogenicity predictions, not 16. The more important data manipulations are discussed below.

Retrospective changes in the COMPACT predictions. For two chemicals, Lewis et al. changed their previously published carcinogenicity predictions from positive to negative. For HC Yellow 4, the authors stated in footnote *b* to Table 1 (p. 179) that while their original prediction was positive, "calculation based on new structure gives negative." For resorcinol, the authors apparently justify the changed prediction by asserting that "the original graphical analysis was clearly negative." However, the paper they cite to justify the negative graphical analysis is their previous paper (*1*), in which they clearly report their carcinogenicity prediction for resorcinol to be positive, not negative. Both changes result in correct predictions, reducing the number of discordant predictions from 16 to 14.

Reinterpretation of NTP's equivocal carcinogenicity results. Equivocal responses often occur in rodent carcinogenicity studies. Lewis et al. state that for assessing concordance "when this single [the carcinogenicity] response is EE (equivocal evidence), the overall response is . . . taken as '-' in the final assessment" (pp. 178–179). This is a reasonable approach, but the authors did not follow this rule when assessing the predictive performance of COMPACT.

There were nine NTP chemicals for which only equivocal evidence of carcinogenicity was observed. For three equivocal carcinogens predicted by COMPACT to be positive (CI pigment red 23, 4-hydroxyacetanilide, and *p*-nitroaniline), the authors reevaluated the results and concluded that these studies were "weak positives/equivocal positives based on pathology reports" (Table 2, p. 180), and thus their predictions that the chemicals would be carcinogens were correct after all. This reduced the number of discordant predictions from 14 to 11.

The source of the "pathology reports" is not given, but clearly Lewis et al.'s interpretation of these studies does not reflect the views of the NTP, which concluded that these three bioassays showed equivocal, not weakly positive, carcinogenic effects (as did the other six chemicals showing equivocal responses). There are several reasonable options for dealing with equivocal carcinogenicity outcomes. One is to regard them all as positive or all as negative (the latter being the rule the authors claim to have followed, as noted previously). Alternatively, chemicals with equivocal or uncertain findings could be excluded from consideration altogether when evaluating predictive methodologies. Less defensible is the strategy used by the authors, who attempted to distinguish between "equivocal positives" (which they considered positive) and "equivocal negatives" (which they considered negative).

COMPACT predicted carcinogenicity outcomes for six of the nine NTP equivocal carcinogens, and these predictions are summarized in Table 2, together with the Lewis et al. interpretation of the carcinogenicity outcomes. The authors concluded that all six chemicals are predicted correctly by COM-

PACT/Hazardexpert. However, by the rule they claim to have used (equivocal carcinogens are regarded as noncarcinogens), only γ-butyrolactone is predicted correctly.

Inclusion of additional related variables in the predictive method. After the carcinogenicity outcomes were known, Lewis et al. found that the predictive performance of COMPACT could be enhanced if they included an additional COMPACT prediction (C2E) and also a predictor variable ("Hazardexpert") that incorporates information about metabolism. While there is nothing inherently wrong with including additional variables in a predictive methodology, this exercise should ideally have been carried out prospectively, not retrospectively. It is much easier to find predictive variables that work once the study outcomes to be predicted are known. The important (and yet to be answered) question is, how will the authors' newly derived, multivariate predictive methodology fare for prospective predictions? Hopefully, it will be better than COMPACT's limited predictive success (56%) for the 44 NTP chemicals.

The combination of COMPACT and Hazardexpert eliminated the apparent discordance for three chemicals: tris(2-chloroethyl)phosphate, 2,3-dibromo-1-propanol, and 1,2,3-trichloropropane, while introducing discordance for another chemical previously predicted correctly (methyl bromide). However, the authors misclassify two other chemicals: chloramine and HC Yellow 4, both of which are reported as successful predictions, but in fact were not predicted correctly (see Table 2). Including additional predictor variables (and not correcting for the misclassification of chloramine and HC Yellow 4) reduced the number of discordant predictions from 11 to 8.

Inclusion of additional, apparently unrelated, variables in the predictive method. The eight chemicals that Lewis et al. conclude are not correctly predicted by COMPACT/Hazardexpert are designated in their Table 4 (p. 182). The authors then carry out further analyses to reduce the number of discordant predictions from eight to five. Frankly, it is unclear exactly how the authors achieve this reduction. It appears that the basis for eliminating the final three chemicals from "discordancy" was an appeal to "structural alert, chronic toxicity studies, and the Ames test," which correctly predicted the carcinogenicity of *o*-benzyl-*p*-chlorophenol, methylphenidate hydrochloride, and diphenylhydantoin, three chemicals "missed" by COMPACT/Hazardexpert. One other chemical (mercuric chloride) not even evaluated by COMPACT/Hazardexpert, but correctly identified by "the metal ion redox potentials for inorganic compounds," was also apparently added in as a correct prediction. The authors should justify how these additional predictions, based on apparently unrelated variables, can be meaningfully interpreted as improving the performance of COMPACT/ Hazardexpert. In any case, the authors include these successful predictions in their calculations and conclude that the concordance for COMPACT/ Hazardexpert when predicting rodent carcinogenicity is 86% (32/37). I leave it to the reader's judgment to determine how much confidence to place in this figure.

I have no objection to the development of techniques designed to predict rodent (or more importantly, human) carcinogenicity, and I suspect that it is possible to develop methods that will be successful in this regard. However, I strongly urge caution in placing too much confidence in COMPACT or in any other predictive method that has little success when applied prospectively and seems to work only when applied retrospectively to the original data set, using extensive (and scientifically questionable) data manipulations and reanalysis.

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REFERENCE

1. Lewis DFV, Ioannides C, Parke DV. A prospective toxicity evaluation (COMPACT) on 40 chemicals currently being tested by the National Toxicology Program. Mutagenesis 5:433–435 (1990).

Response

In response to Joe Haseman's letter, we would like to point out that although our article is retrospective with regard to the rodent carcinogenicity study of the 40 chemicals, the COMPACT data were available at the time of the release of the carcinogenicity assays. The Hazardexpert evaluations for the 40 chemicals were carried out after the NIEHS conference, but the Hazardexpert system (available commercially from Compudrug Ltd) is not part of COMPACT. The following account, hopefully, provides some clarification of the points raised in Dr. Haseman's letter.

Most other systems publish their predictions or analyses without providing any mathematical derivation which can be reproduced by others. In contrast, we show how our predictions/analyses are generated from numerical values (COMPACT parameters) for molecular and electronic features of each chemical. Our attempts to provide a numerical description of the COMPACT plot of molecular planarity/potential chemical reactivity, have not been entirely successful, due to the fact that the training set of chemicals shows a curved line discriminating P4501 specificity from other P450 isozymes, whereas the COMPACT ratio (either area/depth^2/ΔE or area/depth^2/ΔE – 8) gives a straight line relationship. This results in some chemicals (e.g., resorcinol) having a COMPACT ratio and a COMPACT graphical plot which give conflicting results, but the graph is the *original* paradigm. We have recently derived an expression which is more complex (*1*), based on analysis of the COMPACT curve, and this gives more precise results in terms of correlation with the graph, although the actual graphical representation is preferred.

Resorcinol was predicted to be positive in COMPACT using the COMPACT ratio, but the graph of area/depth^2 versus ΔE as presented at the 1993 NTP conference (*2*) clearly shows that this compound should be negative as it is outside the curve. This is the only example in all of the 40 chemicals of a discrepancy between the approximation of the COMPACT ratio and the accurate description of the graph. As the *EHP* paper is retrospective, we feel justified in making this point, even though the graphical description was available in the conference documentation. HC Yellow 4 was changed from positive in COMPACT to negative, due to the fact that the original structure sent to us by NTP was erroneous and was subsequently changed by NTP after our original predictions had been published. When we ran the new (correct) structure through our system, it proved negative, and we feel justified in making this clear in our retrospective study published in *EHP*. However, we provided revised data (including the aforementioned cases) and distributed this at the NTP conference, which, moreover, included our results for the P4502E descriptor, now provided in the February 1995 issue of *EHP* (103:178–184).

The Hazardexpert analyses were generated retrospectively as we had only recently purchased the software. As can be seen from our *EHP* paper, the Hazardexpert results (which utilize the EPA database) give quite good concordances with positive carcinogens, and they are better than the Ames test for negatives and also overall.

Regarding the relatively poor performance of the original computer-based predictions compared with that of Ashby, Tennant, and others, it should be emphasized that the latter employed a combination of mutagenicity, subchronic toxicity, and structural alert tests, which are, therefore, three evaluations combined into one prediction—so it is perhaps not surprising

that this combination of three different tests gives the best overall concordance with rodent carcinogenicity. It is well known that the Ames test gives only just over 50% concordance with rodent carcinogenicity, but it is still extensively used. This is because it has a well-defined endpoint that one can readily understand in biological terms, i.e., genotoxicity. However, there is overwhelming evidence to show that enzymes of the cytochrome P450 superfamily are involved in the metabolism and toxicity of most (~90%) chemicals. P450s play a pivotal role in toxicity and carcinogenicity, and the COMPACT system is designed to identify P450-mediated metabolism and metabolic activation. Although this system (*3*) was originally based solely on the structures of known P450 substrates, we have now generated full three-dimensional structures (*4*) of the mammalian enzymes themselves (including human isoforms) which agree closely with experimental findings. However, we are also aware that there are other mechanisms of carcinogenicity that do not require P450 activation, and structure alert systems can be useful in identifying direct-acting carcinogens, for example (*1,5*).

Nongenotoxic carcinogens are not so easy to predict but we are elaborating models to identify chemicals involved in peroxisome proliferation and other activation pathways such as ß-lyase cleavage. Eventually there will be a battery of tests in place, which we hope will adequately assess the likely risk to *man* from exposure to foreign compounds; so models of human enzymes and receptors which may mediate potentially carcinogenic events will be important. What is crucial is the determination of how readily a chemical is metabolized and whether any reactive intermediates (ROS or metabolites) are sufficiently long-lived to cause irreparable DNA damage. There may well be short-term test procedures developed which can assess these factors *in vitro*, but computer-based systems can be just as accurately predictive; however, these computer systems do not require synthesis of the chemical, are extremely rapid, and, consequently, relatively inexpensive. We appreciate that traditional toxicologists may have been suspicious of replacing biological tests with computer predictions, but there is evidence that attitudes are changing.

One reason for publishing our retrospective study of the 40 NTP chemicals was to show that it is possible for a combination of tests to give reasonable concordances with rodent carcinogenicity, and we did not anticipate that moderating the equivocal results of the rodent study in the light of the pathology report presented at the NTP conference would be controversial. Our *EHP* paper was independently refereed and, as our use of modified equivocal results (reported at the meeting) was not questioned by the referees, one can only presume that NIEHS (which publishes *EHP* and also organized the 1993 conference) did not find this contentious. The problem regarding equivocals is well known, and these are obviously difficult to assess by predictive systems which, in general, do not equivocate. In fact, some systems (e.g., TOPKAT, CASE) tend to exclude equivocal results in the rodent assay when they validate their methods. At the NIEHS conference in 1993 there was lengthy discussion about equivocals, and the prevailing doctrine from NTP was to regard these as negatives, although there was not universal agreement for this view among the delegates. During the presentation of the pathology results it was indicated that a few of the equivocals could be interpreted, on histopathological evidence, as being weakly positive. It did not seem unreasonable to us in our retrospective analysis to take into account the views of the NIEHS pathologist who conducted the examinations. However, if one excludes the equivocals, the concordance between COMPACT and the rodent assay becomes 70% (21/30), which is not much different from the concordance one gets from regarding three of the equivocals as positives. If one regards all of the equivocals as positives, the concordance between COMPACT and the rodent carcinogenicity is 69% (25/36), whereas taking them as negative lowers the concordance further to 64% (23/36). However, it should be noted that if these three equivocals are regarded as positive, most (if not all) of the predictive tests show a similar improvement.

The use of metal ion redox potentials for providing some estimate of carcinogenicity is not currently part of COMPACT, but it is of interest to show that physicochemical parameters may be employed to try to predict the potential carcinogenicity of inorganic compounds. Likewise, Hazardexpert is not part of COMPACT, but the two tests are fairly complementary in the comparisons we have made to date (*1,6*).

In our *EHP* paper we provide explanations of the results for each chemical, including possible reasons why some of these were discordant with the rodent assay. However, we have not altered our results in the light of this additional biochemical knowledge, especially when the predictive methods (e.g., Ames test, Ashby structural alert, etc.) were able to predict correctly the outcome of the rodent carcinogenicity study for those chemicals. The purpose of these developments of predictive systems is to be able to decrease the number of animal experiments and the time required for the safety evaluation of chemicals that are destined for human exposure, and we believe that a combination of several systems represents the best way to achieve this (*6*), with the consideration of P450-mediated pathways of activation and detoxication being most important. Our *EHP* paper merely attempts to show how a combination of systems might work, but we would appreciate advice from a statistician on how to "weight" such tests: perhaps Joe Haseman could help us.

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Drinking Water and Leukemia

Cohn et al. recently expanded (*EHP* 102:556–561) on an earlier ecological study (*1*) of leukemia and drinking water in northern New Jersey. The initial study suggested an association between volatile organic hydrocarbon (VOC) contamination of drinking water and increased risk of leukemia among females (but not males). The authors concluded that 1) the appearance of an association for females only

could not be explained; 2) the limitations of the ecologic study design precluded inferences regarding causality; and 3) the case–referent study would be a useful follow-up in assessing the public health significance of their observations.

The latest study by Cohn et al., while rich in methodological details and rigorous in discussion of its limitations, does not really clarify the discussion of potential health effects of low-level VOC [particularly trichloroethylene (TCE) and perchloroethylene (PCE)] exposures. The addition of 48 municipalities to the study area, extension of the observed period of disease incidence through 1987, and inclusion of non-Hodgkin's lymphomas (NHLs) only increased the size of the study; these changes did not mitigate the weaknesses of the ecologic design. Cohn et al. suggested that since their unit of analysis was the municipality, in which virtually all residents could be presumed to have the same average exposure, their analysis approximated that of a crude individual-level analysis. We believe that this suggestion is based on a misinterpretation of Greenland and Morgenstern (*2*); these authors observed that ecologic bias cannot occur when the geographical units being compared are either 100% exposed or 100% unexposed. In the study by Cohn et al., this condition cannot be said to obtain, as they implicitly recognized in their discussion of exposure misclassification.

In any case, even in the extreme situation described by Greenland and Morgenstern, the analysis approximates a *crude* individual-level analysis, which has not taken individual-level confounding factors, both measured and unmeasured, into account. Such factors (e.g., socioeconomic, occupational, and lifestyle indicators) may still seriously bias the effect estimate in an ecologic study even when they do not appear to be confounders at the ecologic level, especially when the range of estimated group exposure levels is narrow and the effect of exposure on risk appears to be weak (as is true in the study by Cohn et al.) (*2*). Moreover, Cohn et al. analyzed their data in the presence of an apparent effect modifier, sex, which they intended to address as their fourth study hypothesis. Unfortunately, they left unanswered the question of why the effects observed in their study should be specific to females or males only.

More importantly, a closer look at the published results of laboratory and epidemiology studies of TCE and PCE leads to more consistent and persuasive conclusions. TCE was associated with a small increase in immunoblastic lymphosarcoma in two rodent studies, one using the inhalation route (100, 300, and 600 ppm), and one using the oral route (50 or 250 mg/kg) (*3,4*). In neither study, however, was the increase significantly dose related and in neither was the increase considered significant given the high variability of background incidence in control animals. No other rodent bioassays of TCE have resulted in increased risk of lympho-hematopoietic cancer (*5*). PCE was associated with increased incidence of mononuclear cell leukemia in Fisher 344/N rats in a National Toxicology Program bioassay (*6*), using inhalation exposures of 200 or 400 ppm. The significance of this finding was questioned, however, by the U.S. EPA's Science Advisory Board (*7*) because of the "consistently higher control rates of this tumor type in the study laboratory, coupled with widely variable incidence (in comparison with other NTP laboratories)." The evidence for TCE and PCE as rodent lympho-hematopoietic carcinogens is therefore relatively weak, especially since neither has been shown to exhibit mutagenic or genotoxic activity (*5,8*).

Cohn et al. suggested that occupational studies of TCE and PCE exposures "were small, had short follow-up times, and were based on mortality" (p. 560). This inaccurately characterizes these studies, which we believe have provided excellent evidence with which to judge the likely human carcinogenicity of fairly high-level exposure to these materials. Axelson et al. (*9*) recently published the results of an incidence study of cancer among TCE-exposed workers in Sweden (with exposure documented by urinary metabolite measurement and incidence ascertained from 1958 through 1987). No cases of lympho-hematopoietic cancer were observed among the 249 females included in the study (expected number not reported); among 1421 male workers, 5 NHLs were observed, with 3.2 expected (standardized incidence ratio = 1.56, 95% CI, 0.51–3.64). Average exposure levels in this cohort were thought to be on the order of 30–50 ppm in air (with the male workers having longer average duration of exposure than the females); however, the urinary metabolite measurement protocol was not described, and it is conceivable that the biomonitoring data underestimated the actual exposures. Axelson et al. concluded that "the cancer risk to humans from TRI [TCE] exposure is rather small, if any, under the circumstances that have prevailed when using TRI" (*9*: p. 561).

In a large study of aircraft maintenance workers exposed to intermittent TCE levels as high as 400 ppm, U.S. National Cancer Institute investigators followed approximately 7000 subjects from 1952 through 1982 (*10,11*). Among male workers, they observed 9 leukemia deaths compared with 13.1 expected [standardized mortality ratio (SMR) = 69, 95% CI, 31–130] and 10 NHL deaths, compared with 9.8 expected (SMR = 103, 95% CI, 49–189). Among female workers they observed 2 leukemia deaths, compared with 1.9 expected (SMR and CI not calculated) and 4 NHL deaths, compared with 1.4 expected (SMR = 286, 95% CI, 78–731). The small excess of NHL deaths among female workers was not consistently associated with increasing cumulative exposure. Spirtas et al. concluded that "it is only possible to suggest, at this time, that occupational exposure to TCE probably does not pose a strong carcinogenic risk for man" (*10*: p. 528).

The most recent analysis of mortality in a large cohort of dry-cleaning workers, by Ruder et al. (*12*), which was an update of the study by Brown and Kaplan (*13*), studied 1690 workers with follow-up from 1940 through 1990. In a subcohort of workers thought to have been exposed only to PCE, only 2 deaths due to lympho-hematopoietic cancer were observed (about 4.1 expected, SMR = 49, 95% CI, 6–177). n the remainder of the cohort, exposed to PCE and other dry-cleaning solvents, 7 deaths due to lympho-hematopoietic cancer were observed (about 9 expected, SMR = 78, 95% CI, 31–161). These observations are consistent with those of Blair et al. (*14*), who studied a cohort of 5365 dry-cleaners from 1948 through 1979. They observed a small excess of all lympho-hematopoietic cancer deaths, 24 observed versus 20.0 expected (SMR = 120, 95% CI, 80–180). This excess appeared to be related to level of exposure, but only among white males; among females, deaths in this cancer category were limited to the lowest exposure group. Quantitative PCE exposures were not estimated for the workers in the two studies described above. Such exposure has been reported to vary between 28.2 and 88.2 ppm (time-weighted average) in a study of 67 dry-cleaning shops in the United States (*15*).

Cohn et al. assigned estimated VOC exposure levels in their study using categories of < 0.1 ppb, 0.1–5.0 ppb, and > 5.0 ppb. The animal bioassay data and the human occupational data, obtained from large studies of workers exposed daily to TCE or PCE at air concentrations of parts per *million*, do not support the existence of an association between these exposures and lympho-hematopoietic cancer. To believe that the exposures described by Cohn et al., in the low parts per *billion*, could be causally associated with these diseases would be tantamount to standing the concept of dose response on its head. Cohn et al. suggested that human carcinogenicity "could involve different potencies and different organs or cell types from

those in the rodent studies" (p. 560), but they offered no suggestions as to the possible mechanisms that might underlie such differences, nor did they adequately address the substantial body of human epidemiologic data indicating no excess risk of lympho-hematopoietic cancer among workers exposed to TCE and PCE levels approximately three orders of magnitude greater than the New Jersey residents studied.

In summary, we conclude that the evidence from high exposure-level human and animal studies strongly suggests that there is not likely to be any increase in the risk of lympho-hematopoietic cancer in populations exposed to very low levels of TCE or PCE in drinking water. The results obtained by Cohn et al. are perhaps more likely explainable by the limitations of the ecologic study design. While fully supporting all efforts that have been and continue to be made by chemical manufacturers and users to reduce emissions into the environment, we hope that this brief review will encourage Cohn et al. and other public health investigators to view the results of ecological studies of low-level VOC exposures in a wider perspective.

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Response

We thank Dr. Ramlow and Mr. Bloemen for their comments on our epidemiologic study (*EHP* 102:556–561) of low-level exposures to perchloroethylene (PCE) and trichloroethylene (TCE). Our study found associations between PCE and TCE in drinking water and the incidence of leukemia and non-Hodgkin's lymphoma (NHL) in both sexes, especially among females. We believe that our study is an important contribution to the weight of evidence concerning possible hazards of these uniquitous chemicals for the general population.

Ramlow and Bloemen take issue with the design of our study, citing the usual limitations and caveats of ecologic studies. (They note that we also cited these caveats.) However, our study did not use a classic ecologic design. In a true ecologic study, exposures are expressed as proportions or averages of aggregate groups, such as the percentage completing high school in a municipality. In this example, an individual either finished high school or did not. In contrast, we assigned the exposure category of each study subject according to the level of contamination in the water utility serving the municipality of residence. In municipalities served by water systems contaminated with volatile organic compounds, all individuals are exposed by some combination of inhalation, ingestion, and dermal absorption. The potential for exposure misclassification in our study resembled that of many occupational studies for which summary estimates of exposure are applied to all workers in a particular job description or location, irrespective of hot spots, personal protective equipment, or other personal factors that might influence individual exposure.

The relative risk estimates generated in our analyses were adjusted for individual data on age, sex, and race and weighted by population. Race minimally affected the estimates of the rate ratios in the regression analysis and was therefore not included in the report. Standard ecologic municipal socioeconomic indicators were examined qualitatively, but there were no notable differences between the exposure strata, and risk ratios were not adjusted for socioeconomic variables. In short, the most salient limitations of true ecologic designs did not apply to our study.

Ramlow and Bloemen also raise the issue of potential confounding in our study. Confounding occurs when a risk factor for disease is positively or negatively associated with an exposure of interest. Confounding can cause either overestimation or underestimation of the assoication between exposure and outcome. However, unless a confounder is a strong risk factor, the confounder must be strongly associated with the exposure of interest to significantly affect the results. Known strong risk factors for leukemia and NHL include certain genetic traits and DNA-repair enzyme deficiencies, exposure to benzene or radiation, and, for a few histologic types, infection with certain viruses. Smoking is a moderate risk factor for leukemia. There was no *a priori* reason to believe that these risks were differentially distributed among the exposure strata in our study. While we did not have information on smoking status (for malignancies in adults), neither do many occupational studies, including those cited in the letter from Ramlow and Bloeman.

Ramlow and Bloeman also raise the issue of the consistency of our findings with those from the occupational epidemiology and animal toxicology literatures. In evaluating the weight of evidence for potential public health hazards of PCE and TCE, we must consider that the general population includes subgroups that may be more sensitive than "healthy workers" to toxic agents. Additionally, Aschengrau et al.'s recent case–control study found strong associations between leukemia and PCE contamination of drinking water (*1*).

In carefully examining the occupational studies cited by Ramlow and Bloemen, we find that some of these studies, rather than contradicting our results, are consistent with our strongest finding. When duration

and intensity of exposure were included in these occupational analyses, the strength of the associations between NHL and TCE/PCE exposure increased. For example, Blair et al. (*2*) noted 5 lymphohematopoietic cancer cases among men in the highest exposure category compared to 1.2 expected. Spirtas et al. (*3*) found 3 NHL cases among women in the highest cumulative exposure category compared to 0.9 expected, but the odds ratio was not elevated among men. Axelson et al. (*4*) observed 4 cases of NHL among men who were exposed for more than 2 years, compared to 1.5 expected. In the highest cumulative exposure cateogory, they observed 3 NHL cases compared to 0.9 expected. In addition to the cited studies, a recent hospital-based, ocupational case–control study of NHL among men reported an unadjusted odds ratio of 7.2 for exposure to TCE and an odds ratio of 11 for exposure to "degreasing agents" (*5*). Some of the laboratory investigations cited in the letter are also consistent with our findings. In one report, immunoblastic lymphosarcoma incidence in Sprague-Dawley rats was increased by both oral and inhalation routes of exposure to TCE (*6*).

At this time we can only speculate why low levels of these chemicals (relative to laboratory experiments) have been associated with certain lymphohematopoietic cancers (*1*). However, as noted, some of the laboratory data and much of the occupational data are not inconsistent with our result. Until we know more, it is especially important to consider carefully the findings that bear directly on the general population and to follow up with additional studies.

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We cannot say that the continued use of herbicides, insecticides, and other biocides will lead to the eventual extermination of man. Neither, however, can we afford to rest easily.

Raymond F. Dasmann
Vital Issues
April 1969

Forum

Burning Border Health Issues

A small county in Arizona is plagued by unusually high rates of lupus erythematosus and cancer, and residents are looking at air and water pollution from neighboring Mexico as the culprits.

A study released last December confirmed that the population of Santa Cruz county, located along the Mexican border, suffers from 2.4 times the National Cancer Institute's expected rate of multiple myeloma, a form of bone marrow cancer, and almost twice the expected number of lupus cases. The study, conducted by the University of Arizona Cancer Center, found that there were 12 cases of multiple myeloma from 1989 to 1993 in the county of about 30,000, while the expected rate is 5 cases per 100,000 people.

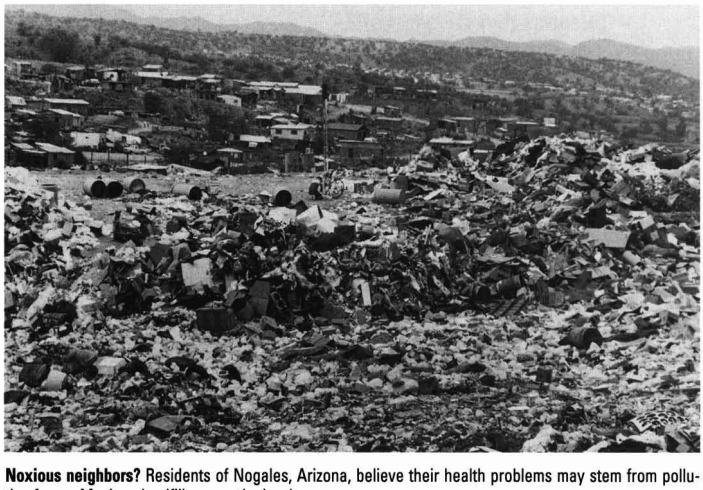
The study also found 94 cases of lupus per 100,000 people, whereas 50.8 cases per 100,000 are expected. According to Brad Christensen, a spokesperson for the Arizona Department of Health Services (ADHS), this is the highest incidence of lupus cases in one area on record in the world.

The study was undertaken at the urging of residents of Nogales, the town most affected by the illnesses, to investigate the health problems. In 1992, a group of cancer victims and their family members who were concerned about the high rates of disease formed a grassroots organization, Living Is for Everyone (LIFE), to lobby state officials and draw attention to the problem.

"The group grew out of the need to address what we thought at the time were only health issues," said Ana Acuna, a 54-year-old lupus patient who helped form the organization. "We had a suspicion that environmental factors were involved, and from there grew the tie to the environment."

In December 1993, the governor, university officials, and ADHS officials responded to LIFE's requests and visited Nogales. "We visited Carillo Street, which is a small neighborhood, and it seemed like every other household was touched by cancer," Christensen said.

The ADHS then committed $100,000 and contracted the university to conduct a study. Although the study did confirm that the disease incidences are unusually high, the researchers did not find an obvious link to environmental problems. However, epidemiologist Larry Clark, who headed the study, said environmental substances were probably a trigger for the lupus increase.



Mario Aguilar/*Green Valley News*

Noxious neighbors? Residents of Nogales, Arizona, believe their health problems may stem from pollution from a Mexican landfill across the border.

The residents believe the source of their health problems lies across the border in a landfill that catches on fire weekly. Burning in dumps is illegal in the United States because the practice causes air pollution and poses a health threat. The residents also blame their poor health on sewage and toxic chemicals that are carried north in a wash that runs through Nogales.

Following the release of the study, the director of the ADHS, Jack Dillenberg, took several copies to the Center for Disease Control in Atlanta to draw federal attention to the problem.

Dillenberg told *Healthlink*, a publication of the ADHS, that he felt the meetings were positive. "I want the CDC to recognize we've got some valuable data now, and I want them to get involved," he said. "Clearly this is a problem that requires solutions beyond what the city, the county, and the state can offer."

According to Christensen, the Santa Cruz community has been very pleased with the response of federal officials to date. On March 22, medical epidemiologist Rossanne Philen, of the CDC, visited Nogales and made a commitment to help further the study of the illnesses, possibly by placing a researcher on the border to look for the causes.

In addition, the Interagency Coordinating Council, which is made up of members of the EPA and the CDC, held a meeting May 2–3 in Rio Rico, which is just north of Nogales, to discuss border issues.

Mexican health officials indicated they would attend the meeting. According to Christensen, Mexican officials have responded well to the issue. Since the study was released, officials closed the dump in question and opened a new dump eight miles away that does not burn waste.

Drink and Diet

Now there's another reason to eat your fruits and vegetables. New research conducted at the Harvard University School of Public Health shows that poor diet combined with a high intake of alcohol increases the risk of colon cancer.

Edward Giovannucci co-authored the

Bellyache up to the bar. A new study shows alcohol and a poor diet may increase risk of colon cancer.

study which appeared in the 15 February 1995 issue of the *Journal of the National Cancer Institute.* Giovannucci and his colleagues examined the diets of 47,931 male health professionals 40–75 years old. In 1986, the subjects, free of diagnosed cancer, filled out questionnaires about their diets. The researchers followed up on the subjects for six years, and during that time they documented 205 new cases of colon cancer.

The researchers were testing the hypothesis that diet plays an important role in the methylation of DNA, which they thought to be important in gene expression and the normal regulation of DNA, Giovannucci said. Diet controls methyl groups, which in turn control the methylation of DNA, he said. Past research has shown that methyl-deficient diets cause various cancers in animals.

The proposed mechanism by which methyl-deficient diets contribute to cancer is best understood from studies of rat hepatocarcinogenesis, the researchers reported in *JNCI.* It has been shown that a methyl-deficient diet in rats is followed by DNA hypomethylation, the overexpression of various genes including several proto-oncogenes, and elevated DNA methyltransferase activity in the liver. Rats eating a methyl-deficient diet for long periods develop liver tumors, and alcohol seems to accentuate this effect. The researchers also cited that abnormal DNA methylation patterns may contribute to carcinogenesis, possibly by influencing both the activation of oncogenes and the inactivation of tumor-suppressor genes.

The key factor in maintaining methyl groups is methionine, an amino acid found in poultry, fish, and low-fat dairy products such as skim milk, Giovannucci said. Folate, which is found in green leafy vegetables, is also important to methyl groups, in that it assists in the production of methionine. Other dietary components such as vitamin B_{12} and choline may relate to methyl-group availability, the researchers said.

Giovannucci and his colleagues looked at the diets to see how much methionine and folate they contained. They also looked at alcohol intake because alcohol has been shown to have suppressive effects on the metabolism of methyl groups.

The results seem to support the researchers' hypothesis. Those who had high alcohol intake combined with low intakes of folate and methionine had a relative risk of 234%, which is over twice the risk of men with low alcohol, high folate, and methionine intakes. High alcohol is defined in the study as 20 or more grams per day, which is about two drinks. Low folate intake is defined as 364 µg per day, and low methionine is 1.75 g per day.

Those who drink, but also have well-balanced diets, have about the same colon cancer risk as nondrinkers, Giovannucci said. All types of alcoholic beverages were related to the risk of colon cancer, and past, presumably heavy, drinkers were also at higher risk of developing colon cancer, the researchers said.

The associations observed for alcohol and methionine were not due to confounding by other dietary factors, smoking, physical activity, body mass, aspirin use, differential surveillance for disease, or family history of colorectal cancer, the researchers said.

The study did suggest that aspirin use modified the risk of colon cancer even with high alcohol and low folate intake, but the researchers said this modification of risk requires confirmation in other populations. Men who took vitamin supplements also appeared to have a lower risk of cancer, but Giovannucci warned that pills are no substitute for nutrient-rich food.

"We think overall that the results support the recommendation to eat lots of fruits and vegetables. Obviously, there are other reasons, but if someone follows these guidelines, he or she will also be benefiting regarding colon cancer," Giovannucci said.

Colon cancer afflicts about 150,000 men and women every year in about equal numbers and kills 60,000 per year, making it the second leading cause of cancer deaths. Giovannucci added that after the age of 65, a woman is just as likely to die from colon cancer as from breast cancer.

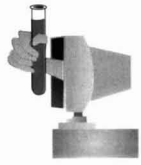
Giovannucci and his colleagues are currently conducting the same study in women, and Giovannucci says the effects appear to be similar so far. In the future, he says, the researchers hope to better understand the mechanism of DNA methylation.

People and plants. A recent conference on biodiversity and human health stressed protection of indigenous people and plants.

Nature's Medicine Cabinet

With support from federal agencies and pharmaceutical companies, scientists are trying to tap nature's medicine cabinet while preserving the plants that stock it. How best to do this was the subject of a two-day conference on Biodiversity and Human Health, held in April in Washington, DC.

Western physicians and traditional healers alike rely on compounds found in plants to treat a wide variety of ills. Pharmaceutical companies now regularly test and develop the ingredients of plants for use in drugs. But plant species are



EHPnet

The summer solstice is a time when people's attention focuses on the sun. A World Wide Web site with the same name shares that focus. Created by the Center for Renewable Energy and Sustainable Technology (CREST), the Solstice site (http://solstice.crest.org) offers a fairly comprehensive introduction to the topics of renewable energy, energy efficiency, the environment, and sustainable development.

One of CREST's primary functions is to explore and demonstrate the use of advanced information and communication technologies. Solstice provides information arranged by subject and type in the categories of energy efficiency, the environment, renewables and alternatives; legislation, policy, and economics; education and social issues, planning, and computers and networking in the context of alternative energy resources.

Solstice also provides two interactive energy education modules that use text and images to teach the theoretical and practical basis of passive solar and renewable energy at an introductory level. The passive solar module provides an overview and information on design, benefits, and resources. Technology areas covered by the renewable energy module are labeled solar, wind power, small hydro, geothermal, and biomass. This module provides a basic overview of the history and theory of each technology, case studies and applications, and economics and global impact. Internet addresses are available for users to direct questions or comments about the modules to experts at Solstice. The Solstice site also offers hyperlinks to related sites including the U.S. Department of Energy's Energy Efficiency and Renewable Energy Network.

Once you have soaked up enough information about renewable resources, it might be time to soak up some sun (with sunscreen, of course). One way to do this is by throwing a frisbee. Users who would like to join pick-up games of ultimate frisbee should take a glance at The Frisbee Page (http://www.sccs.swarthmore.edu/~dalewis/frisbee.html). Everything you ever wanted to know about frisbee is in there and if you don't own a frisbee yet, you can order one through the Internet Disc Shoppe. Pick-up games are listed by state.

going extinct at an increasingly rapid pace, which threatens this supply of healing ingredients, researchers warned at the conference, sponsored by the National Institutes of Health, the National Science Foundation, the Smithsonian Institution, the National Association of Physicians for the Environment, and the Pan American Health Organization.

For the past 40 years, the National Cancer Institute has screened plants for their chemotherapeutic activity, said Thomas D. Mays of NCI's Office of Technology Development. Contractors working for NCI have collected 35,000 plant samples, representing 9,000 to 10,000 species, from Africa and Madagascar, Central and South America, and Southeast Asia. NCI now also looks for plant-based compounds that thwart the AIDS virus, Mays said. The institute is supporting preclinical investigations of three possible anti-HIV agents derived from plants. One, michellamine B, comes from a woody vine called a liana, found in Cameroon. Liana extracts may work against malaria as well, Mays added. Another possible anti-HIV agent is conocurvone, from the Australian smokeweed bush. Two related compounds from a Malaysian rainforest tree, calanolide A and costatolide, may also thwart HIV.

Researchers are also investigating the antiviral potential of prostratin, a molecule in the bark of a Samoan tree, said Paul Alan Cox, a professor of botany at Brigham Young University in Provo, Utah. Samoan healers use prostratin for treating patients with yellow fever. It appears to interfere with viral replication and protect cells from HIV, Cox said. Recently, Cox and his colleagues learned of a compound in the bark of the Samoan tree that appears to stimulate the immune system and double the life span of certain immune cells.

The unique behavior of certain animals may also offer clues for how to prevent or treat diseases, said Eric Chivian of Physicians for Social Responsibility in Washington, DC. For example, understanding why black bears, an endangered species in many parts of the world, can hibernate without losing bone mass may help scientists find ways to prevent bone loss suffered by the elderly, bedridden patients, and astronauts.

Collecting plants, insects, and other natural materials for research or for retail can threaten endangered species, speakers warned. It's a myth that harvesting nontimber products, such as nuts, doesn't harm the ecosystems of rainforests, where many medicinal plants grow, warned Charles Peters, curator of botany at the Institute of Economic Botany of the New York Botanical Garden in Bronx, New York. Local people may reap the benefits of a forest for centuries without causing problems, but increasing that harvest even slightly can prove disruptive, Peters said. For one, although there are many different species in the rainforest, no one species is very abundant. Also, tropical plants have difficulty establishing seedlings. Moreover, species are dependent on one another for survival.

People in the countries where valuable medicinal plants or other species exist, including the indigenous people knowledgeable about the plants, need protecting as well, speakers pointed out. The traditional healers of the world, experts on the medicinal power of plants, are dying off and no one is taking their places, conference speakers warned. One indigenous culture goes extinct every year in the Amazon alone, according to Katy Moran, executive director of the Healing Forest Conservancy in Washington, DC.

Current law fails to ensure that indigenous people receive any benefits when companies develop products that use the fruits of their forests, Mays said. At the same time, countries need incentives to preserve and to provide access to their plants for possible drug discovery. To address these problems, NCI has developed legal agreements that guarantee that countries receive financial rewards and scientific assistance for their contributions to new drugs.

Throughout the meeting, speakers emphasized the importance of taking a holistic approach to preserving biodiversity: saving not just the individual species, but entire ecosystems and cultures. Speakers also warned that medical, scientific, and environmental organizations working on biodiversity issues must strengthen and better coordinate their efforts, particularly in light of new congressional efforts to lift protections for endangered species.

Russian Rivers of Radiation

From 1949 until 1956, workers at the Soviet Union's first nuclear weapons facility in the southern Ural mountains dumped nearly 80 million cubic meters of liquid radioactive waste into the Techa River, a regional waterway shared by 30 villages that dot its shores. Unaware that the river

had been contaminated by plutonium, the 64,000 villagers drank its water, washed their clothes in it, and bathed in it for decades. Among other nuclear accidents at the plant, 217 villages of 272,000 inhabitants were also exposed to 2 million curies of radiation released when a liquid-waste storage tank blew up in 1957. Unlike any other region in the world, at least 400,000 people have been continuously exposed to both external radiation, the gamma rays deposited throughout the area, and internal radiation, the strontium-90 and cesium-137 absorbed from drinking water and contaminated vegetables, according to a February article in *Science*.

Soviet scientists carefully studied the villagers for three decades. Soviet secrecy, however, prevented any results from becoming public; even the villagers were never told why they were being examined. But in early January, a team of radiation biologists from the United States, Europe, and Japan traveled to the city of Chelyabinsk, home of the long-secret nuclear facility Chelyabinsk-65 and its Mayak plutonium production plant, to meet their Russian counterparts and take a look at the research for the first time. Such data represent the only known studies in the world on long-term, low-dose radiation exposure; studies in Hiroshima and Nagasaki, in contrast, were based on short-term, high-dose exposure.

"The Russian scientists have carried out some unique studies, including the only reliable research on the long-term effects of plutonium exposure," writes Michael Balter in his article in *Science*. One epidemiological study of 28,000 Techa River villagers "found a statistically significant increase in leukemia incidence, as well as an overall increase in cancer mortality, compared to control populations that did not live in the contaminated zone. Still, the leukemia risk per unit of radiation dose was at least two times smaller than that of the atomic bomb survivors," he says.

Over the years, several local physicians had tried to gain access to the data being collected on their patients by the Institute of Biophysics Branch Number Four. According to Diahanna Lynch, coordinator of the Russian Environment and Energy Project at the Natural Resources Defense Council, Russian doctor Gulfarida Galimova threatened to prevent the institute's researchers from continuing to examine her patients if they did not provide more information on their condition. In 1993, the researchers gave her a list of 285 patients diagnosed with chronic radiation sickness in her village of Muslyumova, 50 miles downstream on the Techa River from Chelyabinsk-65.

"In 1993, Dr. Galimova determined that of the more than 4,000 residents in the village, about 3,000 were examined by the institute," says Lynch. "Of these, she says, 92% had some kind of chronic illness, ranging from circulatory problems to birth defects such as missing kidneys. Dr. Galimova has also been a local activist in the Chelyabinsk Movement for Nuclear Safety, encouraging people to lobby the government to resettle the village in a cleaner area, and to demand compensation for the damage to the villagers," said Lynch.

Traces of plutonium have been found in the organs and tissues of the villagers and local animals, according to a recent article in *Surviving Together*, published by the environmental organization ISAR (formerly the Institute for Soviet-American Relations), in Washington, DC. In addition, an article distributed by the Japanese Kyodo News Service after the January 1995 meeting in Chelyabinsk reported that villagers along the Techa River have more lymphatic genetic mutations than people who suffered radiation from the atomic bombing of Hiroshima. Scientists also discovered a buildup of strontium-90 and other radioactive isotopes in the livers and in other organs of the local residents, as well as an increasing incidence of mutations of the gene responsible for T-cell antigen receptors in lymphocytes in peripheral blood, according to the article.

In January, President Boris Yeltsin's former environment adviser, Alexei Yablokov, now in charge of environmental matters for the country's top policy-making body, the Security Council, warned that radiation from the Chelyabinsk site could ultimately spread to the North Pole. He said that radioactive groundwater was now contaminating the Tobol River, which feeds into the Ob River system. The Ob system empties into the Barents Sea, which flows toward the North Pole. He also said that total radiation around Chelyabinsk-65 is 22 times the radiation released in the 1986 explosion at the Chernobyl nuclear reactor in Ukraine. Although the Mayak facility's five industrial uranium-graphite reactors have been shut down, the plant is still used for reprocessing spent fuel.

Paula Garb

Downriver risk. A family in Muslyumovo grows vegetables on the banks of the contaminated Techa river, 50 miles from Chelyabinsk-65.

As the Cell Cycles

Scientists have known for decades that exposure to certain environmental agents can lead to cancer, and many have suspected that this occurs through the alteration of cell cycle controls. Until recently, however, not enough was known about the molecular basis of cell growth and division to understand the specific pathways by which such agents could alter cell growth in a way that leads to cancer. In the last few years, a large number of specific control points in the cell cycle have been identified, as have the individual genes and proteins that regulate these checkpoints. Researchers have observed that alteration of such controls can disrupt normal cell cycle regulation, but the mechanisms by which chemical treatment or exposure affects these critical functions are largely unknown. Recent research in this area, however, has shed some light on how environmental agents and external cell signals affect cell cycle regulation.

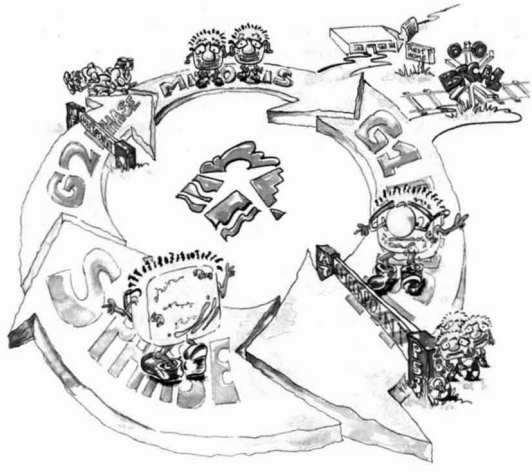
All eukaryotes, from yeast to humans, share many features in the process of cell division. Cells that are actively growing and dividing pass through four stages: G_1 (gap), followed by the S-phase in which the chromosomal DNA replicates, G_2, and finally M (mitosis), in which the chromosomes move to opposite ends of the cell

and the cell then divides. Research has recently indicated that the transitions between cell cycle states are regulated at checkpoints by a family of protein kinases, the cyclin-dependent kinases (CDKs), and their activating partners, the cyclins.

One of the most important checkpoints is START in late G_1, at which the cell commits itself to another round of DNA replication and at which both positive and negative signals are integrated into the cell cycle. Many checkpoints are deregulated in oncogenesis, and this is often due to changes in cyclin-CDK complexes. In particular, the deregulation of START may allow cell growth and division to become insensitive to external cues. Research has shown that this insensitivity can be a consequence of either the aberrant expression of positive regulators, such as the cyclins, or the loss of negative regulators, such as the cyclin-dependent inhibitor proteins (CDIs). Another consequence of abnormal START checkpoint control is that cells can bypass the normal restriction on entry into the S-phase that is normally imposed by damaged DNA, and this may allow the cells to replicate unrepaired mutations and thus accumulate genetic changes that contribute to carcinogenesis.

Much current research is focused on identifying factors internal to the cell nucleus that regulate cell growth, and how the over- or underexpression of those factors perturbs the cell cycle. At the NIEHS, Richard Paules heads a growth control and cancer group that is conducting *in vitro* studies on mouse and human cells. By overexpressing an oncoprotein, called *mos*, that can affect the *ras*/*raf*/MAP (mitogen activated protein) kinase pathway in mouse fibroblasts, Paules's group has observed that cells cannot exit G_1 and go into resting mode. Rather, the cells are pushed by abnormally high levels of cyclin A and the cell division cycle gene, *CDC2*, and become unstable and thus vulnerable to further genomic alteration that can lead to uncontrolled growth. Similar studies are underway with the MAP kinase (*MEK1*) and v-Ha-*ras* transformed mouse fibroblasts.

Paules's team, in collaboration with William K. Kaufmann of the University of North Carolina-Chapel Hill School of Medicine, is also investigating checkpoint responses to the kind of damage that may result from exposure to environmental toxicants. Previous research has shown that a lack of the p53 tumor-suppressor gene can lead to genomic instability. Paules's team has shown that one consequence of this may be the loss of the G_2 checkpoint function. G_2 provides a protective delay, preventing entry into mitosis when there is DNA damage. Without this checkpoint, cells are vulnerable to the chromosomal aberrations frequently seen in cancers.



Richard Paules/Elizabeth West

The Cell Cycle

"We are very excited about the possibility of understanding the molecular consequences of exposure to a variety of environmental agents that impact normal cell cycle control," Paules said. "The hope for the future would be to develop intelligent approaches for early detection and better chemotherapeutic strategies exploiting these pathways."

Other researchers are examining chemical interactions with the cell cycle. Thomas Goldsworthy and his colleagues at the Chemical Industry Institute of Toxicology have teamed up with NIEHS researchers George Lucier and Robert Maronpot to examine pathways by which certain environmental agents affect the cell cycle. Goldsworthy's team is particularly interested in how nongenotoxic, carcinogenic agents affect the cell cycle.

"We know that genotoxic agents can cause direct mutation of some of the key cell cycle regulators, but we also believe that nongenotoxic agents can indirectly lead to these changes," said Goldsworthy. "Our hypothesis is that exposure to certain chemicals can cause aberrant expression of certain genes, such as the p53 tumor suppressor, which in turn prompts certain cell cycle events. Once you have an altered response to the growth signals, that can lead to cell cycle dysregulation. This can allow the cell to proceed to DNA synthesis and replication without repairing any DNA damage, and that in turn leads to altered growth, genomic instability, and the accumulation of DNA mutations—the hallmarks of cancer."

Goldsworthy has investigated unleaded gasoline and its mechanism of carcinogenesis in mouse liver and observed that precancerous cells exposed to gasoline lose their response to inhibitory growth factors and exhibit aberrant growth. The challenge now is to understand the dose and species susceptibility to these processes. "Although specific cell cycle genes may not be identical between mice and humans, we can say that the processes for controlling cell growth are similar, and certain chemicals, such as gasoline, do appear to affect these processes," Goldsworthy says. "Are these the critical changes that result in cancer? We don't know."

Goldsworthy's work with Maronpot is focusing on identifying the growth factors and oncogenes that are involved in chemically induced mouse liver neoplasms and relating those changes to cell proliferation and cell death. Chemicals being studied are mainly agents shown not to directly interact with DNA, including chlorinated hydrocarbons, furan, and phenobarbital. The team has identified a number of novel genes that have the potential to affect the regulation of the cell cycle and appear to be involved in mouse hepatocarcinogenesis.

Goldsworthy and Lucier have teamed up to study receptor-mediated carcinogenesis, particularly in response to dioxin exposure. The team has been examining dose–response effects, hormonal effects on receptor binding, gene expression, cell growth, and the induction of liver cancers.

"I believe this is the future of toxicology," says Goldsworthy of research examining the interaction of environmental agents and cell cycle controls. "We've been characterizing the cancer process with respect to altered cell growth, but we don't really understand the exact interaction between the chemical and the growth process and its role in inducing the cancer. The tools are now available to really understand the interactions of chemicals with the critical cellular and molecular components of the cell cycle, which will lead to better species extrapolations and, ultimately, improved risk assessment."

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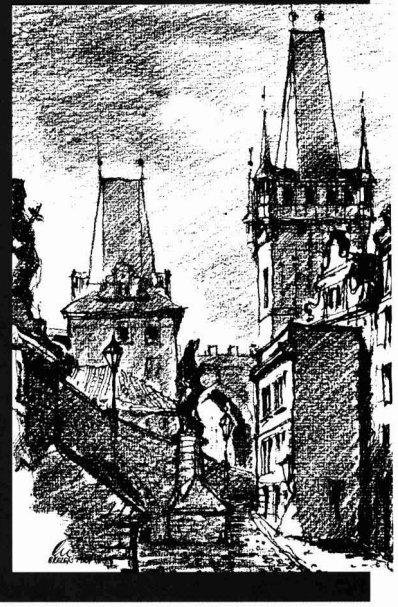
INTERNATIONAL CONFERENCE

on

ENVIRONMENTAL MUTAGENS IN HUMAN POPULATIONS



PRAGUE, CZECH REPUBLIC

AUGUST 20–25, 1995

Symposium Topics

1. *Biomarkers of exposure and effects of mutagens and carcinogens*
2. *Genetic-environmental interactions on cancer susceptibility (polymorphism inductionladaptation)*
3. *Characterization of exposure to environmental mutagens in highly polluted areas*
4. *Genetic and cancer risk from accidental exposures*
5. *Reproductive and developmental effects of environmental mutagens*
6. *Advances in detection of genetic damage in germ cells and genetic risk estimation*

Forum Topics

1. *Identification of human populations at risk*
2. *Methodological problems in the design of epidemiological studies focused on environmental mutagens and carcinogens*
3. *Does exposure to environmental mutagens add significantly to the cancer burden*
4. *Should population monitoring andlor screening be routinely done on exposed workers*
5. *Future of human population monitoring*

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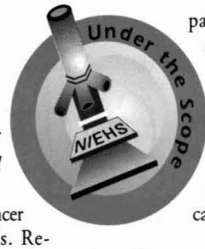
Molecular Pathology: Unlocking the Cell's Secrets



Editor's note: This "Under the Scope" is the first in a series of profiles on areas of intramural research at the NIEHS.

The secrets to the origins of cancer are hidden deep within cells. Researchers in the Laboratory of Experimental Pathology (LEP) at the NIEHS are using the techniques of molecular biology to uncover the role of environmental agents in changing the molecular structure of cells that may lead to cancer.

Traditional pathology consisted of using stained tissue sections for diagnosis and for identifying specific tissue alterations such as identification of infectious organisms and demonstration of specific cellular enzymes. A second generation of specialized stains based on immunohistochemistry provided information about the presence and localization of specific proteins for which polyclonal and monoclonal antibodies were available. Today, molecular pathology laboratories are using molecular methods such as *in situ* hybridization staining to detect specific messenger RNAs. These methods allow a researcher to show whether a cell has actually produced a protein, rather than just stored it. Because changes in gene expression (indicated by changes in mRNA) are associated with induction and progression of tumors, *in situ* hybridization offers scientists a means of teasing out the underpinnings of the cancer process. Other dimensions of molecular pathology including extraction of cellular DNA and RNA as well as proteins from tumors and identification of this material using electrophoresis and other molecular biology techniques have led to identification of several oncogenes in human and animal cancers.

Oncogene Clues

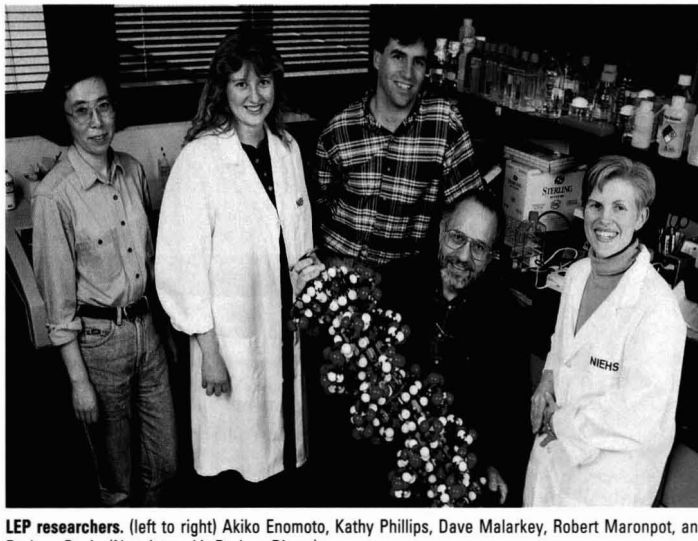
Lung cancer is one of the most common cancers in the United States. Worldwide, liver cancers are the most common. Most animal carcinogenicity studies have been conducted in mouse lung or liver cells because mice readily develop tumors in these sites when exposed to carcinogenic agents. Efforts led by Robert Maronpot, chief of the LEP, are ongoing in the laboratory to better understand these tumor endpoints and their utility in hazard identification and human risk assessments. "We hope that there are bridges between tissues and across species that will enable us to transfer this information to humans," Maronpot said. "If something is occurring molecularly, then we might find a way to block it, thus blocking cancer, or provide methods for better therapies."

Early molecular pathology studies conducted by the LEP in collaboration with other NIEHS researchers looked at DNA from tumor cells and adjacent tissues in mice for alterations to proto-oncogenes, normal genes that mutate slightly to become oncogenes and cause cancer. After identifying a gene, the investigators treated the animals with an environmental agent to determine if the gene's activity changed, such as causing more tumors to develop than in normal mice or causing tumors to develop sooner. For example, researchers found that tetrachloroethylene and trichloroethylene, widely used industrial solvents and common contaminants of surface, ground, and drinking water, and dichloroacetic acid, a metabolite of trichloroethylene and a major organic contaminant of chlorinated drinking water, activated oncogenes in induced liver tumors. This activation led to an early increase in liver tumor incidence in one strain of mice. Some of the activated oncogenes in these tumors differed from those found in tumors that occurred spontaneously.

Past studies have shown that oncogene activation is more frequent in tumors induced by low doses of hepatocarcinogens than by high doses and provides evidence that the mechanisms or pathways of tumor induction differ as a function of the dose of carcinogen. Dave Malarkey is currently studying the dose–response relationship to oncogene activation. The implications of identifying a dose-dependent effect on the mechanism of tumor induction directly challenge the risk assessment assumption that chemical carcinogens at low and high doses produce cancer by the same mechanisms.

Most of the oncogene studies in mouse liver tumors have been done at NIEHS, says Maronpot. However, the focus is shifting away from these studies because not all induced or spontaneous liver tumors have been shown to carry an activated oncogene, making it difficult to draw definitive conclusions. Some of these types of studies will continue though, in the hopes of discovering new oncogenes. "We determined that some agents that cause cancer in animals, and presumably in man, do so by activating oncogenes, which may tell us something about risk assessment. Also, some of the specific mutations in activated oncogenes identified in animal tumors match what is seen in human tumors, which offers the hope that the basic fundamental molecular mechanisms may be similar," said Maronpot.

In addition, retrospective studies are being designed to identify specific genetic alterations in neoplasms from some of the 450 previous two-year chemical bioassays. These studies will attempt to correlate chemical-specific properties including structural features, genotoxicity, and metabolism with characteristics of genetic alterations in preneoplastic and neoplastic lesions of specific target organs. These studies will allow scientists to compare classes of chemicals, evaluate structure–activity relationships within classes, and determine the response of target tissues



Steve McCaw

LEP researchers. (left to right) Akiko Enomoto, Kathy Phillips, Dave Malarkey, Robert Maronpot, and Barbara Davis. (Not pictured is Darlene Dixon.)

to different chemicals without having to do long-term studies. Chemicals for which genetic alterations (oncogene activation) have been determined include oxazepam, vinyl carbamate, chlordane, trichloroethylene, tetrachloroethylene, dichloracetic acid, furan, and butadiene.

The Transgenic Track

A new focus is on transgenic mice. The mice are genetically engineered to carry human or mouse oncogenes or genes for certain growth factors. "They offer a unique approach to identifying and investigating factors that may influence tumor development by allowing us to directly assess what these genes do in target organs and how they are influenced by environmental agents," Maronpot says. Some transgenic mice develop tumors in as little as 6 months when exposed to certain chemicals, as opposed to the normal 18- to 24-month time span. The mice hyperrespond to test agents, enabling the investigators to obtain answers to their questions much faster than with traditional models.

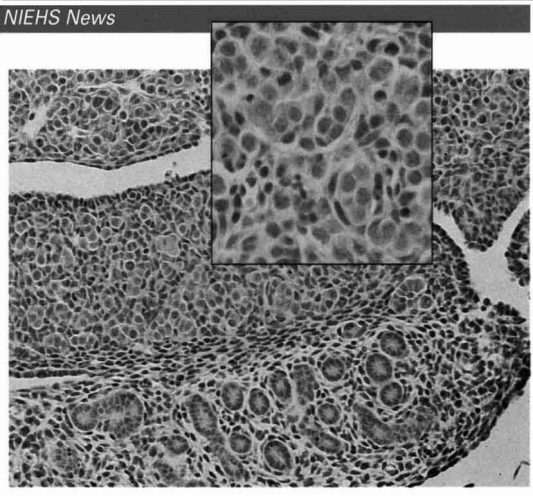
LEP investigators will soon begin studying a strain of transgenic mice developed in Japan that carries a normal human H-*ras* proto-oncogene. When these mice were exposed to potent carcinogens, this gene was activated in the induced tumors. Maronpot and his colleagues will expose the mice to environmental chemicals to which people may be exposed such as chloroform, chlordane, ethyl acrylate, and furan, which have previously been shown to cause cancer in laboratory animals. If the mice respond as expected, they will be used to test agents whose effects are unknown. "The questions are, however, Are we going to miss something with this technique or will we be overpredicting which agents cause cancer?" Maronpot says. "So far, these mice show a lot of promise. If we show that environmental agents produce tumors containing an activated human H-*ras* gene, then we could warn people about their exposure."

Sleuthing *in situ*

A relatively new area of investigation involves *in situ* hybridization, which allows researchers to identify proteins produced by specific genes in the tissue where these proteins are normally found.

Most of the current LEP *in situ* studies involve mouse ovarian toxicity and cancer. Barbara Davis, a guest researcher in the LEP, and researcher Kathy Phillips are studying estradiol, a hormone neccesary for reproduction, in ovarian granulosa cells, which line the egg-containing follicles, to determine the effect of environmental agents on estradiol production. Because estradiol production is the same in animals and humans, it may be a good system for comparing toxicity.

Specifically, Davis is investigating how environmental agents act on aromatase, an enzyme that converts testosterone to estradiol and also acts on the estrogen and androgen receptors. The studies are performed in whole tissue and then *in situ* in specific rat ovarian cells. The primary agents under investigation are di(2-ethylhexyl)phthalate, which rat studies have shown suppresses estradiol production, and other phthalates to see if this class of agents has the same actions. "Phthalates are ubiquitous in the environment," Davis says. Phthalates are components of plastics and readily leech into the environment.



Akiko Enomoto

Ovarian variation. Using *in situ* hybridization, researchers can study expression of the oncogene *WT1* in ovarian cells.

LEP and other NIEHS researchers will also study mercury vapor using *in vivo* rat studies and *in vitro* rat and human granulosa cells. "We will look at these cells *in vitro*," Davis said. "If we determine that mercury disrupts the pathways, then it implies that these pathways are likely to be disrupted in humans also." A 1994 study by NIEHS epidemiologist Andrew Roland found that female dental assistants with high occupational exposure to mercury (by making mercury amalgam tooth fillings) were less fertile than unexposed dental assistants.

Davis is using *in situ* hybridization studies to generate markers to identify the genes affected in ovarian cancer. There are not many studies on agents that cause ovarian cancer, although ovarian cancer is the fifth leading cause of cancer death in women. "If we better understood the underlying mechanism that causes ovarian cancer, we could develop preventative measures," Davis said. Davis and Phillips are studying the *WT1* marker, an oncogene expressed in immature and adult ovarian cells, using an *in situ* method developed by pathologist Akiko Enomoto. They are also investigating the tumor-suppressor gene p53, and *BRCA1*, an oncogene involved in inherited breast tumors as well as in ovarian cancer. These studies are being conducted using molecular probes, synthetic complementary copies of DNA sequences used to look for messenger protein expression.

These studies use *in situ* polymerase chain reaction (PCR), which allows the researchers to make millions of copies of any DNA sequence.

Signs of Promise

Other ongoing research areas in the LEP include the development of immunohistochemistry methods for cell products such as p53, estrogen receptors, fibroblast growth factors (FGF-ß), and other products related to cell growth and proliferation. The reproductive pathobiology group of the LEP, led by Darlene Dixon, is using immunohistochemical staining of mouse and human tissues to assess the presence of several growth factors in uterine tumors. Preliminary findings show that one particular growth factor, TGF-α is found in uterine smooth muscle tumors and expression increases with malignancy. Immunolocalization of a mature form of this growth factor has been found exclusively in malignant uterine smooth muscle tumors in mice.

Other techniques are under development such as *in situ* polymerase chain reaction (PCR) which will allow the amplification of specific sequences of DNA and RNA present in small amounts in individual cancerous cells. The technique enables researchers to amplify or make copies of a gene inside a cell, without destroying it, attach a color marker, and identify where a "bad" gene is located. "We want to understand how genes are activated, and when that occurs in the process in intact tissue, not tissue extract." Maronpot says. "This technique will allow us to identify damaged cells. Although many cells are exposed to the environmental agent, only some change, others die or are repaired. But the cell that did not die or repair itself, perpetuates the damage." Maronpot stresses that the contributions of molecular pathology must be combined with those of scientists in many disciplines including chemistry, toxicology, and physiology to unlock the secrets of how environmental agents affect human cells.

Barbara Proujan

Barbara Proujan is a freelance journalist in Graham, North Carolina.

What's Hiding Under the Sink:

Dangers of Household Pesticides

In the war against home and garden pests, over 70 million American households make more than 4 billion pesticide applications per year. Indeed, 85% of America's 84.5 million households maintain a home arsenal averaging three to four pesticide products, ranging from pest strips, bait boxes, and bug bombs to flea collars, pesticidal pet shampoos, aerosols, granules, liquids, and dusts. There are over 20,000 different household pesticide products containing over 300 active ingredients and perhaps as many as 1,700 inert ingredients, according to the *National Home and Garden Pesticide Use Survey*, which was prepared for the EPA by the Research Triangle Institute in 1990. Seventy-five percent of American households use insecticides, with cockroaches and ants the leading targets.

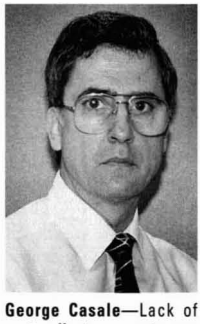
It's not just a disgust for bugs that prompts such widespread use. According to Tim Maniscalo, a company spokesperson for DowElanco, a manufacturer of chlorpyrifos, a leading pesticide ingredient, about 40% of the population is allergic to shed cockroach parts. Stings and bites from venomous pests such as fire ants and brown recluse spiders can be life threatening. Fleas, ticks, and mosquitoes are potential vectors of a wide range of diseases, ranging from bubonic plague to lyme disease to malaria. Thus, there are strong reasons for having household pesticide products available. Still, the pervasiveness of household pesticides makes the potential acute and chronic health effects of these products a matter of practical concern.

Nationwide in 1993, 140,000 pesticide exposures, 93% of which involved home use, were reported to poison control centers. About 25% of these reported exposures involved pesticide poisoning symptoms. Over half the exposures involved children under age 6, according to EPA health statistician and incident data officer Jerome Blondell. But Blondell worries that acute pesticide poisonings and poison control center statistics may be only the tip of the iceberg when it comes to the impact of household pesticide use on human health. Blondell says we could be misdiagnosing or overlooking chronic effects from some of today's common household pesticide products.

Carbamate Insecticides

Carbaryl and propoxur, carbamate insecticides introduced in 1956 and 1963, respectively, like most widely used household pesticides, have relatively low acute mammalian toxicities. "However," asks George Casale, research assistant professor at the University of Nebraska Medical Center's Eppley Research Institute, "are you deceiving yourself to think that because there is not acute exposure toxicity that you are safe?"

"The very safety of some of these pesticides could spell rather significant danger, because you can be exposed to quite a lot of pesticide with no concern," says Casale, who studies the immunological effects of common anticholinesterase pesticides, ranging from organophosphates like dichlorvos to carbamates like carbaryl. "People are too comfortable with some of these low acute toxicity pesticides, and they shouldn't be."



U. of Nebraska Med. Ctr.

George Casale—Lack of acute effects may not mean a pesticide is safe.

Anticholinesterase pesticides inhibit breakdown of the neurotransmitter acetylcholine by inhibiting acetylcholinesterase, a serine hydrolase enzyme. The possibility that carbaryl and other anticholinesterase insecticides affect more than the nervous system, for example, also impairing immune processes dependent on serine hydrolase activity, is usually ignored. However, in a series of experiments over the last several years, Casale and co-workers demonstrated that carbaryl and other common anticholinesterase insecticides inhibit serine hydrolase-dependent immune processes, such as interleukin 2 (IL-2) signaling.

Concentrations of carbaryl below those causing acute toxicity inhibit human natural killer cells *in vitro*. Natural killer cells are particularly effective against leukemias and lymphomas, which epidemiological studies have correlated with farm use of anticholinesterase pesticides around the world. In Casale's high-dose pilot study of mice, carbaryl, which is metabolized similarly in mice, rats, and humans, inhibited natural killer cells. Whole-animal studies of carbaryl and other anticholinesterase pesticides to determine whether natural killer cell inhibition occurs at low doses from repeated exposures, as would be the case in chronic household pesticide use, have yet to be concluded.

The next logical research step, says Casale, is to develop a whole-organism model to determine where at the cellular level the pesticide is affecting the immune system. Neither the kind of esterases inhibited by carbaryl and other anticholinesterase pesticides nor the actual esterase targets have been identified. Scientists do not know why carbaryl, despite its exceptionally low acute toxicity to the nervous system, is more toxic to the complement system than paraokon, the pri-

mary metabolite of the more acutely toxic pesticide parathion. "There has been very little in the way of a systematic approach to studying biological interactions with these chemicals," says Casale. Even in regard to cancer, it is hard to come up with general conclusions about these pesticides. "The support has been helter-skelter, not systematic."

"The problem that I have with transient acute effects," says Casale, "is that people are not exposed once to a chemical, but rather are exposed repeatedly." Even pesticides with very low acute toxicities can be so highly reactive with body proteins that crude pilot tests show 100% bonding with proteins within 24 hours. "There are probably quite a few chemicals out there, that . . . will modify proteins that the body will then recognize as foreign," he says. Casale decries the paucity of immunologic research on the many pesticides causing dermatitis and rashes, as these are likely candidates in processes related to allergy and autoimmune reactions.

According to Rudy Richardson, director of toxicology at the University of Michigan, much of the work on the immunologic effects of pesticides is difficult to interpret and equivocal, and there is not much in the way of controlled studies in humans. Richardson is hopeful that more immunologists will go into toxicology in the future. "We have spent an enormous amount of time in pesticides with cancer assessments," says John Bucher, acting chief of the toxicology branch of the Environmental Toxicology Program at NIEHS. "[But] we could be missing the boat on the potential effects on the immune system. What we see is an increasing number of reports on multiple chemical sensitivity, which anecdotally has been set off in people by one large exposure to a pesticide or multiple pesticides." Bucher believes that there is some immune system involvement in multiple chemical sensitivity and that the role of pesticides needs more study. Also insufficiently studied are subtle nervous system effects from pesticide exposures. "We almost never see anything on learning, memory, and potential psychological effects of exposures," adds Bucher. "You can't ask a test animal for the kind of information that you can ask people. So you can't adequately study some of these things with animal models."

Organophosphate Insecticides

Chlorpyrifos, an anticholinesterase organophosphate that is among the 10 most commonly used household insecticides, has been in use since 1966. Neonatal animals generally show a higher sensitivity to organophosphate insecticides than older animals, and chlorpyrifos is no exception. Human newborns have very low concentrations of the serum enzyme needed to detoxify chlorpyrifos, says Clement Furlong, director of the toxicology program at the University of Washington. Furlong and graduate student Wan-Fen Li found that newborn rodents require several weeks to develop the enzymes needed to detoxify chlorpyrifos. Furlong is currently studying how long it takes human newborns to develop the serum enzyme needed for chlorpyrifos detoxification.

NCI

Sheila Zahm—The whole idea is prudent avoidance to minimize pesticide exposure.

Detoxification of organophosphate pesticides is genetically controlled in humans and other species, and at least 15-fold differences exist among humans in their ability to hydrolyze the toxic metabolite of chlorpyrifos. This biochemical individuality may help explain the variation in symptoms from similar pesticide exposures. When Furlong studied New York City pesticide applicators applying chlorpyrifos full-time, all had the resistant phenotype. Others are less fortunate in their genetic inheritance. Permanent cognitive damage manifest as a substantial drop in IQ to below normal was the outcome for a physician accidentally poisoned by chlorpyrifos. In other cases, including many instances of neurodegenerative disorders liked delayed neuropathy, the damage is more transient, and the person eventually recovers.

"I have some concern regarding the over-the-counter sales of these compounds because the average householder does not have a clue how damaging these pesticides can be if misused," says Furlong, who half-jokingly concludes seminars by telling those with low levels of detoxification enzyme to switch to fly swatters. Actually, the genetics of detoxification are quite complicated: a two-step activation and breakdown pathway is controlled by different genes. Also, genetic resistance to one pesticide does not necessarily mean resistance to others. For example, people with the genotype most resistant to parathion are most susceptible to diazinon and vice versa. There are also environmental influences, such as smoking and drugs, which may increase sensitivity by speeding up P450 microsome activation of chlorpyrifos into its neurotoxic oxon metabolite.

"One possibility with an insecticide such as chlorpyrifos that can cause extensive neurochemical changes in the absence of overt signs is that significant exposures can occur with less indication of exposure," says Carey Pope, director of the toxicology program at Northeast Louisiana University. Long-term neurochemical and behavioral effects of chlorpyrifos on the brain may be cryptic, easily overlooked, and persist in adults after a single exposure without overt signs of toxicity.

"The adult brain appears more sensitive to persistent neurochemical changes, compared to the neonatal brain," says Pope, citing chlorpyrifos experiments using the maximum tolerated dose, the highest dose of a chemical causing no lethality. About 50% of the maximum tolerated dose of chlorpyrifos inhibits brain neurochemicals in young animals, whereas about 15% of the maximum tolerated dose causes a similar 50% inhibition of acetylcholinesterase activity in adult brains. Richardson, who recently reviewed the literature on the neurotoxic potential of chlorpyrifos in the *Journal of Toxicology and Environmental Health*, emphasizes that chlorpyrifos exhibits only moderate acute toxicity in most mammalian species because the active oxon metabolite is detoxified. When problems like delayed neurotoxicity occur, it is usually associated with extremely large doses of the insecticide, well above those encountered in normal household use.

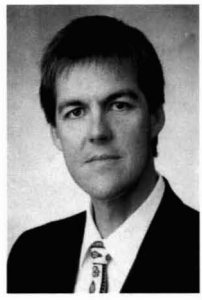
Another organophosphate insecticide with possible brain effects, dichlorvos, is found in 8.3 million households and applied

Top Ten Home and Garden Pesticides

Active ingredient	Thousands products	Percentage products	Thousands households	Percentage households	Thousands applications indoors	Thousands applications outdoors
Piperonyl butoxide (synergist)	41,729	12.76	27,335	34.01	294,013	58,991
Pyrethrins	34,609	10.58	22,739	28.46	244,328	39,289
MGK-264) (synergist)	27,558	8.43	19,532	24.51	203,328	13,249
Propoxur	21,484	6.57	18,749	23.71	209,528	53,594
DEET	21,544	6.59	17,227	21.78	238,433	14,134
Aliphatic petroleum hydrocarbons	18,652	5.70	14,480	18.27	110,701	32,750
Carbaryl	18,437	5.64	12,494	15.77	28,591	31,735
Phenylphenol	17,618	5.39	16,227	20.63	537,048	1,452
Bleach	16,266	4.97	15,591	19.95	672,959	10,397
Chlorpyrifos	16,652	5.09	13,993	17.81	174,322	41,900

Source: U. S. Department of Commerce

72 million times per year, mostly indoors via foggers and pest strips. "Indoor air use of pesticide products in the home is the main source of exposure for children," says toxicologist William Pease of the University of California-Berkeley School of Public Health. Pease asserts that exposures from household use exceed those from pesticide residues on food. His review of San Francisco Bay area poison control center records revealed cases of children becoming sick after crawling on freshly sprayed floors and carpet. "Basically, pesticide products do not need active ingredient coating all spaces in the home," says Pease.



DowElanco

Eric Wintemute—Industry has made great improvements toward safer products.

Pease also expresses concern about *para*-dichlorobenzene moth balls and other products designed for continuous pesticide release in closed spaces, where concentrations may become elevated. Pease cites regulatory EPA calculations that a child who is active during 6 out of 24 hours of confinement in a room treated with a dichlorvos fogger can absorb a dose exceeding that causing cholinesterase inhibition in chronic animal experiments, resulting in symptoms such as runny eyes, diarrhea, or nausea.

Though dichlorvos is hardly unique in its anticholinesterase mode of action, it has been unfairly singled out by the media and antipesticide groups for criticism on the basis of health effects studies that have been misinterpreted and even later been shown to be wrong, says Eric Wintemute, president and CEO of Amvac Chemical Corporation, which makes dichlorvos. "Industry as a whole has made great improvements towards more environmentally friendly products," says Wintemute.

Dichlorvos and other organophosphates replaced the older, more persistent organochlorine insecticides like DDT because they break down relatively quickly and do not have long-lasting residues. "As far as household pest control, we certainly agree that the first line of defense should be screens and mechanical control of flying pests," says Wintemute. Baits and traps, which are alternatives to spraying a whole area with pesticides, are becoming more popular and work well against crawling insects like roaches, but are not so effective for flying insects, he says. "At the point where pests become a nuisance, because of the health risk, as insects do spread disease, this is when to consider pesticides," says Wintemute. "Unlike aerosols, pest strips are controlled release to decrease levels of the target pest. Pest strips can be formulated so that there is a lower level of exposure than with aerosols, so there is less possibility of misuse."

EPA

Louise Mehler—There are inert ingredients of real toxicological significance.

Use of dichlorvos for controlled release from pet flea collars was stopped in the mid-1980s, following reaction to a "Today Show" vignette on network television that showed a child petting a cat while a voice cited a study saying that children had a 100 times greater chance of getting cancer when the pet had a flea collar containing dichlorvos. "It was emotional, an overreaction, fear-driven," said Wintemute, in describing how Hartz, and then other pet flea collar companies, stopped using dichlorvos and switched to competing active ingredients, such as chlorpyrifos.

The positive cancer study in question was conducted by the National Toxicology Program (NTP) and conflicted with 10 published studies that were negative. According to Wintemute, a review by the EPA Science Advisory Panel found serious problems with the NTP study and interpretation of the data. For instance, there was no allowance for the fact that rat tumors in the study were benign, not malignant, or that, unlike mice, humans do not have a forestomach subject to feeding tube irritation, or that doses were relatively high and the aging rats were tumor-prone. But the political climate in 1987 was such that the EPA reclassified dichlorvos as a probable carcinogen on the basis of one partially positive and 10 negative studies, said Wintemute. In 1989, after new studies and a special review, the EPA reclassified dichlorvos from a probable carcinogen to a possible carcinogen. "We are working to move dichlorvos to a group d classification [insufficient evidence to determine potential carcinogenicity] or group e [not a proven carcinogen], where we feel it belongs," says Wintemute.

In 1992, Japan completed its review of dichlorvos and concluded that it was not a human carcinogen. In 1993, the World Health Organization concluded that dichlorvos was not a chronic health hazard. In 1994, The United Kingdom concluded that no classification was required for human carcinogenicity and that dichlorvos was not a mutagen. Yet dichlorvos on pest strips was associated with childhood leukemia in a recent epidemiological case–control study in Denver, Colorado, published in the *American Journal of Public Health*. However, Wintemute questions the scientific value of answers obtained when parents of leukemia victims are asked if they had used pest strips.

In a separate case–control study in Missouri by James Davis, an epidemiologist with the Missouri Department of Health, dichlorvos and other insecticides were associated with an elevated odds ratio for childhood brain cancer. "The true extent of exposure and health problems associated with consumer pesticide use are currently unknown," says Davis, noting that the epidemiological studies "raise some red flags that should be looked at," particularly since no primary cause has been identified for childhood brain cancer.

"If epidemiological studies are detailed enough to indicate certain agents and correlate with laboratory studies, that can certainly be very powerful evidence," says Sheila Zahm of the National Cancer Institute, referring to Davis's studies. However, Zahm is also quick to add that epidemiological leads are not always confirmed by animal studies. A major case in point is the widely used lawn herbicide 2,4-D, which is found in 10.5 million households. Epidemiological studies with farmers linked 2,4-D use with lymphomas. However, animal studies later vindicated 2,4-D as not being a carcinogen.

There are many limitations and variables which must be kept in mind when evaluating epidemiological studies, says Richardson. Results can be influenced by how questions are asked, and human memory can be selective. People may remember a pesticide spray but forget dietary or other potential causal factors, or vice versa. Also, epidemiological studies typically lack exposure data at the part-per-million level and lack blood cholinesterase measurements that would be of most value to toxicologists. "We need more basic toxicology and more controlled studies," says Richardson. "If we had double-blind prospective studies, instead of retrospective studies, then we could draw some real conclusions."

In a recent Norwegian study published in *Neurochemical Research* in 1994, researchers fed dichlorvos to pregnant guinea pigs and found a dose-dependent reduction in brain weights of offspring unrelated to either body weight changes or specific neurotransmitters or brain regions monitored. Dichlorvos alkylation of neuronal DNA early in development, such as during the brain growth spurt period (days 40–50 of gestation in guinea pigs) before DNA repair enzymes become active, is suspected.

The best way to deal with fetal and infant pesticide sensitivity, contends

Wintemute, is to have a 100-fold buffer zone above the no-effect level so that adverse effects are unlikely even if the product is abused. Though protocols exist for exposing pregnant animals to a chemical and examining the second generation for mutagenesis and teratogenicity, says Zahm, "there is nothing specific if a pesticide is a suspected child carcinogen. There are standard testing protocols, but often the target sites in animals are not the same as in humans." So it would be difficult, barring breakthroughs in testing protocols, to check, for instance, for brain cancer risk in children using current standard animal testing protocols. "The whole idea is prudent avoidance to minimize exposure, especially to children, and certainly if pregnant to try not to use anything," says Zahm, noting that rapidly growing fetuses may be more susceptible to mutagenesis, chromosomal aberrations, and carcinogenesis. Zahm also points out that infants crawling around on carpets can be affected by lawn and other outdoor pesticides tracked indoors. When these chemicals are brought indoors, the residues last much longer than outdoors, where water and sunlight promote biodegradation. Both Zahm and Davis believe that more studies need to focus on the fetus and on infants from birth to age 6 months, as these are critical periods of susceptibility.

Petroleum Hydrocarbons

Aliphatic petroleum hydrocarbons are the sixth most common active ingredient in household pesticides today. Highly refined horticultural oil has a relatively low acute oral toxicity, though it is a skin and lung irritant. Petroleum oils vary greatly in terms of refining, and hence in amounts of aromatic hydrocarbon impurities, which are potentially toxic benzene-ring compounds. Thus, petroleum oil-based pesticides are complex mixtures of varying quantities of aromatic and aliphatic hydrocarbons with potentially diverse toxicological profiles and health effects.

Among the few toxicological effects of aliphatic petroleum hydrocarbons mentioned in a 1988 petroleum industry review in *Occupational Medicine* are central nervous system depression manifested as dizziness and incoordination. Petroleum hydrocarbons and solvents of various sorts, including aromatic compounds such as benzene, toluene, and xylene, are also among the unnamed "inert ingredients" formulated into household pesticide products.

Synergists and Pyrethrins

The synergist piperonyl butoxide (PBO), the number one active ingredient in household pesticides, is commonly formulated with the number two active ingredient, pyrethrin compounds, in household pesticide products. Synergists by themselves have little pesticidal activity, but increase the effectiveness of other pesticide active ingredients. PBO has an extremely low acute oral toxicity. By itself, PBO has, at least until recently, been considered neither mutagenic nor carcinogenic, though liver and kidney damage has been noted over the years in animal studies. However, Japanese researchers at the Tokyo Metropolitan Laboratory of Public Health have recently published a series of chronic toxicity studies that shows a dose-dependent relationship between hepatocellular carcinoma and PBO when doses are increased to exceptionally high levels, well above what human beings are ever likely to encounter.

Ironically, if a synergist like PBO were banned as a carcinogen, higher amounts of other pesticide active ingredients would be added to the environment because synergists allow dramatic reductions in quantities of active ingredients needed for the pesticide to be effective.

The third most common active ingredient is the synergist MGK-264, which is applied over 200 million times per year. The *Hazardous Chemicals Desk Reference* refers to MGK-264 as being of moderate toxicity, with central nervous system and reproductive effects in experimental animals. Surprisingly, for such a widely used household pesticide ingredient, MGK-264 is not currently the subject of much toxicological research.

Pyrethrins are the collective name for a group of six pesticidal compounds derived from pyrethrum flowers in the genus *Chrysanthemum*. Pyrethrum flowers and refined pyrethrin extracts with varying amounts of floral impurities, some of which are allergens, have been used in pest control for several centuries. Though pyrethrum extracts are relatively low in terms of acute toxicity, there is concern that pyrethrins and their synthetic counterparts, pyrethroids, can trigger allergic reactions, particularly among the nation's estimated 15 million persons with asthma.

A 1994 report by Paul Wax, a physician at the Strong Memorial Hospital in Rochester, New York, published in *Clinical Toxicology,* reported the death of a 37-year-old woman with a history of mild asthma after inhaling a pyrethrin pet shampoo. Minutes after applying the shampoo, the woman developed fatal lung symptoms, went into cardiopulmonary arrest, and died. However, said Wax, the 0.06% pyrethrins in the pet flea shampoo were not proven to be the cause, as there were neither immunological studies of the event nor subsequent animal studies trying to reproduce the result. The report was strictly observational, as is often the case in pesticide exposure incidents. Still, after the shampoo ingredients and emulsifiers listed on the label were excluded as allergens, pyrethrins were the only known allergen the woman could have been exposed to. However, 54% of the flea shampoo was labeled inert ingredients, which are considered trade secrets not divulged even to the medical profession.

At play among pesticides. Infants and children are at greater risk from exposure to pesticides on lawns and pets.

Several derivatives of natural pyrethrin molecules, known as synthetic pyrethroids, are also widely used household pesticides and are suspected to be allergens. Some of these include tetramethrin, resmethrin, and allethrin, cumulatively found in over 30 million households. Thus, pyrethrin and pyrethroid products may need to be labeled with bronchospasm warnings for asthmatics.

Inert Ingredients

The EPA estimates that there are at least 1,700 chemical compounds collectively listed under the rubric "inert ingredients" on pesticide labels. A recent walk down a supermarket insecticide aisle revealed many products labeled as over 99% inert ingredients. Section 2m of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) states: "The term 'inert ingredient' means an ingredient which is not active." In actual practice, pesticide manufacturers decide what to call inert and what to designate as an active ingredient subject to EPA regulation. This has produced a situation where ingredients considered active and regulated by the EPA in some pesticide products are unregulated, inert ingredients missing from the labels of other pesticide products. The EPA has produced several categories of inert ingredients, which include several of the top household pesticide active ingredients. According to the EPA's Office of the Inspector General, "EPA knows little or nothing about the adverse effects of most of

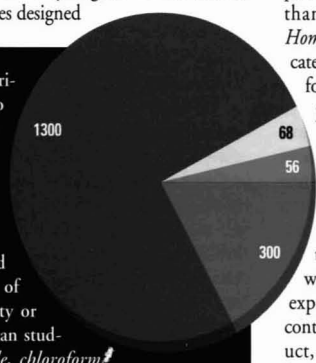
these inerts [inerts of unknown toxicity]. Some data may exist for the inerts of unknown toxicity, but EPA has not yet evaluated the data to determine the effects."

Inert ingredients are low priority, accounting for under 1% of the Office of Pesticide Programs budget, as the EPA still has many older (pre-1972) active ingredients that need to be reregistered and evaluated for health effects under FIFRA. Also, EPA has no specific procedures or timeframes for ensuring that these inerts are reviewed, according to the EPA's Office of the Inspector General. "Until these reviews are completed, users are unaware of potentially toxic inert ingredients contained in certain pesticide products. The use of these pesticide products may be jeopardizing human health and the environment," states the office.

"Inert ingredients are confidential information," adds California-EPA's Louise Mehler, a physician and program director of CAL-EPA's Worker Pesticide Illness Surveillance Program. "If we were to disclose that information we could be prosecuted for it and imprisoned. There are inert ingredients that are sometimes of real toxicological significance. It could also be just water." Though inerts are trade secrets protected by law from disclosure, it is widely believed that pesticide companies know their competitors' inert ingredients, as reverse engineering is relatively simple with today's technology. "The chemists here say that since the invention of the mass spectrometer anybody who wants can really find out," says Mehler.

Not all chemical companies are rigorously secretive, and some reveal their inert ingredients upon request. For example, DowElanco makes no secret in its technical literature that its liquid formulations of chlorpyrifos are "usually solutions of chlorpyrifos in a petroleum fraction" referred to as "xylene range aromatic solvent." The major components of this solvent are nine-carbon aromatic hydrocarbons with some xylene. Xylene is sometimes registered as an active ingredient, as it is has pesticidal activity, but it is more commonly used as an inert ingredient to keep the pesticide active ingredient in solution, prevent clumping, and as a delivery vehicle.

Xylene, toluene, and ethyl benzene are among the inerts found in common household products studied by John Wurpel, associate professor at St. John's University College of Pharmacy. Potential health effects of these inert ingredients include nonspecific depression of the central nervous system. Benzene is a known carcinogen. Xylene, at least in the case of chlorpyrifos, also has synergistic health effects. In rat studies designed to examine behavioral effects of low pesticide doses similar to those found in homes, "We were surprised to see birth effects [embryotoxicity] because we used a low dose," said Wurpel. Usually chlorpyrifos is a teratogen only at high doses. But in combination with xylene there is a synergism, probably because the xylene carrier allows the pesticide to enter the fetus. Thus, inert ingredients in household pesticide formulations can complicate interpretation of health effects based on pure active ingredients.



Potentially toxic—Inert ingredients with a high priority for testing. Many of these are structurally similar to other chemicals that exhibit toxicity. Either testing is already underway for these potentially toxic ingredients, or the existing data suggests potential adverse effects. *Examples: toluene, xylene, petroleum hydrocarbons, methyl bromide*

Toxic—Inert ingredients with known adverse effects and of toxicological concern. These ingredients have evidence of carcinogenicity, adverse reproductive effects, neurotoxicity or other chronic effects, or birth defects in laboratory or human studies. *Examples: aniline, asbestos, benzene, carbon disulfide, chloroform, formaldehyde; hexachlorophene, lead, cadmium, mercury oleate, pyrethrins, and pyrethroids*

Generally recognized as safe—Inert ingredients for which EPA has no reason to expect adverse effects to occur. These include ingredients of minimal concern and for which sufficient information is available to conclude that adverse effects are not expected. *Examples: alfalfa, cardboard, castor oil, dextrose, ethanol, fish meal, gypsum, lard, latex, nylon, olive oil, onions, pine oil, polyvinyl chloride resin, rubber, silicone, sodium fluoride, urea, water, wintergreen oil*

Unknown toxicity—EPA knows little or nothing about the adverse effects of most of these inert ingredients . Some data may exist for the inert ingredients of unknown toxicity, but EPA has not yet evaluated the data to determine the effects. These ingredients presented no cause for suspicion. An inert ingredient was put in this group if there was no basis to put it in any of the other three groups. *Examples: barium sulfate, epoxy resin, aluminum powder, styrene acrylic copolymer, sodium nitrite, sulfuric acid, salicylic acid, limonene, thymol, menthol, lithium chloride, napthalene, polyethylene terephthalate, D and C Red # 37, saccharin, malathion, kerosene, coal tar, asphalt, lanolin, camphor, boric acid, Freon 114*]

Source: U.S. EPA Office of the Inspector General, *Inert Ingredients of Pesticides* (audit report no. E1EPF1-05-0117-1100378), 27 September 1991.

Future Directions

A 1994 study of pesticide labels published in the *Journal of the American Optometric Association* found that it requires an 11th-grade cognitive reading level to understand a pesticide label, which means that 40–50% of the general population cannot read and understand the directions on a pesticide product label, assuming they have the necessary 20/30 visual acuity to read the fine print. This study suggests that labeling may not be even minimally effective in protecting the common user of household pesticides from adverse health effects.

In addition, some experts suspect that there may be a lot of avoidable urban pesticide exposures because people may be using pesticides out of annoyance or fear, rather than actual need. Indeed, the *National Home and Garden Pesticide Use Survey* indicates that 37% of all U.S. households treat for insects even when there is not a major problem. However, almost 39% of households use insecticides because they have a major insect problem, often of pests of potential public health importance.

There is a trend toward use of less toxic alternatives, like baits and traps, which minimize household pesticide exposures. "Because of the difficulties in controlling how the end-user uses the product, and knowing that at least some will become ill, as we are currently seeing adverse effects, the question in our minds, since there are alternative means of treating many pests, is if we should even recommend some of these products when we know that there are alternatives," says Pease. Experts agree, however, that it would be premature to call a truce against pests and jettison household pesticides, despite potential health risks, until we have effective alternatives available. Until that time, efforts in the war against bugs should include systematic research into the potential chronic health effects of the most widely used household pesticides and their potential replacements.

Joel Grossman

Joel Grossman is a freelance journalist in Santa Monica, California.

Third Congress of Toxicology in Developing Countries
Cairo, Egypt
November 19-23, 1995
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What's Eating Us about What We're Eating

Most people know the old adage: an appple a day keeps the doctor away. Increasingly, Americans are paying heed to the adage and taking it many steps further, eating their greens and downing their multivitamins in the hope of staving off all types of cancer. But the daily bombardment of conflicting advice about what to eat to stay healthy is enough to kill your appetite.

The connection between nutrition and cancer prevention is still controversial. The Food and Drug Administration will not allow labeling to the effect that food, food supplements, and vitamins prevent disease because it hasn't been proven. Almost all cancers of epithelial origin, such as prostate, colon, breast, and lung, are believed to be affected by diet, however, and scientists are struggling to pinpoint exactly how diet contributes to the development and progression of these cancers. In particular, researchers are investigating the contribution of fat and calories to a variety of cancers, including those outside the digestive tract, and the roles of fiber, nutrients, and antioxidant vitamins in cancer development. People are eager to hear the results of such research, hoping for a dietary prescription to prevent cancer.

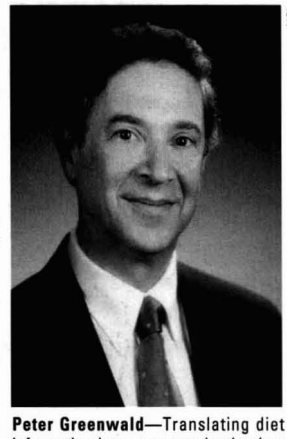
The growth of the National Cancer Institute's diet and cancer budget is evidence of the increasing interest in the diet–disease connection. Diet and cancer research began at NCI in 1974 with less than $3 million and grew by 1990 to more than $67 million. This funding was boosted by a series of scientific review reports, such as the one in 1980 by the National Research Council that suggested that many common human cancers, including cancers of the esophagus, stomach, liver, colon/rectum, lung, breast, and prostate are influenced by dietary patterns.

Follow-up reports by the U.S. Public Health Service and the National Research Council emphasized that further basic and applied nutritional research is needed, including clinical prevention trials. According to Peter Greenwald, director of the Division of Cancer Prevention and Control at NCI, the challenge that the agency and investigators face is huge: "to effectively translate diet and cancer information into a significant reduction of cancer incidence and mortality."

There is conflicting information about the precise role of dietary factors, and cause-and-effect relationships have not been established: for every confirmatory finding, another study finds no association. And many of the research questions are still fundamental; for example, when studying the contribution of fat in diet, should researchers really be looking at calories, since fat is so laden with calories? Most disturbing to some researchers is that in most cases the mechanisms behind diet and cancer have not been detailed. And the preliminary models that exist have been disputed.

Furthermore, there has been a reluctance to base recommendations for the modification of human diets on observations in experimental animals. Too often, some researchers say, laboratory animals are obese, so the contribution of nutrients to their health cannot be separated out.

"Teasing apart nutrition is a long row to hoe, and we have only just gotten started," said Bernard Weinstein, director of the Comprehensive Cancer Center at Columbia-Presbyterian Cancer Center. "With the thousands of compounds people put in their mouths, the study of diet is unbelievably complex."


NCI

Peter Greenwald—Translating diet information into cancer reduction is a huge task.

After years of study costing millions of dollars, NCI's Greenwald says that the knowledge at hand can suggest only general advice on how to cut your chances of getting cancer. "There is enough strong evidence to say that eating patterns affect your risk, not only of cancer, but of heart disease, and diabetes, and that you should cut your fat and stay trim," he said. "Although we have no answers yet on how specific constituents of food contribute to cancer, there are no studies that show you can be worse off by eating more vegetables and fruits."

Contradictory Evidence

Critical dietary factors implicated in the development of breast, colon, and other epithelial cancers consist of macronutrients, such as fat and fiber; micronutrients, such as vitamins and minerals; and the hundreds of non-nutritive constitutents in vegetables and fruits. For example, a diet rich in micronutrients found in fruits and vegetables appears to be protective for several types of cancer, including cancers of the lung, colon, rectum, bladder, oral cavity, stomach, cervix, and esophagus. Increased body weight is associated with postmenopausal breast and endometrial cancer. But the most vocal debates swirl around the contribution of fat in the diet for colon, rectum, breast, and prostate cancers.

This debate centers on the relative value of diet–disease associations depending on what type of study is done—epidemiological reviews, case–control studies, or randomized clinical trials. A major problem with most epidemiological studies is that they rely on the recall of the eater. Few randomized trials are conducted because they are expensive and difficult to manage. Problematic in all of these studies, researchers say, is the question of what other lifestyle factors may play a role. For example, a person who doesn't eat much fat is likely to eat more fruits and vegetables and be committed to other health measures such as exercise and reduced alcohol consumption. So the question remains: how can the separate effects of each of

these variables be determined?

Cancer researcher Cheryl Ritenbaugh of the University of Arizona says that in general such studies need to be more structured. Speaking at the Fourth International Conference on Prevention of Human Cancer, held in Tucson, Arizona, in June 1992, Ritenbaugh said: "There is a need for prospective, placebo-controlled clinical trials to test the low-fat, high-fiber, and increased numbers of fruit and vegetable servings hypothesis in specific high-risk populations for breast, colon, lung, and prostrate cancer."

Breast cancer. Breast cancer research may be the most contentious area of research and illustrates the difficulties in drawing connections between nutrition and malignancies. Greenwald summarizes the state of research on nutrition and breast cancer this way: "[Regarding] fat, there is a fair amount of agreement, but strong views the other way. Antioxidants are less clear, but need to be studied. Estrogen contribution is a hypothesis, but it is important. There are contradictory studies on pesticides. The contribution of exercise is debated. More study is needed on alcohol as a contributing factor."

The primary support for the proposed link between dietary fat and cancer is based on studies comparing countries such as Japan and China which have low fat intake and low rates of breast cancer, as well as cancers of the colon and prostrate, with countries such as the United States where fat intake is high and there are high rates of breast cancer. Similar correlations have also been observed in regions within countries, like Italy, in which the fat-consuming north has higher levels of breast cancer than the south, where the diet is leaner. But results of such epidemiological studies have different implications to researchers who question whether other variables may be responsible.

For example, scientists question whether low breast cancer rates in women in some countries are due not to eating less fat and its associated calories, which can trigger cell division, but due to having less body fat, a genetic factor contributing to cancer. Other researchers hypothesize that less fat consumption in childhood delays the onset of menstruation, and thus exposure to estrogen (prolonged estrogen exposure is considered a risk factor in breast cancer). Also, short stature has been positively correlated with low cancer rates in developing countries. Another factor to consider is that many rural populations have low breast cancer rates, where foods are often grown without harmful pesticides and residents may not be exposed to industrial contaminants or electromagnetic fields. Researchers are also studying the beneficial effects of fresh air and exercise in these populations, as well as lower alcohol consumption.

Some studies do seem to confirm the connection between fat and breast cancer. A 1990 meta-analysis of 12 case–control studies among postmenopausal women by the National Cancer Institute of Canada showed a 50% relative increase in breast cancer among women ingesting high intakes of saturated fat. Another analysis of postmenopausal women in Hawaii, by the Cancer Research Center of Hawaii, estimated that 10–20% of breast cancer could be prevented by significantly decreasing saturated fat intake.

Then a study appeared in October 1992 that rattled the accepted theories. The largest study of its kind, it offered convincing evidence that dietary fat and fiber do not play a role in breast cancer. Walter Willett and his colleagues at Brigham and Women's Hospital in Boston studied 89,494 women for 8 years, asking detailed questions about their diets and health. During the study period, 1,439 women developed breast cancer. But the researchers reported that no matter how they analyzed their data, they could not find any relationship between what the women ate and their chances of getting breast cancer. The fifth of women who ate the least fat, those for whom fat accounted for less than 25% of total calories, were just as likely to get cancer as the fifth of the women who ate the most fat, for whom fat accounted for more than 49% of their calories.

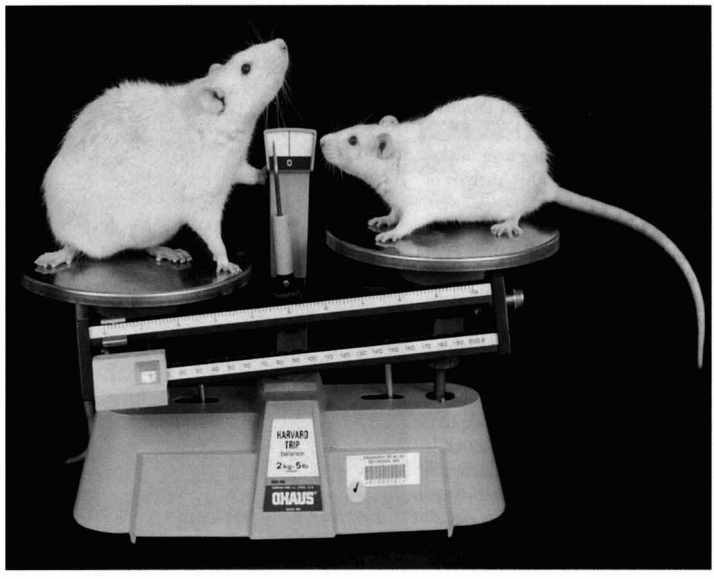
The fat factor. Researchers are now agreeing that fat plays a major role in many cancers but don't know precisely what that role is.

Criticism of Willett's study was intense and continues today because he claims no large study, epidemiological or randomized, will find any different result. Greenwald says Willet's study relied on the recall of participants, and there were "methodological and design problems," said Ernst Wynder, director of the American Health Foundation. "The totality of evidence, including a half century of animal model data, ecological data, the meta-analysis of 12 case–control studies, and plausible biological mechanisms which support the fat hypothesis" should be considered before drawing conclusions from this single study, said Wynder.

The NCI has launched a large trial to reconcile the positive correlations from international studies with the lack of positive findings from Willett's study and other case–control and cohort studies. But the $140-million, 15-year Women's Health Trial has provoked a storm of controversy because of concerns about the study's statistical power to detect an effect. Ross Prentice, head of the division of Public Health Sciences at the Fred Hutchinson Cancer Research Center in Seattle which is leading the Women's Health Trial, countered that the study is meant to answer "the public health question." Said Prentice, "The purpose is to identify a practical strategy for women to reduce their risk of cancer and other common diseases through dietary modifications that the general public can adhere to. . . . It is much less important to know exactly which change caused what degree of risk reduction, although it is of intellectual interest."

What about the contribution of food nutrients, particularly antioxidant vitamins E and C and beta-carotene (vitamin A) in reducing the risk of developing breast cancer, and indeed any cancer? Results from a 1993 study in China showed that people who took vitamins A and E had a 13% lower risk of dying from cancer and raised hopes that disease prevention was as close as a multivitamin. But, that same year, Willett reported that large intakes of vitamin C or E didn't protect against breast cancer. He did, however, observe a significant inverse association of vitamin A intake and breast cancer risk.

Colon cancer. There is perhaps a less ambiguous association between dietary fat and colon cancer, which, along with rectal cancer, is the most common form of cancer in the United States. Positive associations between animal (but not vegetable) fat consumption and colon cancer rates have been seen in many, but not all, studies. The question here has largely been which kind of fat is implicated. In the 1992 Harvard study of 89,000 nurses, those whose diets were high



Steve McCaw

A shift in the balance. Experiments on obese rats suggest weight may play a large role in cancer risk.

in red meat and animal fat were more likely to develop colon cancer than those who ate poultry and seafood. Another study of 49,000 men, published in 1992 by the Harvard School of Public Health, showed that those who ate a high-fat, low-fiber diet quadrupled their risk of developing precancerous colon polyps. But in this study, the risk was said to be due to the consumption of saturated fat (corn oil or corn/safflower oil), rather than polyunsaturated or monounsaturated fat intake (coconut oil, olive oil, marine fish oil). A further analysis of the same data earlier this year found that men with a high alcohol intake and a diet low in fruits, vegetables, and whole-grain foods are particularly vulnerable to colon cancer.

A review of the epidemiological literature concerning the contribution of fat, fiber, and calories to colon cancer by Bandaru Reddy, a researcher in the division of nutritional carcinogenesis at the American Health Foundation, found that most epidemiological models suggest that fat intake may be even more important than calorie intake in colon carcinogenesis. "However, the literature remains confusing, although the majority of these researchers agree that diets low in fat, high in dietary fibers, and high in fruits, vegetables, and calcium content are inversely associated with colon cancer risk," Reddy wrote in the journal *Preventive Medicine* in 1993.

Because many studies of fiber have shown a protective effect against colon cancer, the question arises whether it is fiber or fat that is a primary risk factor for colon cancer. Johanna Dwyer, a Tufts University cancer researcher, says, "I think it is both fat and fiber, but researchers generally fall into one camp or another."

To answer the question, the NCI is undertaking the Multisite Polyp Prevention Study to study the effect of decreasing dietary fat intake and increasing dietary fiber intake, both which can be achieved through eating more fruits and vegetables. The randomized, controlled study is based on the assumption that because there is a strong association between colon polyps and the development of colon cancer, an intervention that reduces the recurrence of large-bowel polyps has a strong likelihood of reducing the incidence of large-bowel cancer. The study is being conducted at 10 academic medical centers across the United States and is enrolling 2,000 male and female colon cancer patients over the age of 35. Half of the patients will be randomized to a control group with no intervention except for information on basic nutrition, and the other half will be assigned to the diet intervention group with target goals of eating 20% of calories from fat, 18 grams of fiber per 1,000 calories and 5–8 servings of fruits and vegetables daily. The recurrence of polyps in both groups at the end of years one and four will determine the effectiveness of dietary intervention. Initial results from an Australian Polyp Prevention Project of 400 colon cancer patients show no difference in the incidence of new cancers in a group randomized to a low-fat diet, but do show a trend for reduction of cancer spread in the group randomized to a high-fiber diet, according to Reddy.

Meaningful Mechanisms

If human studies can't answer the question, can laboratory experiments? Some researchers believe the mechanisms by which fat affects cancer risk have been neatly worked out, while some argue that most animal nutritional experiments have no relevance to humans because the animals are generally obese, thus skewing the contribution of calories to carcinogenesis.

David Rose, associate director of the American Health Foundation, has conducted numerous animal studies that he says show fat can be associated with cancer in two ways. According to one theory, fat intake can change specific fatty acids on the cell membrane, altering their function and the production of prostaglandins, which can then suppress the functioning of the immune system. High-fat diets and omega-6 polyunsaturated fatty acids, such as corn oils, have these effects, but omega-3 fatty acids, such as fish oil, do not, Rose says.

The second mechanism involves the way the body handles estrogen. One of the least controversial notions about breast cancer is "that estrogen plays some sort of promotional role," Rose asserts. Dietary fat can alter the production, metabolism, and excretion of estrogen. High-fat diets alter the type of bacteria and enzymes found in the intestinal tract, leading to an increased capacity to break down estrogen, allowing more estrogen to be reabsorbed into the body. "It [estrogen] may not initiate the tumor, although some people think that's possible, but it helps the cancer develop," says Rose. "High fiber in a diet has the reverse effect by decreasing the ability of estrogen to be reabsorbed."

This "gut story" may play a role in many cancers, including colon and prostate cancer, Rose says. While estrogen may not be involved in these other cancers, the ability of the intestinal tract to eliminate potential carcinogens is.

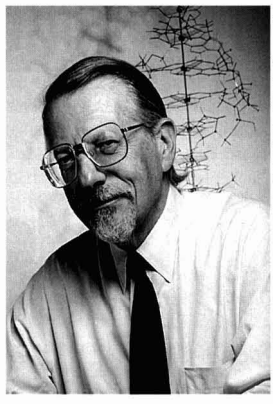
Willett believes estrogen may be important, but not specifically for the reasons Rose cites. He believes elevated levels of estrogen cause women to menstruate earlier, and therefore heightens the degree to which estrogen is active. Observational studies have shown that early menarche is associated with earlier onset of breast cancer. Willett also postulates that "energy restrictions" or low caloric intake in early life could confer a protective effect on breast cancer, whether or not the energy is derived from fat or calories. He notes a high association between tall women and breast cancer, saying that rapid growth in youth may set in motion the wheels of uncontrolled cancerous cell division. "Energy restriction during growth has emerged as a promising hypothesis which may explain much of the international variability—but it doesn't suggest a feasible intervention," Willett says.

Studies on the role of calories in breast cancer have centered on body mass because caloric intake contributes to obesity. But

study findings have been puzzling, according to Louise Brinton, of the NCI's Environmental Epidemiology Branch. Although increased body mass has now been fairly consistently shown to increase the risk of the development of postmenopausal breast cancer, "there has been a surprising lack of attention on weight loss as an intervention for lowering breast cancer risk," she says.

But here animal studies may provide some insights. Like a growing number of scientists who study diet and cancer in laboratory animals, Angelo Turturo of the Division of Biometry and Risk Assessment at the FDA's National Center for Toxicological Research believes control of calories is the key to many types of cancer. "Just as an effect of calorie restriction, live tumor incidence in lab animals can go from zero to seventy percent. You can shut it off with low calorie intake." When baby mice are given doses of a carcinogen and high calories, "they can get a liver tumor at one year," Turturo says. "But calorie restrict other mice at four months who are also receiving the same carcinogen and they won't get cancer."

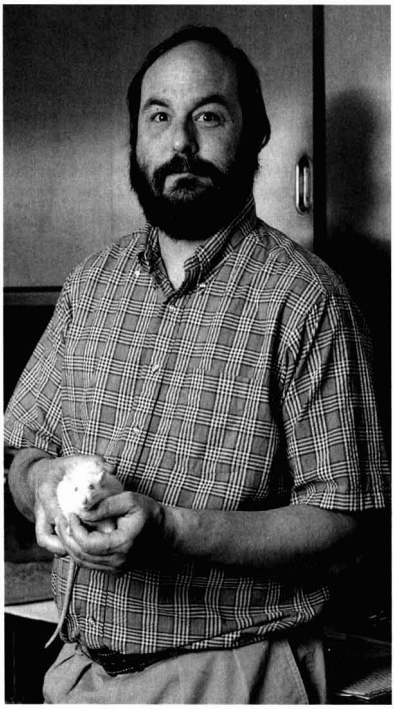
According to Turturo, tumorigenesis is often the result of a promotional effect on endogenous hormones and the stimulation of growth factors. The job of the endocrine system is to regulate growth and the development of organs based on available energy and physiology. "The question is not if calories promote cancer, but why wouldn't they promote cancer?" he says. "Some people have the bizarre notion that normal growth and carcinogenesis are not related. Calorie restriction can affect physiological, cellular, biochemical, and such molecular processes as endocrine homeostatsis, promotion, oncogene expression, progression and the immune response, which affect all steps in the induction of toxicity." Turturo says that most epidemiological studies are "useless" and all interventional studies have failed because they rarely control for calorie intake. "We've known since the 1930s that calorie intake can significantly affect life span and that the most efficient modulator of cancer is total calories."



Columbia Presbyterian Cancer Ctr.

Bernard Weinstein—We need to develop dietary biomarkers.

Animal experimentation can answer questions about cancer risk, but not if the animals are obese—as most are, maintains Frank Kari, a nutritionist at the NIEHS. Kari has found that some chemicals shown to be carcinogenic in these overweight animals do not produce cancer in calorie-restricted animals. "I noticed over the last decade that the average weight of rats and mice was increasing. Most of these animals eat and drink as much as they want and consequently are obese. I also noticed a relationship between lesions and weight and found that the heavier animals tend to die spontaneously of a lot of different chronic diseases," said Kari.



Steve McCaw

Frank Kari—We may be setting ourselves up through diet to be at risk from potential carcinogens.

Kari designed a set of experiments, the results of which will be presented later this summer, that show that certain chemicals now regulated as carcinogens are not carcinogenic in rats and mice that are just 5–7% lighter than most laboratory animals. These chemicals include two commonly used pharmaceuticals, a food additive, and an industrial pollutant. "I found I could turn a carcinogen into a noncarcinogen just depending on how heavy the host is," said Kari. "What this means to me is that it calls into question how we now regulate chemicals. The big picture that we do not look at is the wide range of outcomes available in the host. It may mean we can set ourselves up nutritionally to be at risk to potential carcinogens."

Animal studies by the Health Protection Branch of National Health and Welfare in Ottawa, Canada, looked at the effects of dietary modifications on cell proliferation. They found that diet- and calorie-restricted mice showed less cell division in seven tissues, including the mammary gland, which was the most affected in nonrestricted animals. "If a cell doesn't proliferate, it doesn't produce a tumor," says biologist Eric Lok. On the other hand, Lok adds, when a cell divides at a high rate given excess calories and energy, there may be a greater chance a somatic mutation will occur, possibly as a result of environmental chemicals, and will become fixed in the genome.

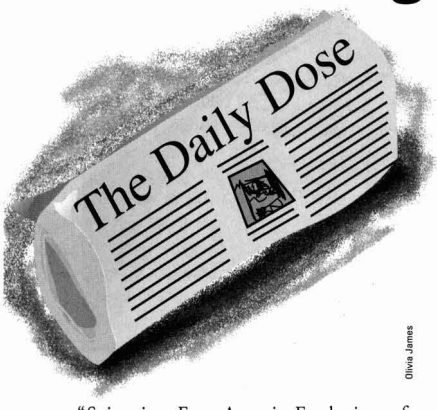
But Lois Gold, a biochemist at the University of California-Berkeley, maintains that animal studies such as those by Lok cannot answer the specific question of which dietary nutrients promote which cancer. "In rodents, we never get more than a 50% chance that a tumor will occur in the same site twice in these studies," she said. "All we are finding is that obese rats have more cell division." Weinstein disagrees with Gold's assertion that animal studies have little value. "Gold underrates the predictive value of the assays. There is a unity of biology across rats and humans that tells us valuable things. Dose responses may be a problem, but if you abandon them, you are left with nothing." What the field needs now is "more objective markers of the action in the body of what we eat. We have made too many inferences and associations," Weinstein says. "We need to take our cue from cardiovascular disease studies that routinely measure serum cholesterol, HDL, LDL, and other markers. We just cannot stay in the old rut of dietary history. We need to know what is happening in tissues, in DNA." Weinstein says that although such biomarkers will be expensive to develop, widespread use of them in interventional studies will reduce costs.

"We are at an exciting point where the revolution in our knowledge of the cellular and molecular basis of cancer can start to be applied with nutritional studies," Weinstein continues. "And we need to double our efforts because the public is already deciding what to do, in the absence of proof from us."

Renee Twombly

Renee Twombly is a freelance journalist in Durham, North Carolina.

Making Headlines in the Lab



Olivia James

"Scientists Fear Atomic Explosion of Buried Waste" was the eye-opening headline that dominated the front page of *The New York Times* on March 5 of this year. Myriad wire services, radio, and TV broadcasts picked up the story about the possibility that a proposed nuclear dump site at Yucca Mountain, Nevada, might explode. On the surface it seemed the safety and health concerns of the public and the environment were being well-served. But the story behind the story raises some thought-provoking questions about how science is communicated.

According to a year-long internal peer review of the explosion thesis by 30 scientists at Los Alamos National Laboratory in New Mexico, the probability that such a high-level waste site would explode is "essentially zero." The review was not made available, however, to the *Times* reporter at the time he was preparing the story.

What's more, in an account about a week later in the journal *Nature,* one of the two particle physicists championing the notion that a series of chain reactions could trigger a nuclear explosion openly admitted that "in the world that we live in, you look for the weakness in your competition and try to exploit it." Both the *Times* and *Nature* stories disclosed that the physicist is also the leading proponent of a rival nuclear waste disposal technology still vying for federal funding.

Bringing a sophisticated scientific debate to the public arena is no easy task. And the challenges are not getting any easier as science continues to evolve. The public is increasingly interested in—and demanding—better and more immediate access to all kinds of health and environmental information. Emerging communication technologies and media, such as the Internet, hotlines, and TV talk shows, are further clouding the issues. But with credibility as the currency and equalizer of science communication, the experts say the issues are surmountable. Time will tell whether the stakeholders are up to the challenge.

Fact and Fallacy

The Yucca Mountain case illustrates the multifaceted problems involved in communicating scientific information to a general audience. In addition to the inherent difficulties in simplifying highly technical information for public consumption, there are a host of external forces shaping how information is communicated, including the varied interests of scientists and journalists, the limitations of the science itself, the dynamics of public perception, and competing political interests.

In today's increasingly complex society with ever-expanding technological capabilities, greater potential than ever exists for the message to get fouled up and frustrate not only the public, but scientists, policy makers, and research institutions. To begin with, few scientists are capable of describing their work in laymen's terms, explains Laurie Garrett, president of the National Association of Science Writers, and those who can often resort to being patronizing, which is a turn-off to the public. "Even journalists who are fluent in the scientific language often have a problem obtaining usable quotes from some scientists," says Garrett, a science writer with *Newsday* in New York.

Finding experts in a given field who can be trusted to give an objective viewpoint ranks as another leading difficulty, according to Richard Stone, the environmental science reporter for *Science,* which is published by the American Association for the Advancement of Science. "It's easy to find people who are going to exaggerate the importance or relevancy of a finding," Stone says. Michael Jacobson, executive director of the Center for Science in the Public Interest (CSIPI), agrees. "Researchers or organizations naturally exaggerate. It's a problem of being human."

Ronald Begley, Washington bureau chief of *Chemical Week,* argues that the problem is more involved than that. There's a tendency for various groups to feed on studies that support their views and dismiss studies that don't, Begley says. "Industry and environmental groups often use science or pseudo-science to argue for things, and they don't necessarily use it correctly," he says.

For these reasons, it's important to accurately portray the position a news source is coming from, including their financial interests, Stone says. "It's unfair to write about results hyped by industry or environmentalists without getting the other side."

Samuel Silverstein, president of the Federation of Applied Science and Experimental Biology, says that a lot of the communication errors are only natural because the various parties, as well as the information, are imperfect. "Even federal agencies have limitations. No one group can opine on everything," he says. Silverstein compares communication of conclusions based on scientific information to a trip to the doctor. "When you go to a doctor, the doctor makes decisions on the basis of imperfect information."

Nelson E. Fabian, executive director of the National Environmental Health Association, says that people who formulate and communicate health and science policies to the public may intentionally as well as inadvertently introduce bias into

the communication process: "Policy makers are human beings. If they calculate positions, there are a host of factors that range from science to the read on the constituency. Science is just one component. The financial issues are there, too, but again they are just one issue of several." In the final analysis, Garrett adds, "very few policy makers give science much weight," though it varies radically by politician.

Anne Thomas, associate director of the National Institutes of Health Office of Communications, says reporters "should look at sources and motives; it's definitely part of their job," especially with so many groups and institutions now offering science information. For example, a single medical advance may be promoted by the journal it's published in, several funding agencies, the university's medical school, and sometimes voluntary organizations affiliated in some way with the findings, Thomas says.

Often, part of the problem is an inherent limitation in the scientific process: scientific results may lend themselves to a variety of interpretations. For parties involved in communicating science, getting across the conditional nature of discovery is a major challenge. "People often think 'this is the truth,' when it really is a hypothesis backed up by data," says Thomas. "Inherent in science is mutability. It changes as science grows."

In trying to meet the expectations of a public that wants hard and true answers, science communicators may risk jumping to conclusions to meet their audience's demands. For example, extrapolating from wildlife to humans or from the test tube to humans entails a broad range of uncertainty. A rash of alarming stories in the mainstream media in the past year about environmental estrogens reducing sperm counts are a prime example of how findings can be misinterpreted. According to Stone, neither the scientists who released the information nor the journalists who reported the story looked closely enough at the doses of estrogens encountered by humans. On closer examination of the data, says Stone, assertions that estrogenlike compounds pose a threat to human reproductive health are, for now, theoretical.

Silverstein maintains, however, that instead of overselling their latest findings to the media, scientists are actually more reluctant to speculate with journalists than with their colleagues. "I know my peers know when I leave the data and start to speculate. Reporters frequently don't."

In the past, both scientists and journalists have overstated claims and released preliminary findings in the race to be first. One example of a race to "get the scoop" occurred in the early AIDS coverage, Garrett observes. "Quite a number of theories were put forward and got a lot of play well before there were any data to back up the claims." Still, says Silverstein, just the focus of attention on an issue may result in a benefit. For example, Silverstein says though Robert Gallo may not have really been the first to isolate the AIDS virus, he still helped the world develop an HIV test faster and safeguard the blood supply at an earlier date. "Sometimes, you have to look at the big picture."

Journalists are under constraints as well, including space limitations, deadlines, and pressures to not only get the story first, but to convince their editors why it belongs on the front page or at the top of the TV news hour. "If reporters miss a crucial story, they will be accused of being asleep at the wheel," Garrett says.

In an effort the make science news more marketable, television, as well as the print media, relies on elements of drama, conflict, and visually alluring pictures, which according to media watchers can lead to distorted perceptions in the public. As Silverstein puts it, "sound-byte science is not good science."

The result, Fabian observes, is that people may believe a five-second expert on a TV talk show who says something outrageous, but they won't believe the county professionals who testify for 30 minutes at a hearing. "It's fascinating that Oprah has more credibility than the experts," he says.

Some experts worry that too many cries of alarm may desensitize the public's interest and attention to science issues. Says *Chemical Week's* Begley, "The public does have a weariness with health and environmental reporting. It's so ephemeral. Everything causes cancer—grilled hamburger, hot dogs, peanut butter . . . [it gets blown] out of proportion."

Experts agree that journalists and other science communicators need to put science in the context of other research, instead of, for example, placing too much weight on the most recent study when dozens of earlier studies may really be more important. "That's journalism for you. This is what's new and then it's forgotten," says Jacobson.

Despite the challenges reporters face on a daily basis, they get high marks on the whole from most scientists, researchers, fellow journalists, communications experts, and policy makers. Jacobson says, "in general, the media, journalists, do a good job. You can quibble with how much importance is given a story or who is interviewed, but gradually priorities are conveyed."

Government Gab

Federal science agencies and academic institutions are encountering many of the same problems that reporters battle, as pressure mounts for information to be made immediately available to citizens, health providers, health educators, and decision-makers. "We certainly see that at NIH," Thomas says. "The agency is playing a more direct role in communicating and educating people about health ideas." Says Kenneth Olden, director of the NIEHS, "unless information from our laboratories and our scientists is accessible to a wider public, NIEHS cannot fulfill its mission."

NIH launched one of the first and most successful federal hotlines in the early 1980s called 1-800-4-CANCER. Thomas says, "For the public, it is a major challenge to try to figure out what is credible and what isn't." But Thomas cautions that just because a patient is given a statistic over a hotline, it doesn't mean it is relevant to their case.

Finis Cavender, director of EnviroHealth, the toll-free (1-800-NIEHS94) environmental health information clearinghouse established by the NIEHS last October, says that, the NIEHS has to avoid placing value judgments on the information it conveys, even when that information may be disturbing. Cavender says, "We hand out information we'd just prefer not to give out. We try to steer people in a way that will actually be beneficial to them."

Cavender adds that it is important to have a place where people can call back and get some explanation if they need it. "If all you do is send the fact sheet, there is some problem with that." Sometimes the public needs things translated into practical terms, Cavender explains. They often don't understand the concept of relative dosing, for example.

Communicating information to the public is the most difficult when the scientific evidence is inconclusive. Cavender says the NIEHS tries to provide a balanced view, but also tries to encourage the public to be cautious about believing scientific claims, such as those that promise a cure for incurable diseases. "There are people willing to take their money out there," he says.

Popular Perception

One unchangeable obstacle that scientists and journalists encounter is the fact that an individual's frame of reference will always affect the way the message is perceived. "Their perception is always going to have emotional and cultural components that have nothing to do with probability," Garrett says.

Fabian adds that "dealing with the public is never easy. Once the public is aroused, you're dealing with people who are frightened, people who are angry." In part, this is because Americans have a modest level of scientific literacy. Yet the medium of transmission is also to blame. Silverstein explains, "the public is given a sensational headline, but insufficient information to evaluate it, especially with TV."

The bottom line, Begley says, is that "the public has a responsibility to decide whether they believe everything the media tells them. People have to be smart media consumers."

How the current state of science communication is impacting the American public at large is still in question, mainly because it's difficult to assess. Some insist that the media continues to be too alarmist in its reports with doomsaying accounts on everything from global climate change to skin cancer. Others think science journalism wouldn't be so popular if the public thought it was being continually hoodwinked.

Newsday's Garrett says that doomsaying stories are for the most part "ancient news." The trend in the public has gone completely in the other direction toward a free market in terms of the environment, she says.

Yet Begley maintains that overall many journalists still aren't getting it right on the environment. "They're goofing by not telling the real story. Neither the original scare stories nor the backlash were based on a good understanding of the science." After a while, Begley says, the public becomes inured and stops trusting environmental, science, and health reporting. How information is communicated partially determines whether the public becomes jaded, Fabian adds.

Either way, all sides seem to agree that credibility is the best check on science communication. At NIH, Thomas says, "we put credibility upper-most. We realize the only thing we have to offer is credibility. If we put out bad information, any message on top of that will be dead on arrival."

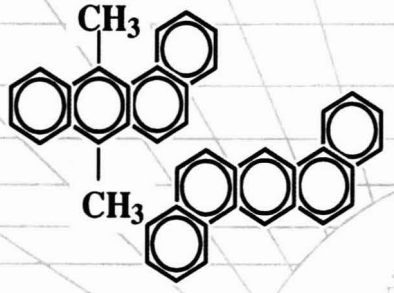
CSIPI's Jacobson says the same is true of special interest groups. "If they lose credibility, they might as well close up shop." Silverstein adds that credibility is paramount for scientists, too.

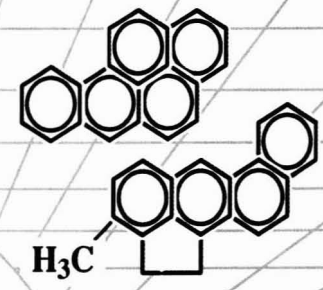
The responsibility for accurately and effectively communicating science information is not on the media alone. The public, scientists, policy makers, industry, academic institutions, and federal officials are all key players in the effort to improve science communication with the goal of changing the adage to: "You *can* believe everything you read."

Julie Wakefield-Albers

Julie Wakefield-Albers is a freelance journalist in Arlington, Virginia.

Meeting Announcement





Risk Assessment of PAHs in the Environment

June 26 - 28, 1995

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This conference will provide state-of-the-science data on health risk assessment of complex mixtures of polycyclic aromatic hydrocarbons (PAHs) in the environment. The conference will focus on new data relevant to hazard identification and dose-response assessment, and will also address issues relating to the occurrence and bioavailability of PAH mixtures in the environment. Both cancer and non-cancer risk assessment will be considered. The conference will target government, academic, and industry scientists involved in research and regulatory areas applicable to PAH risk assessment. The conference will include technical presentations, panel discussions, breakout sessions, and a poster session/reception.

For more information please contact: **Alex Taylor**
c/o JACA Corporation, 550 Pinetown Road, Fort Washington, PA 19034
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Like Sugar for Poison: Glucose as a Substitute for Benzene

Joseph Tart / Olivia James

Scientists searching for a replacement for toxic substances in chemical manufacturing may have come up with a sweet solution: glucose.

Glucose, the body's main fuel, is found in certain foods and also formed by the breakdown of sugars and starches. It may one day be a replacement for benzene, a highly regulated compound that is ubiquitous in the chemical industry (12 billion pounds were produced in the United States in 1993). Benzene helps make jeans blue—it's the feedstock for indigo dye—and ice cream vanilla flavored—it's the source of vanillin. It's also the starting point for a number of important industrial chemicals including hydroquinone, used in film developing, phenol, used to make solvents, and adipic acid, which is used to make nylon. Benzene is also a potent carcinogen.

"Benzene is quite toxic. It's nobody's friend. Companies don't like it because they have to comply with [benzene] regulations, and they're expensive. Chemists just don't like to handle it. Whereas with glucose, nobody has any concerns about it," says Stephen DeVito of the EPA's Office of Pollution Prevention and Toxics.

Living Catalysts

The basic research on replacing benzene with glucose is being carried out at Michigan State University by chemist John Frost, whose work is funded primarily by the EPA and the National Science Foundation.

Frost's research is an example of "green chemistry," an attempt to incorporate environmental concerns into the chemical manufacturing process. It focuses on creating processes that avoid toxic emissions and harmful by-products. Glucose-based chemistry, says Frost, needs only water and temperatures typically no higher than body temperature. Benzene-based processes, on the other hand, are energy-intensive and demand high temperatures.

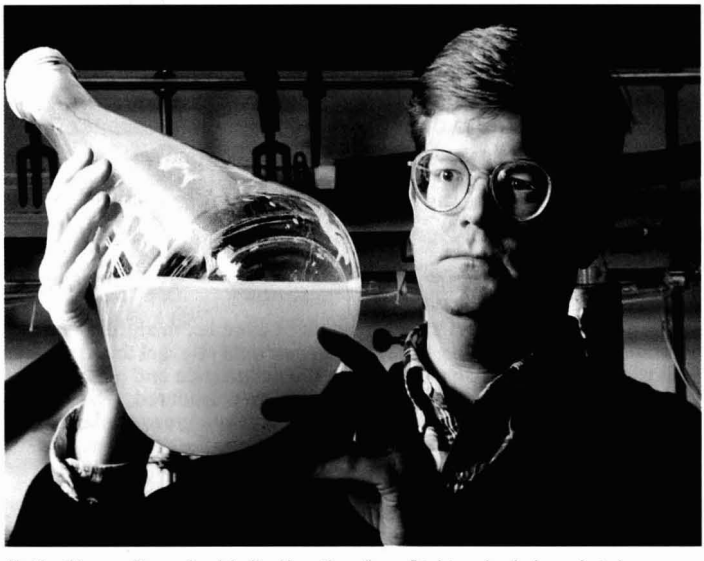
The process of substituting glucose for benzene involves biocatalysis: using bacteria to make chemicals. It capitalizes on the fact that both benzene and glucose have similar chemical structures—they are both carbon-ring compounds, explains Donald Paul, president of Bio-Technical Resources, a genetic engineering firm in Manitowoc, Wisconsin.

Frost mixed bioengineered *E. coli* bacteria with glucose to replace the link in the chemical reaction that uses benzene to make adipic acid. "What you can do by mixing genes from one organism to the next is to create pathways, which have bits and pieces of other pathways, but in their entirety don't exist in nature," said Frost.

To make adipic acid conventionally, benzene is hydrogenated to yield cyclohexane, which is then oxidized in the presence of metal catalysts to produce a mixture of cyclohexanone and cyclohexanol. When the mixture is oxidized with nitric acid, it produces adipic acid and releases nitrous oxide, an environmentally harmful chemical.

To provide a more benign way to produce adipic acid, Frost inserted genes from two bacteria, *Klebsiella pneumoniae* and *Acinetobacter calcoaceticus*, into *E. coli*. *E. coli* converts glucose into 3-dehydroshikimate (DHS), which is a normal product in the process of making amino acids. The added genes produce enzymes, one of which, in effect, hijacks DHS from amino acid formation, and two others which help convert it to *cis,cis*-muconic acid, a constituent of adipic acid, which is hydrogenated to yield adipic acid. Unlike benzene, glucose contains oxygen atoms that eliminate the oxidation step, eliminating nitrous oxide emissions.

Using genes from *Klebsiella pneumoniae* and the same process, Frost has produced



Michigan State U.

Shaking things up. Researcher John Frost is putting a "green" twist on chemical manufacturing.

catechol, another important chemical produced by benzene. Catechol is used to make vanillin, some drugs, and agrichemicals. In this process, not only does glucose replace benzene, but the toxic chemical intermediate, phenol, is eliminated, as is corrosive hydrogen peroxide.

Hydroquinone, which is produced in the catechol-manufacturing process, can also be made using glucose. In this case, genes from *Klebsiella pneumoniae* are inserted into *E. coli*, allowing it to synthesize quinic acid. Oxidation with manganese produces benzoquinone, which can then be reduced to hydroquinone. Approximately 88 million pounds of hydroquinone are produced annually. Although this technology avoids the use of benzene, it produces manganese salts that can present environmental problems.

Ecology and Economics

Using benzene to produce adipic acid not only requires petroleum, a nonrenewable resource, it also spells trouble for the atmosphere. The manufacturing process produces carbon dioxide, a greenhouse gas. The nitrous oxide given off by the process also thins the stratospheric ozone layer and plays a role in global warming. Chemists Mark Thiemans and William Trogler of the University of California-San Diego reported in 1991 that production of adipic acid is responsible for one-tenth of the annual increase in atmospheric nitrous oxide. Four billion pounds of adipic acid are made globally each year, 1.75 billion of it in the United States, according to Frost.

"Glucose is renewable, and anything you make from starch or glucose is pulling CO_2 from the atmosphere. It's using plant-fixed carbon dioxide," says Frost.

Glucose is available from a variety of sources. While crops such as corn may be one source of glucose, plants like switchgrass and prairies grasses are also possible sources, as well as fast-growing hybrid poplar trees. "A lot of marginal land could be made productive for this type of application," says Frost. It would involve what he describes as "very low input agricultural techniques," meaning minimal use of chemical fertilizers and irrigation. Cellulose-containing waste products can also be used as glucose sources. "When farmers are done harvesting their crops, there's stubble left over. That is useful stuff," says Frost. And, according to Frost, the waste streams for the use of glucose as a starting material are no different from what an ordinary municipal sewage treatment plant handles.

Frost argues that stricter EPA regulations are a driving force in pushing chemical companies away from using benzene. Last year the EPA told chemical manufacturers they had three years to slash toxic air emissions by nearly 90% from 1990 levels. Benzene is one of the chemicals covered by the rule. The costs of eliminating these emissions is estimated between $450 million and $1 billion. While not all the costs can be attributed to benzene, this is another argument for an alternative.

"There are literally thousands of theoretical pathways toward making our complex chemical products," says Paul Anastas of EPA's Office of Pollution Prevention and Toxics. "Always the way of approaching chemical products is to the use lowest cost feedstock and convert as much as possible to product. What has not traditionally been a consideration is the substances generated as by-products or the inherent toxicity of those products. With the added costs of regulatory compliance, waste disposal, and waste treatment, the economic equation has changed. Rather than looking at simply the lowest cost feedstock in terms of highest percentage conversion into products, it will be economically beneficial to also consider the toxicity of feedstocks, by-products, and so forth, to lower all of these other costs such as regulatory compliance."

"[Frost is] setting the paradigms for this kind of work so hopefully in the future, when industry starts to think about going commercial with this type of thing, they can use this type of methodology," says George Rubottom, the National Science Foundation's program manager for research in synthetic organic chemistry.

Lauren Blum, a chemist with the Environmental Defense Fund, thinks it's a worthwhile approach. "Anytime you move from petrochemicals to biomass it should make sense from an energy perspective," she says. One concern she does have is ensuring that the energy demands to supply the glucose are not excessive.

Frost's research has generated some industrial interest and funding. The DuPont Company is partially funding his research, though David Anton, DuPont's research manger for bioprocessing development, won't divulge the amount. But he says that "DuPont has made a relatively major effort to look at this type of an area."

Even though DuPont announced in 1991 it would develop technology to capture and recycle nitrous oxide formed in making adipic acid, substituting glucose for benzene may still make economic sense. "The thing that drives every business is cost. Glucose has the potential to be cheaper than benzene. Benzene is thirteen cents per pound; glucose is five and a half to six cents," says Anton.

"What this really is, is an opportunity to make more money," says Allan Ford, a member of the American Chemical Society's Committee on the Improvement of the Environment. Ford, who now heads the Gulf Coast Hazardous Substance Research Center, a multi-university consortium based in Beaumont, Texas, was a former director of environmental science for Monsanto Company in St. Louis.

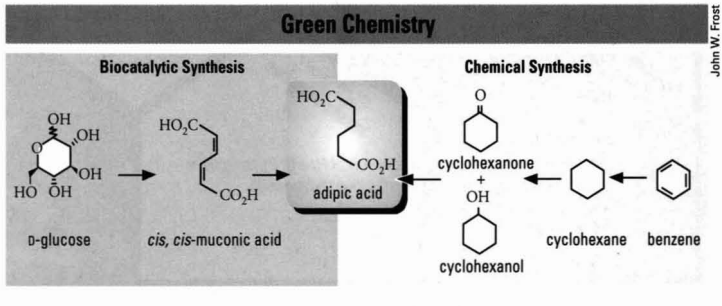
Surmountable Obstacles

Despite its promise, there are questions that may cloud the future of glucose as a bioengineered industrial feedstock. One problem that must be solved is how to use glucose on a large scale. Frost has been able to produce "shake flask" amounts of adipic acid in the laboratory, in the range of three to five grams. Obviously, far larger amounts will be required by industry. Even though drug firms produce insulin with genetically engineered bacteria, nothing of the size required to produce vast amounts of industrial chemicals using bacteria has been done before, says DeVito.

It will take some convincing to show people the advantages of Frost's technology, says Anastas. These advantages include less waste and a cleaner way of achieving the end product.

Frost, who optimistically describes the adoption of such technology as "inevitable," notes that its use will require changes in the way chemical companies do business. "The companies that are basic in glucose feedstock are not basic in chemicals. Chemical companies don't know anything about fermentation. So you have to have acquisitions or strategic alliances for this thing to find its course," he says.

Anton suggests that such possibilities are on DuPont's mind. "DuPont does not produce all the raw materials it uses. To the extent that we can gain access to the raw



materials that we need to run our processes in an economically favorable way, glucose will be another raw material that we will go after," he says.

Marion Bradford, a research scientist at A. E. Staley Manufacturing Company of Decatur, Illinois, says Frost's work has attracted interest from grain processing companies such as Staley.

Another question is how much benzene could this new technology replace? Frost estimates about 20%, pointing to adipic acid as the major product glucose could be used to make. Said Anastas, "I do think replacing a couple of billion pounds of benzene is significant, especially when you consider the workers who have to deal with this carcinogenic material."

DuPont's Anton is more cautious. "We believe there will be cases where glucose will displace petroleum as the preferred feedstock for some chemical applications. We don't know what those are going to be because the way things will ultimately play out, we'll find in some cases it will be cheaper with glucose, in other cases it will be cheaper with petroleum." And if the cost of glucose is low enough the technology could "offer some relief from benzene in some applications," says David Kurtz, professor of chemistry at Ohio Northern University.

Frost himself acknowledges that the current methods of making adipic acid are cheaper than a glucose-based technology. But he argues that factoring in costs of controlling nitrous oxide and benzene emissions will make the new technology more appealing.

"It really is the front end of a whole philosophy of developing a manufacturing system that has minimal environmental impact," says Ford. "I think there are real possibilities, once you start recognizing that damaging the environment costs money."

Harvey Black

Harvey Black is a freelance journalist in Madison, Wisconsin.

Suggested Reading

Draths KM, Frost JW. Microbial biocatalysis: synthesis of adipic acid from D-glucose. ACS symposium series 577. Washington, DC:American Chemical Society, 1994.

Frost JW. Green chemistry at work. EPA J 20:22–23 (1995).

Frost JW, Lievense J. Prospects for biocatalytic synthesis of aromatics in the 21st century. N J Chem 18:341–348 (1994).

Patnaik R, Liao JC. Engineering of *Escherichia coli* central metabolism for aromatic metabolite production with near theoretical yield. Appl Environ Microbiol 60:3903–3908 (1994).

The Use of Trout and Zebra fish in Biomedical Toxicology

July 10 and 11, 1995
Oregon State University, Corvallis, Oregon

Objectives of the Workshop

This workshop is designed to provide hands-on experience in the care and use of trout and zebrafish, and in the design and performance of toxicology studies employing fish as animal models. The information will be state-of-the-art, but it will be targeted dso that researcher with no prior experience in the use of fish models will also benefit.

The workshop will consist of morning lectures followed by afternoon laboratory sessions. Registration will be limited so hands-on experience is available to all participants.

Lecture topics will include:

- Facilities and procedures for spawning and rearing trout and zebrafish, including water quality, computerized facility monitoring, and photoperiod manipulation to produce biannual spawning in rainbow trout.
- Developmental toxicity studies using chlorinated hydrocarbons as the example.
- Production of genetically altered fish, such as isogenics, transgenics, and triploids

Combination laboratory-lecture topics will include:

- Exposure protocols (embryo microinjection, water bath, and dietary exposures) and the design of tumor studies.
- Pharmacokinetic studies in fish
- Phase I and Phase II metabolism in fish
- Histopathology in fish, with emphasis on phenotyping neoplasms
- Techniques for assessing genetic damage including DNA adduct analysis and determination of oncogene and tumor suppressor gene mutations.

For more information contact:

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Marine/Freshwater Biomedical Center
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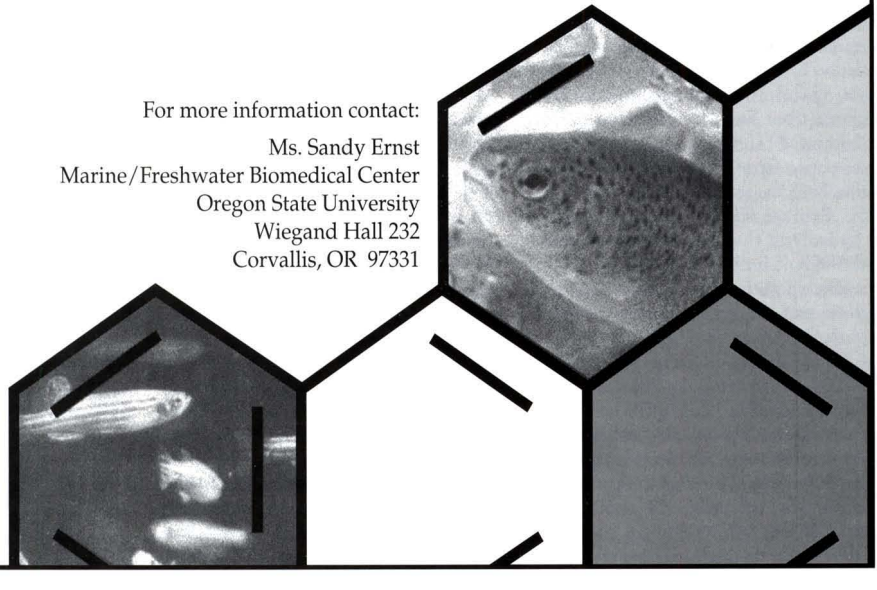
Wiegand Hall 232
Corvallis, OR 97331



An intensive two day workshop sponsored by the Oregon State University NIEHS Marine/ Freshwater Biomedical Sciences Center and the Society of Toxicology

Ethylenebisdithiocarbamates and Ethylenethiourea: Possible Human Health Hazards

Paul Houeto,[1] Gabriel Bindoula,[2] and Jerome R Hoffman[3,4]

[1]Laboratoire de Toxicologie, Hopital Fernand Widal, Paris, France; [2]Institut Gustave Roussey, Villejuif, France; [3]Reanimation Toxicologique, Hopital Fernand Widal, Paris, France; [4]Department of Emergency Medicine, UCLA School of Medicine, Los Angeles, CA 900241 USA

Humans are exposed to ethylenebisdithiocarbamates (EBDCs) from environmental sources. Exposure to EBDCs is chronic for workers in a variety of industries, where EBDCs are used for their properties as slimicides, vulcanization accelerators, antioxidants, and scavengers in waste-water treatment. EBDCs, and particularly the EBDC metabolite ethylenethiourea, have clearly defined, important toxic effects in various animal species, and there is reason to suspect they are carcinogenic in humans. In the absence of definitive information regarding human risk, further studies need to be done. In the interim, regular surveillance of workers with high levels of exposure to EBDCs, with specific attention to markers of thyroid and hepatic pathology, should be considered. *Key words:* dithiocarbamates, ethylenebisdithiocarbamates, ethylenethiourea, hepatocellular carcinoma, thyroid cancer, zineb. *Environ Health Perspect* 103: 568–573 (1995)

Dithiocarbamates are widely used as fungicides because of their efficacy against a broad spectrum of fungi and their associated plant diseases. Dithiocarbamates are also used in industry as slimicides in water-cooling systems, in sugar, pulp, and paper manufacturing, and as vulcanization accelerators and antioxidants in rubber. Because of their chelating properties, they are also used as scavengers in waste-water treatment.

Dithiocarbamates can be divided into two groups: the ethylenebisdithiocarbamates (EBDC), such as maneb, zineb, and mancozeb, and the dimethyldithiocarbamates (DMDC), including ferbam, ziram, and thiram. In recent years there has been increasing awareness of the hazards of various synthetic organic chemicals to living organisms.

Ethylenethiourea (ETU) is one of the principal metabolites of EBDCs and is thought to be the source of most of the toxicity associated with EBDCs. ETU is a degradation by-product of the manufacture of EBDCs (in tobacco, cooked foods, commercial beverages, etc.), formed during product storage (*1–4*). ETU is also the major identifiable product of ultraviolet irradiation, according to Gruiskshan and Jarrow, who studied its photolysis and hydrolysis (*5*). EBDCs are metabolized by mammals to ETU (*6,7*). Extensive methylation of ETU occurs in the cat, but not the rat (*8*). Although no studies of ETU formation have been conducted in humans, ETU has been found in the urine of potato farmers exposed to EBDCs (*9*), implying that such metabolism occurs.

Because ETU can be produced by hydrolytic thermal decomposition of EBDCs, it is difficult to analyze. Several rather cumbersome techniques that have been used and the best currently available technique are reviewed below (*10–13*).

Even though the acute toxicity of pure EBDCs is low, the health of workers may be jeopardized by inhaling ETU or EBDCs (*14*). ETU is hydrosoluble and has raised concern because of its thyroid toxicity and tumorigenicity (*15–18*). In this review, we summarize and analyze the available data on EBDCs used as pesticides to indicate their impact on mammals.

Presence of ETU in Commercial Food Products

Several foods purchased locally were found to contain small quantities of ETU (*10,19*). Pease and Holt (*20*) reported that tomatoes (including tomato juice, soup, canned tomatoes, and catsup), potatoes, cucumbers, summer squash, and cantaloupes taken from 17 different locations throughout the United States where maneb was applied according to label directions showed no residue of ETU (<0.05 ppm), even in the presence of 4 ppm of maneb (1 day after the last of three applications on tomatoes).

Wine contained 0.037 ppm ETU and no residue of EBDC. ETU residues in beer ranged from 0.026 to 0.07 ppm (*21*). Studies with [^{14}C]ETU show that more than 80% of the applied dose remained in the beer after fermentation (*19*).

ETU is produced by burning cigarettes, at a quantity of about 50% of the EBDC residue in the tobacco (determined by measurement of carbon disulfide). For instance, 32 ppm of EBDC in tobacco produced about 16 µg ETU/g, of which one-tenth was absorbed by the smokers (*19*). Such direct absorption by smokers, and absorption by nonsmokers through passive smoking, along with absorption from consumption of cooked vegetables containing EBDC residues, may be the most important source of exposure to ETU among the general public (*19,22*).

Kinetics and Metabolism

In general, dithiocarbamates can be absorbed via the skin, the mucous membranes, and the respiratory and gastrointestinal (GI) tracts. Whereas dithiocarbamates are absorbed rapidly from the GI tract, metal-complexed alkylene bisdithiocarbamates are absorbed poorly from both the GI tract and through the skin (*23*).

ETU is rapidly absorbed from the GI tract and cleared from the body in all mammalian species that have been tested. After only 5 min, ETU appeared in the blood of rats administered an oral dose of 100 mg/kg body weight of [^{14}C]ETU. Within 48 hr, 82–99% of an oral dose was eliminated via the urine and about 3% via the feces (*24,25*). Newsome (*6*) and Ruddick et al. (*26*) found that approximately 70% of ETU was eliminated in the urine and 1% in the feces; comparable results were found for mice, while in monkeys 55% was eliminated via the urine within 48 hr and less than 1.5% via the feces (*27*).

ETU and its metabolites have a half-life of about 28 hr in monkeys, 9–10 hr in rats, and 5 hr in mice (*25*). In a study of the accumulation and elimination of radioactivity by the thyroid gland of rats dosed with ETU, dose levels of 2 and 200 µg ^{14}C-labeled ETU were administered daily for 14 days (*28*). In a second experiment rats were dosed with 0, 0.1, 1, 10, 50, or 100 mg [^{14}C]ETU/kg body weight in food daily for 7 days (*28*). The first experiment showed that the concentration of ETU and/or its metabolites in the thyroid is dose dependent, and the second experiment showed that the level of ^{14}C in the thyroid did not increase appreciably when the daily dose was increased above 50 mg/kg diet. Withdrawal of ETU from the diet led to an 80–94% reduction in the radioactivity in the thyroid after 17 days (*28*).

In a recent study conducted in potato growers, ETU, used as a biological indicator of EBDC exposure, had a long half-life (32–100 hr) and could therefore be detected in urine even several days after exposure (*9*). Other investigators (*29*) reported that

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Received 17 January 1995; accepted 16 March 1995.

in a sample of 10 farmers exposed to dithiocarbamate, monitored thoughout the entire exposure period (approximately 5 hr), ETU was identifiable in urine samples collected immediately after exposure in only three, and persistence of ETU in urine over the entire 40-hr period after exposure was observed in only one subject. However, Kurttio and Savolainen (*9*) reported an elimination half-life for ETU of close to 100 hr in 38 potato farmers exposed to EBDCs.

The metabolic decomposition of EBDCs in mammals is complex and results in the formation of carbon disulfide, ethylene diamine, a few ethylene bisthiuram disulfides, ethylene bisthiocyanate, hydrogen sulfide, and ETU. Dithiocarbamates and their metabolic products are found in certain organs, such as the liver, kidneys, and especially the thyroid, but accumulation of these compounds does not take place because of their rapid metabolism (*23*).

With regard to the hepatic metabolic system, liver monooxygenases in rats are inhibited by oral administration of ETU (*30*). In mice, however, these same researchers found opposite effects: ETU treatment caused an increase in cytochrome P450 content and aniline hydroxylase activity. In addition, a dose 20 times that producing inhibition of aminopyrine *N*-demethylase activity in rats had no effect on this enzyme in liver microsomes of mice.

Lewerenz and Plass (*31*) suggested that the different response of hepatic microsomal enzymes may contribute to the difference in acute toxicity and teratogenicity between mice and rats. This implies that ETU is likely metabolized by different enzymatic pathways. Compounds identified after oral administration of ETU in the urine of mice were 2-imidazolin-2-ylsulfenate, ethylene urea, and unchanged ETU (*32–34*). The S-oxidation of ETU occured in the microsomal fraction of mouse liver (*32*). Imidazole, ethylene urea, imidazolone, and unchanged ETU were detected in the urine of rats (*8*).

Hui (*35*) noted that although flavin-dependent monooxygenase metabolism and binding is a major pathway for ETU biotransformation in the mouse, the toxicological significance of this fact is not clear. Siddiqui et al. (*36*) comparatively evaluated the acute effects of the EBDC fungicides mancozeb and zineb on microsomal mixed-function oxidase (MFO) in male albino rats. The differences in the inhibitory effectiveness of mancozeb and zineb toward MFO may be attributed to the presence of both Mn^{2+} and Zn^{2+} in mancozeb and zineb molecules, differences in their absorption and disposition kinetics, and/or in their metabolic products. In addition, the tendency of these fungicides to chelate metal-containing enzymes such as cytochromes (*37*) appears to be another likely mechanism of their toxicity. Mn^{2+} (*38*) and Zn^{2+} (*39*) have been shown to elicit a significant inhibition of hepatic microsomal hydroxylation of aniline and *N*-demethylation of aminopyrine. It has been suggested that this inhibition of monooxygenase occurs as a result of denaturation of cytochrome P450 brought about by an interaction of EBDC with the sulfhydryl groups of cytochrome P450 and by some other unknown mechanism, probably related to its lipophilic character.

Analytical Methods

Since EBDC tolerances for zineb were first established in 1955, the official methodology used by the U.S. Food and Drug Administration (FDA) for analyzing EBDC residues on food has been a nonspecific method based on the colorimetric determination of a yellow complex formed by reaction of evolved carbon disulfide (CS_2) with a reagent after acid hydrolysis of the sample (*40*). Because this method cannot distinguish between residues of individual EBDCs, all EBDC tolerances are based on zineb.

A further limitation of this method is that it cannot distinguish EBDC residues from those of another major subgroup of dithiocarbamate fungicides, the dimethyldithiocarbamates (ferbam and ziram). Today, refinements of this basic methodology can separate and measure residues of individual EBDCs on crops.

The analytical methodology for ETU has been more actively developed because of concern about its presence in food. The FDA methodology for residues does not detect ETU, since ETU cannot evolve CS_2. However, several reports have described analytical methods for ETU (*1,2,12,41–46*).

Gas chromatography is still the major technique for the residue analysis of ETU, using precolumn derivatization to achieve sensitivity and specificity (*2,6,47,48*). Under suitable clean-up and GC conditions, the procedures allow a detection limit of 10 ppb in various foodstuffs. The derivatization procedures are, however, time consuming, and additional ETU may be formed during reactions at elevated temperatures as a result of decomposition of EBDC residue present in the sample.

Column liquid chromatography (LC) has also been applied using either nonselective UV absorbance detection with a rather complicated sample clean-up (*11,49*), or selective electrochemical detection, for which sample clean-up is much simpler (*46,50,51*). This method can be done with much simpler sample clean-up. In fact, for determination of ETU in wine and beer, down to a level of about 50 ppb, no clean-up is necessary at all (*52*).

These GC and LC methods share the disadvantages of laboriousness and/or insufficient sensitivity. Hogendoorn et al. (*13*) therefore investigated the potential of reverse-phase LC column switching for trace-level determination of ETU in water samples. They found that column switching was adequate for the clean-up and concentration of chloroallylalcohol (CAAL) from aqueous samples, even in combination with relatively nonselective detection (UV, 205 nm). Because ETU is considerably more polar than CAAL and is consequently even less retained on a C_{18} column, development of a reverse-phase column switching procedure is inherently more difficult. This study reports such a development for the trace-level determination of ETU in ground waste samples down to about 0.05 ppb.

Other analytical methods of determining ETU have also been reported, including thin-layer chromatography (TLC), polarography, and radioisotope dilution. A variety of absorbents and developing solvents (TLC) have been used to detect ETU in plants (*1,12,53,43*). Semi-quantitative determinations are possible by comparison with ETU standards run simultaneously (*54*). Polarography involves clean-up on an alumina column, followed by paper chromatography and determination of the nitroso derivative by polarography (*1*). Radioisotope dilution, a reverse isotope dilution method, has been used to determine ETU in the presence of its metabolites and is useful in the low milligram range (*41,54*)

Animal Studies

ETU generally has low acute toxicity, but it does induce a wide spectrum of anomalies in many test animals fed significant doses for various lengths of time. The most prominent aspect of ETU toxicology is its action on the thyroid gland, with resultant hyperplasia and decrease in thyroid hormone levels. ETU has also been shown to have teratogenic, carcinogenic, immunotoxic, and mutagenic effects in various animals studies (*55–58*).

Carcinogenicity. Several studies have shown that the prolonged administration of ETU to rats causes thyroid neoplasms (*17,41,56,59–61*). Ulland (*17*) included 350 and 175 ppm ETU in the diet of male and female rats, which led to a dose-related induction of follicular and papillary thyroid cancers, and of related lesions such as thyroid solid-cell adenomas and hyperplastic and simple goiter. The first tumor was found after 68 weeks, and most cancers occurred after 18–24 months, when the study was terminated.

Recently Chhabra et al. (*61*) studied the perinatal and adult exposure of ETU induction of thyroid neoplasms in rats and identified similar thyroid effects in mice. They confirmed that ETU was carcinogenic in both male and female rats at dietary concentrations of 83 and 250 ppm, regardless of the level of perinatal exposure. Males were more sensitive than females, as demonstrated by a higher incidence of follicular cell neoplasms (adenoma or adenocarcinoma).

The possible mechanism of thyroid follicular cell carcinogenesis in rodents has been reviewed recently (*62–65*). Exposure to ETU, for either 9 months or 2 years, led to a general decrease in serum T_4 levels and increase in serum thyroid-stimulating hormone levels in both rats and mice. These hormonal changes correlated with morphological changes in the thyroid glands, suggesting that ETU carcinogenesis may be due to an imbalance of thyroid–pituitary homeostasis.

Chronic ETU administration produces hepatocellular carcinoma in the mouse and rat (*16,56,61*). Innes (*16*) reported induction of liver neoplasms in mice exposed to dietary concentrations of 646 ppm ETU for 18 months. Two strains of mice fed ETU in the diet had an increased incidence of hepatomas (*56*). Centrilobular hepatocellular cytomegaly occurred in male and female mice and rats receiving ETU at concentrations of 500 ppm or greater. Hepatocytes surrounding the central venules were enlarged, with homogeneously staining, finely granular eosinophilic cytoplasm (*61*).

Thus, toxicological studies have generally shown that although the thyroid is the major site of ETU-induced carcinogenicity in rats (*17,59,60*), the liver is also a major target of ETU-induced carcinogenicity in mice (*16*).

While the incidence of neoplasms and non-neoplastic lesions after exposure to EBDCs or ETU is only clearly proven to be increased in the thyroid gland or liver, several isolated studies suggest a possible effect on other sites as well. Chhabra et al. (*61*) showed statiscally significant increases in neoplasms of the Zymbal gland and of the hematopoietic system in ETU-treated rats, as compared to control animals. There was also a statistically insignificant trend toward a slight increase in rare renal tubular cell neoplasms. In addition, Chernov and Khistenko (*66*) and Balin (*67*) have reported that zineb and maneb induced pulmonary adenomas in mice after oral administration. Finally, Ulland et al. (*17*) described pulmonary metastases in rats with oral exposures.

Mutagenicity. With regard to the mutagenicity of EBDC, Seiler (*68*) found that maneb was negative in tests with *Salmonella* strains HisG46, TA1530, TA1531, and TA1532, and doubtful in TA1534. In addition, maneb and zineb given intraperitoneally to mice at 100 mg/kg body weight caused chromatid aberrations in bone marrow cells (*55,69*).

For ETU, tests with a large number of *S. typhimurium* strains gave mostly negative results (*70,71*). However Seiler (*72*) reported weak (dose unrelated) mutagenicity in *S. typhimurium* strain G46. ETU was also mutagenic in a host-mediated assay of *S. typhimurium* TA1530, when mice were dosed with 6000 mg ETU/kg body weight, but not at doses of 2000 mg/kg or less (*73*). Schüpbach and Hummler (*73,74*) also reported that ETU appeared to induce base-pair mutations but not frameshift mutations in *S. typhimurium* TA98, TA 1537, and TA1538 exposed to ETU in the presence of dimethysulfoxide and/or liver microsomes (*25*).

Teratogenicity. EBDCs are teratogenic in rats, mice, and hamsters, but not in cats. In rats, the major reproduction indices were unaffected by maneb dietary levels of ≤ 250 mg/kg diet. There was no histological evidence of congenital anomalies in a variety of tissues and organs of male and female rats of the F_{3b} litter subjected to histopathological examination (*75*). Maneb, zineb, and mancozeb exert dose-dependent damaging effects on the gonads of rats of both sexes. The dose levels used were 96–960 mg/kg body weight of zineb, 140–1400 mg/kg of mancozeb, and 14–700 mg/kg of maneb, each given twice a week for 4.5 months. Both reproductive and endocrine structures were affected at all dose levels, leading to decreased fertility (*76,77*). In a 4-month inhalation study on rats using maneb at 4.7 mg/m^3, no effect on sperm motility was detected (*78*). Administration of 100 mg/kg body weight of zineb to rats for a period of 2, 4, or 6 months produced delayed insemination, sterility, resorption of fetuses, and developmental anomalies (*79*).

In both rats and hamsters exposed to EBDCs, malformations of the brain predominate (*80,81*). The major teratologic effect is the development of hydrocephalus and other central nervous system defects postnatally, resulting in a high mortality rate among the offspring (*56*). In studies by Larsson et al. (*82*), maneb was administered to Sprague-Dawley rats at dose levels of 0, 400, 770, or 1420 mg/kg body weight, by gavage, as a single dose on day 11 of gestation. Gross malformations occurred in all surviving animals at 770 and 1420 mg/kg, but no malformations were observed in the single litter of the low-dose group. CDI mice administered 0, 375, 750, or 1500 mg maneb/kg body weight on days 7–16 of gestation showed a decrease in fetal caudal ossification centers at all dose levels (*83*). Abnormalities included cleft palate, hydrocephalus, and other serious defects. Abnormalities found at the highest dose level may have been due in part to the presence of ETU in the formulation (*84*).

In contrast to these findings in other species, teratogenicity has not been observed in cats given daily doses of 0, 5, 10, 30, 60, or 120 mg ETU/kg (*85*). The explanation for this exception is not certain, but it may be that extensive methylation of ETU occurs in the cat, but not the rat (*8*).

Human Studies

The acute toxicity of dithiocarbamates is low, and acute intoxication in human beings is therefore unlikely. There is a report, however, of a 62-year-old man who developed acute renal insufficiency after applying maneb. A causal relationship of the exposure is not clear, however, because the patient had a history of hypertension, cerebral infarction, and gastrectomy and chemotherapy (for stomach cancer). The patient was treated with hemodialysis and survived to hospital discharge (*86*). Temporary alterations in central nervous functions (*87*), diarrhea, and acute renal and transient heart failure (*86*) have also been reported after acute exposure to EBDCs.

In numerous studies EBDCs and ETU have been strong skin sensitizers (*88–93*). The irritant and allergic potential of most dithiocarbamates is evident upon occupational exposure.

Cases of diffuse erythema and eczematoid epidermatitis of the eyelids and inguinal regions, probably with elements of sun sensitization, were observed among agricultural workers (grapes and tobacco) in contact with zineb (*94*) or maneb (*95–98*).

In a rarely cited epidemiologic study, which is the only such study currently available, von Meyer (*99*) found trends toward increasing incidences of human liver and thyroid cancers in several geographic areas of the United States, in relation to the degree of exposure to dithiocarbamates, as determined by sales and crop production statistics for dithiocarbamate-containing pesticides. The study was conducted in four states (New York, Florida, Maine, and Pennsylvania), with an overall sample size of more than 27 million people in the countries surveyed. In comparison to very low baseline national incidences of these cancers, the slight increases in the exposed communities (over a 20-year period, following introduction of widespread agriculture use of these pesticides) were for the most part not statistically significant.

In 1978, Lybarger (*100*), in a study of workers at a plant that produces ETU in the United States, found only slight differences in levels of T_4 and T_3 in 43 workers and 7 controls subjects. Smith (*101*) carried out a study of thyroid function which was somewhat limited by the small number of workers and by the intermittent nature of the mixing work. He found no evidence that thyroid function is severely affected by exposure to ETU at the levels experienced by these workers, nor was there evidence of any clinical effect. Only one worker was considered hypothyroid upon biological testing, although T_4 results in the exposed workers were generally somewhat lower than those in control subjects.

A Parkinsonian syndrome has also been reported in two young, previously healthy agricultural workers after chronic exposure to maneb (*102*). Subsequent evaluation of 50 other workers similarly exposed on a chronic basis found significantly increased incidences of various neurologic effects (including cogwheel rigidity, fatigue, and complaints of memory loss) and increases in a variety of other Parkinson-like symptoms (including tremor, ataxia, and bradykinesia) compared to a control group of workers not exposed to this pesticide. Although such toxic effects are probably attributable to the manganese contained in this pesticide, the possibility of an independent contribution of the EBDC itself cannot be entirely eliminated.

Discussion

Human exposure to EBDCs, and the toxic EBDC metabolite ETU, is not insignificant. Although these compounds affect primarily workers in specific industries, ETU is also present in relatively small quantities in a number of common foodstuffs. It is therefore important to analyze the risks incurred by such exposures, particularly in light of the known toxicities of these chemicals in animals.

The toxicokinetics of EBDCs have been well studied. EBDCs are absorbed slowly through the GI tract and skin and are metabolized via hepatic microsomal enzymes to produce ETU, which appears to be the major cause of toxicity of these chemicals. ETU is rapidly absorbed via the GI tract (*23*). The elimination of ETU is largely renal (*27*), and its elimination half-life is variable, ranging between 32–100 hr, according to the species involved (*9,25*). EBDC residues accumulate preferentially in the thyroid, and secondarily in the liver (*28*).

Differences in acute toxicity as well as in teratogenicity are seen in mice and rats exposed to ETU directly. In addition, teratogenicity does not occur in the cat, which unlike other species extensively metabolizes ETU to its *S*-methyl derivative. Thus, interspecies differences in hepatic microsomal enzyme activity, regarding both the formation of ETU from EBDCs and its subsequent transformation, are most likely responsible for the variable toxicity in different species (*8,31–33*). Since these same metabolic pathways for EBDC and ETU may also be different in humans, there may also be major differences in the toxicity of EBDCs in humans, in ways that cannot be easily predicted.

It is possible today to chemically evaluate the presence of EBDCs and ETU with a fairly high degree of accuracy. Although many methods initially proposed in the literature are neither adequately sensitive or specific, a method described by Hogendorn in 1991 (*13*), based on a reverse-phase LC column-switching technique, has proven to be quite specific and sensitive enough to allow detection of residual quantities on the order of parts per trillion.

It is clear that EBDCs and ETU are potentially toxic in chronically exposed animals. Although it remains difficult to evaluate the effects of acute intoxications with these agents because only a few studies of such toxicity have been done, the toxicity of prolonged exposures is definitive. Adverse effects include a variety of acute symptoms, as well as evidence of carcinogenesis, mutagenesis, and teratogenesis. Although several authors have described adverse effects on the liver (*16,56,61*), and one investigator has actually stated that the liver is the primary target organ (*60*), the bulk of evidence suggests that it is in fact the thyroid which is most adversely affected by these chemicals (*17,41,56,59–61*). Thyroid and hepatic neoplasms both occur in animals exposed to EBDCs and ETU, but the teratogenicity of ETU appears to be limited to rats and hamsters (*8,26,33,71,80*), among species thus far studied. The major target organ of this teratogenicity, it is generally agreed, is the brain (*56,80,81*).

Since the different toxic response seen among various animal species is best explained by differences in their metabolism of EBDCs, such that one cannot easily extrapolate these findings directly to humans, and since few studies of exposure to EBDCs have been conducted in humans, our current knowledge of the subject must be considered quite limited. Nevertheless, there is enough evidence to conclude that these chemicals exert at least some toxic effect, the maximum degree of which is not yet certain.

EBDCs and ETU definitely have an irritant effect on human skin (*88–93*), but information regarding more important types of toxicity is inconclusive. With regard to pathologic changes in the thyroid and the liver, no direct relation has been established vis-a-vis exposure to EBDCs, although one large epidemiologic study may suggest a possible relationship between use of dithiocarbamates and human liver and thyroid cancers. In this study there were statistically insignificant trends toward increased incidence of these neoplasms in four geographic areas of the United States where dithiocarbamate-containing pesticides are most commonly used, as evidenced by sales and crop production statistics (*99*). Although von Meyer downplays the significance of the small differences noted, it remains possible that excess cancer rates were not proven statistically only because of insufficient power of the study, despite its size and 20-year duration. In the presence of extremely low baseline rates of these cancers in the general community, and given the diluting effect of surveillance of all individuals in a geographic area, including many who are not exposed to the putative causative agent, it would require enormous sample sizes to find even relative increases in cancer incidence. Because no other studies have been done on the subject, the question of possible carcinogenicity of EBDCs in humans remains unanswered.

Conclusions

Humans are exposed to EBDCs, especially certain classes of workers for whom this exposure is chronic. Such EBDCs, and particularly the EBDC metabolite ETU, have clear and important toxic effects in various animal species, and there is reason to at least suspect possible carcinogenicity of these agents in humans. It is therefore desirable that further human studies be done to better define and assess this risk. Such research is feasible today, in light of available methods to quantitate degrees of exposure to these chemicals. Furthermore, pending the results of such studies, it would clearly be desirable to undertake regular surveillance of humans exposed to EBDCs, with specific attention to markers of thyroid and hepatic pathology.

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Effects of Coumestrol on Estrogen Receptor Function and Uterine Growth in Ovariectomized Rats

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Isoflavonoids and related compounds such as coumestrol have classically been categorized as phytoestrogens because these environmentally derived substances bind to the estrogen receptor (ER) and increase uterine wet weight in immature rats and mice. Assessment of the binding affinities of isoflavonoids for ER and subsequent effects on uterine growth suggest these compounds are less active estrogens than estradiol and therefore may reduce the risk of developing breast or prostate cancer in humans by preventing estradiol binding to ER. With the renewed interest in the relationships between environmental estrogens and cancer cause and prevention, we assessed the effects of the phytoestrogen coumestrol on uterotropic response in the immature, ovariectomized rat. Our studies demonstrated that in this animal model, coumestrol is an atypical estrogen that does not stimulate uterine cellular hyperplasia. Although acute (subcutaneous injection) or chronic (multiple injection or orally via drinking water) administration of coumestrol significantly increased uterine wet and dry weights, the phytoestrogen failed to increase uterine DNA content. The lack of true estrogenic activity was characterized by the inability of this phytoestrogen to cause cytosolic ER depletion, nuclear ER accumulation, or the stimulation of nuclear type II sites which characteristically precede estrogenic stimulation of cellular DNA synthesis and proliferation. In fact, subcutaneous or oral coumestrol treatment caused an atypical threefold induction of cytosolic ER without corresponding cytosolic depletion and nuclear accumulation of this receptor, and this increased the sensitivity of the uterus to subsequent stimulation by estradiol. These results in the immature, ovariectomized rat contrast with studies of intact, immature animals and suggest that ovarian estrogens may be a component in the estrogenic response to phytoestrogens such as coumestrol in intact animals. Consequently, the potential estrogenicity of phytoestrogens requires careful reassessment in intact and ovariectomized animals before the impact of these environmentally derived substances on reproductive function and cancer can be realized. *Key words*: coumestrol, estrogen receptor, phytoestrogen, rat uterine growth, type II [^{3}H]estradiol binding sites. *Environ Health Perspect* 103:574–581 (1995)

Bioflavonoids represent a class of naturally occurring plant pigments that humans consume daily in gram quantities (*1,2*). A high correlation exists between the intake of bioflavonoid-rich diets and a lower incidence of stomach, colon, breast, and prostate cancer in man (*3–7*). For these reasons, some investigators have suggested that consumption of weakly estrogenic isoflavonoids such as equol, daidzein, and coumestrol may prevent estrogen-dependent breast and prostate cancers by competing with more active, endogenous estrogens such as estradiol for estrogen receptor (ER) in these target tissues (*5–7*). Conversely, studies by our laboratory and others have shown that flavonoids such as luteolin and quercetin bind with high affinity (K_d ~1–5 nM) to nuclear type II [^{3}H]estradiol binding sites, but not ER, and this is correlated with the antagonism of estrogenic response in the rat uterus, the inhibition of breast, ovarian, pancreatic, and colon cancer cell proliferation *in vitro* and estrogen-independent mammary tumor growth in mice (*8–13*). Therefore, it is not surprising that a naturally occurring flavonoid metabolite, methyl *p*-hydroxyphenyllactate (MeHPLA), has been identified as an endogenous cell-growth-regulating agent and the natural ligand for the type II site (*14*). On the basis of these studies demonstrating mixed agonist/antagonist (estrogenic/antiestrogenic) activities of flavonoids and isoflavonoids in estrogen-responsive tissues, it is likely that dietarily derived flavonoids and/or their metabolites which interact either with ER or type II sites may profoundly affect reproductive function and the incidence of estrogen-dependent breast and prostate cancer (*3–7*).

Although isoflavonoids such as coumestrol have demonstrated estrogenic activity in a variety of experimental systems, most of these studies involved feeding these compounds to intact animals for periods of time ranging from days to weeks and subsequently determining uterine wet and/or dry weights (*15–19*). Consequently, the uterotropic response profiles to phytoestrogens have not been extensively evaluated in ovariectomized animals, in which the contribution of ovarian steroids to the overall net uterine hypertrophy, hyperplasia, and DNA synthesis has been eliminated. Recent studies have shown that oral administration of coumestrol to intact, immature rats failed to antagonize estrogenic stimulation of uterine growth, and, in fact, coumestrol treatment increased uterine weight in these studies, suggesting that this isoflavonoid possessed estrogenic activity (*15–17*). However, because these studies involved the treatment of intact, immature female mice with coumestrol over a period of days, it is possible that coumestrol modulated gonadotropin secretion and/or ovarian steroidogenesis, which may have been partially responsible for the observed estrogenic response. A recent report by Yamazaki (*20*) demonstrating that the isoflavonoid ipriflavone is estrogenic in intact, but not ovariectomized, immature rats also supports this hypothesis (*20*).

Our studies described here were designed to assess the estrogenic activity of parenterally and orally administered coumestrol in the immature, ovariectomized rat to rule out the effects of ovarian estrogen on uterotropic response patterns. These data demonstrate that at the dose levels and treatment conditions used, coumestrol behaved as an atypical estrogen, failing to cause significant cytosolic-depletion and nuclear accumulation of ER or uterine hyperplasia and DNA synthesis in the ovariectomized rat, even though uterine wet and dry weights were elevated above control levels. Therefore, the true estrogenic activity of coumestrol, and perhaps other well-known phytoestrogens, requires careful evaluation in ovariectomized animals, particularly in view of the fact that emphasis is currently being directed toward defining the relationships between phytoestrogen exposure and neoplasia in estrogen-responsive tissues such as the mammary gland, uterine endometrium, and prostate.

Materials and Methods

Chemicals. Coumestrol was purchased from Eastman Kodak (Rochester, New York) and genestein and daidzein were purchased from Indofine (Somerville, New

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This research was supported by grants from the National Institute for Environmental Health Science (ES-05477) and the National Cancer Institute (CA-35490 and CA-55590).

Received 3 October 1994; accepted 27 February 1995.

Jersey). The purity of the flavonoids was determined to be greater than 99% by HPLC analysis using a μBondapak C_{18} column (Waters/Millipore, Milford, Massachusetts) eluted with water:methanol by standard procedures in our laboratory (*9*). Estradiol and diethylstilbestrol were obtained from Sigma (St. Louis, Missouri) and 2, 4, 6, 7-[^{3}H]estradiol (112 Ci/mmole) was purchased from Amersham Radiochemicals (Boston, Massachusetts).

Animals and treatment. Immature (21-day-old) Sprague-Dawley female rats (Holtzman Laboratories, Madison, Wisconsin) were ovariectomized under Metofane anesthesia by standard procedures and allowed to recover 7–10 days before treatment. Animals were housed in stainless-steel cages under controlled conditions consisting of 12 hr of light daily (lights on at 0700 hr), and food and water were provided *ad libitum*. We separated the rats into treatment groups consisting of five or six animals per group and injected them subcutaneously or treated them orally with the indicated dose levels of estradiol or coumestrol dissolved in saline-2.0% Tween 80 (injection) or tap water-2.0% Tween 80 vehicle (oral dosing studies) under the conditions described in the text and figure legends. This method of oral administration is remarkably consistent throughout the treatment period, and phytoestrogens were delivered for weeks without significant effects on body weights or other signs of generalized, nonspecific systemic cytotoxicity. In these experiments, the 30- to 40-day-old rats typically consumed 25.8 ± 4.4 mL of vehicle or coumestrol solution per day. At a concentration of 50 μg coumestrol/mL of tap water-Tween-80 vehicle, this represented a dose of 1.29 ± 0.22 mg of coumestrol per day per animal and doses in excess of ~13 mg/rat/day (500 μg/mL drinking water; ~286 mg/kg body weight) can be readily delivered by this procedure. Animals were sacrificed by cervical dislocation and the uteri were removed, stripped of extraneous tissue, weighed, and stored in saline at 4°C for biochemical analysis. To obtain dry weights, uteri from some of the animals (five to six per treatment group) were dried in an oven at 70°C for 16–24 hr until constant weights were obtained.

Tissue homogenization and fractionation. For biochemical analyses, we homogenized uteri from the control and treated animals in ice-cold TE buffer (10 mM Tris, 1.5 mM EDTA, pH 7.4 at 22°C) in Kontes ground-glass homogenizers in a volume equivalent to 100 mg fresh uterine wet weight equivalents per milliliter and centrifuged the homogenate at 800*g* for 20 min in a Beckman GH-3 rotor to obtain the low-speed cytosol (supernatant) and nuclear pellet fractions (*21,22*). The cytosol was centrifuged at 40,000*g* for 30 min in a Beckman JA 20 rotor to obtain the high-speed cytosol fraction, and equivalent results are routinely obtained with 200,000*g* cytosol preparations. We washed the nuclear pellet fraction three times by resuspension and centrifugation (800*g* for 7 min) in TE buffer before resuspension in the same buffer and analysis for ER or type II sites by [^{3}H]estradiol exchange as described below.

Measurement of cytosolic and nuclear ER by [^{3}H]estradiol exchange. We diluted cytosol and nuclear fractions from control and treated animals to 20 mg fresh uterine wet weight equivalents/mL in TE buffer and brought them to 10 mM with dithiothreitol (*21,22*). The preparations were incubated at 4°C for 140 min in the presence of the reducing agent to eliminate interference from type II sites (*21,22*). Aliquots (250 μL) of the cytosol or nuclear suspensions were incubated (cytosol, 30°C for 30 min; nuclei, 30°C for 30 min) in the presence of a wide range of [^{3}H]estradiol concentrations (0.4 to 10 nM, total binding) ± 300-fold excess diethylstilbestrol (0.12–3.0 μM, nonspecific binding). After this incubation, we incubated cytosol fractions (4°C for 15 min) with hydroxylapetite (HAP) and washed the HAP bound protein by resuspension and centrifugation (800*g* for 5 min) to separate bound and free [^{3}H]estradiol. Nuclear suspensions were also washed by resuspension and centrifugation to remove free [^{3}H]estradiol (*21,22*). Bound [^{3}H]estradiol was extracted from the final, washed HAP or nuclear pellets with ethanol and specific [^{3}H]estradiol binding evaluated by Scatchard analysis (*21,22*). Results were expressed as ER sites per cell assuming that mammalian tissues contain approximately 7 pg of DNA per cell nucleus (*23*). Uterine DNA content was estimated by the method of Burton (*24*).

In studies where the binding affinity of the various phytoestrogens for ER was assessed, uterine cytosol fractions from ovariectomized rats were prepared exactly as described above and diluted to 20 mg/mL in TE buffer containing 10 mM dithiothreitol. After preincubation (140 min at 4°C) in the presence of reducing agent to eliminate interference from cytosol type II sites (*21*), aliquots of the cytosol were incubated (37°C for 30 min) in triplicate in the presence of 10 nM [^{3}H]estradiol ± the indicated concentrations (0.1 nM–10.0 μM) of daidzein, genistein, coumestrol, or diethylstilbestrol, and bound and free steroid were separated by HAP adsorption as described above. We determined [^{3}H]estradiol binding to ER in these studies in the absence (100% bound) or presence of the competitor as previously described (*8*). In a typical experiment, 100% bound represented approximately 5000 cpm.

Assessment of isoflavonoid binding affinity for nuclear type II sites. Since nuclear type II site stimulation appears to be involved in target cell response to estrogenic hormones, and flavonoids such as luteolin, quercetin, and pelargonidin appear to inhibit cellular proliferation through type II site binding interactions (*8*), we determined the binding affinities of isoflavonoids such as coumestrol, daidzein, and genistein for type II sites in rat uterine nuclei. For these studies, uterine nuclear fractions from estradiol-implanted rats (*8*) were prepared in TE buffer, diluted to a final volume equivalent to 20 mg uterine wet weight equivalents/mL, and incubated (4°C for 60 minutes) in the presence of 20 nM [^{3}H]estradiol ± the indicated concentrations of competitor (1.0 nM–20.0 μM) as described in the figure legends. After incubation, the nuclear suspensions were washed by resuspension in TE buffer and centrifugation to remove free steroid, and bound [^{3}H]estradiol was determined by liquid scintillation counting exactly as previously described (*8*). A value of 100% bound in the absence of competitor represented approximately 25, 000 cpm.

Measurement of cytosolic and nuclear type II sites by [^{3}H]estradiol exchange. In experiments where it was necessary to quantitate treatment effects on the levels of cytosolic and nuclear type II binding sites, these subcellular fractions were prepared exactly as described above for the type II site competition assays (*21,22*). We quantified cytosolic type II sites by saturation analysis using the hydroxylapetite adsorption–[^{3}H]estradiol exchange assay (HAA–[^{3}H]estradiol exchange) previously developed by our laboratory for measurement of type II sites without interference from endogenous ligands such as MeHPLA (*22,25*). Briefly, cytosol preparations from controls and coumestrol-treated animals were incubated with hydroxylapetite (HAP) at 4°C for 15 min to allow the type II site to bind to the HAP. The pellet bound protein was washed three times by resuspension in TE buffer and centrifugation (800*g* for 7 min), and the final washed pellet was resuspended in TE buffer and aliquots incubated (22°C for 16 hr) in the presence of a wide range (0.4 nM–40 nM) of [^{3}H]estradiol concentrations in the absence (total binding) or presence (nonspecific binding) of 300-fold excess diethylstilbestrol (*22,25*). Similarly, the washed nuclear pellet fractions from these uteri were incubated (4°C for 60 min) with [^{3}H]estradiol ± diethylstilbestrol, and specific binding to type II sites in cytosol and nuclear fractions was deter-

mined on the basis of uterine DNA content (sites/cell) as previously described in detail (*23*). DNA was estimated by the method of Burton (*24*).

Statistical analyses. Where indicated, the experimental results are expressed as the mean ± SEM. The data presented in the various figures in this manuscript were analyzed statistically by the appropriate one-way or two-way analysis of variance (fixed treatment models) and Duncan's new multiple range test on the treatment means as described in detail (*26*).

Results

To correlate phytoestrogen binding interactions with biological response, we evaluated the binding affinities of these compounds with ER and type II sites in rat uterine nuclear fractions. The data in Figure 1 demonstrate that coumestrol (K_d ~180 nM), daidzein (K_d >1000 nM), and genistein (K_d ~180 nM) bind to the ER (Fig. 1A) with relatively low affinities as described by numerous laboratories for various tissues (*16,26*). This is consistent with the fact that these compounds are weak or short-acting estrogens with substantially less biological activity than long-acting estrogens such as estradiol (*28*). Coumestrol (K_d ~10 nM), daidzein (K_d ~5 nM), and genistein (K_d ~5 nM) displayed higher binding affinities for type II sites than for the ER (Fig. 1), and the apparent binding affinities of these three isoflavonoids for nuclear type II sites are similar to those determined for luteolin and quercetin, which bind to nuclear type II sites (but not ER) with very high affinity (K_d ~1–5 nM) and inhibit estrogen stimulation of uterine growth in the rat and mammary tumor growth in the mouse (*8,9*).

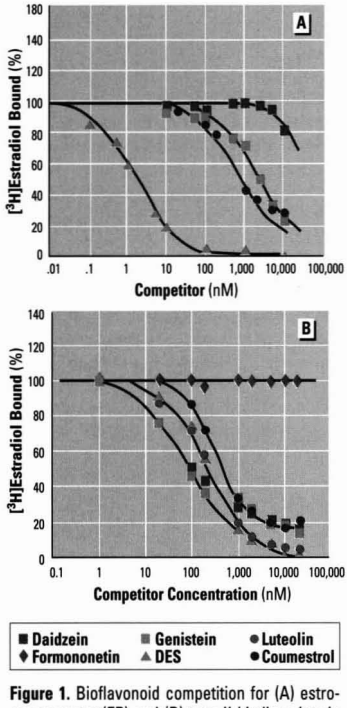

Figure 1. Bioflavonoid competition for (A) estrogen receptor (ER) and (B) type II binding sites in rat uterine cytosol and nuclei. Uterine cytosol and nuclear fractions were incubated in the presence of [^{3}H]estradiol (10 nM, ER; 20 nM, type II) plus or minus the indicated concentrations of competitor under conditions optimum for each of the respective binding sites as described in methods. DES, diethylstilbestrol.

Because coumestrol displayed higher affinity for ER than daidzein and has recently been described as an estrogen in the intact, immature female rat, we focused our efforts on the characterization of the estrogenic activity of coumestrol in the immature, ovariectomized rat uterine model system. Dose–response studies demonstrated that a single injection of coumestrol in doses ranging from 50 to 200 µg resulted in a significant increase in uterine wet weight relative to control (Fig. 2), and the response obtained with 100–200 µg of coumestrol was equivalent to that obtained after a single injection of 1 µg estradiol-17ß. Therefore, it appeared that in the immature, ovariectomized rat, coumestrol treatment increased uterine wet weight 24 hr after a single injection in a manner similar to that described for long-acting estrogens such as estradiol (*28,29*).

The data in Figure 3 represent the temporal effects of coumestrol on uterine wet weight and DNA content after the injection of 100 µg of this phytoestrogen. As expected, based on previous studies suggesting that coumestrol has estrogenic activity (*15–17*), uterine wet weight was increased within 4 hr after coumestrol treatment, and this response was sustained for 24 hr, declining to control levels by 62 hr after injection. This observation was consistent with that reported for active estrogens such as estradiol, where sustaining uterine wet weight beyond 24 hr after injection is typically associated with the stimulation of cellular DNA synthesis and true uterine growth (*29*). Much to our surprise, even though coumestrol treatment increased uterine wet and dry weights in a manner similar to that obtained with estradiol, it failed to increase uterine DNA content at 24 hr after injection. These findings suggest that in the immature, ovariectomized rat, coumestrol behaves as an atypical estrogen, which stimulates cellular hypertrophy and perhaps protein synthesis without stimulatins cellular hyperplasia, reflected by a doubling in DNA content (*29*). Therefore, coumestrol mimics estriol, estrone, and estradiol-17α in this model system by behaving as a short-acting estrogen, capable of stimulating cellular hypertrophy and not hyperplasia when administered as a single injection (*28–31*).

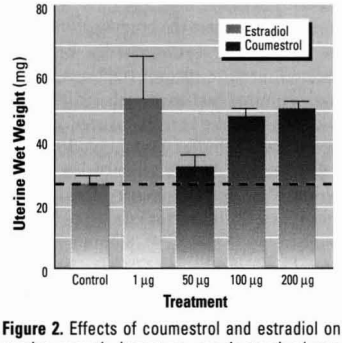

Figure 2. Effects of coumestrol and estradiol on uterine growth. Immature, ovariectomized rats received a single subcutaneous injection of vehicle (controls) or the indicated concentrations of estradiol or coumestrol. Uterine wet weights were determined 24 hr after injection (*40*). No greater response was subsequently obtained with a single subcutaneous injection of 500 µg coumestrol. Data are means ± SEM.

We also assessed the effects of multiple injections of this phytoestrogen on uterine wet weight and DNA content 24 hr after the last injection (Fig. 4) because estriol and estradiol-17α have been shown to stimulate uterine cellular hypertrophy and hyperplasia in the intact immature (*29*) or adult-ovariectomized (*28,30,31*) rats when administered by multiple injection or pellet implant. Therefore, we expected that similar results would be obtained after multiple injections of coumestrol. The data in Figure 4 clearly demonstrate that this was not the case. Although multiple injections of coumestrol increased uterine wet weight relative to control, this response was not equivalent to that after following a single injection of 1 µg estradiol (Fig. 4A). More importantly, estradiol treatment nearly doubled uterine DNA content (Fig. 4B), whereas neither single or multiple injections of this phytoestrogen increased uterine DNA content in this study. In fact, two injections of coumestrol may have slightly reduced uterine DNA content relative to control (Fig. 4B). These data further confirm that in the immature, ovariec-

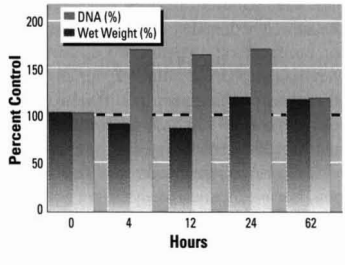

Figure 3. Temporal effects of coumestrol on uterine growth. Ovariectomized rats (four to six per group) were injected with vehicle (controls) or coumestrol (100 µg), and uterine wet weights and DNA content were determined at the indicated times following treatment. The data represent the means for two separate experiments.

tomized rat, coumestrol behaves as an atypical estrogen.

To explain the inability of coumestrol to stimulate uterine cellular hyperplasia (increased uterine DNA content) in the ovariectomized rat, we assessed the effects of this isoflavonoid on the intracellular compartmentalization of ER in the uterus at various times after treatment. Although it is likely that ER is localized in the nucleus and cytosolic ER is most likely an artifact generated during tissue homogenization (*32–34*), the assessment of ER dynamics (cytosolic ER depletion and nuclear ER accumulation) after estrogen administration can be used to assess the estrogenicity of a variety of compounds (*29,34*). In the present studies, we injected immature, ovariectomized rats with a dose (100 µg) of coumestrol, which substantially increased uterine wet weight without causing cellular hyperplasia (Fig. 4) and the levels of cytosolic and nuclear ER were measured as a function of time after treatment (Fig. 5). Again, coumestrol behaved as an atypical estrogen, failing to cause measurable cytosolic ER depletion or significant nuclear ER accumulation. In fact, cytosolic ER levels were elevated above the time zero control level within 4 hr after coumestrol treatment and reached a maximum two- to threefold induction by 24 hr.

To ensure that the atypical effects of coumestrol on ER dynamics were not a characteristic of the model system, we compared the temporal effects of estradiol and coumestrol on ER compartmentalization in the rat uterus in immature, ovariectomized rats. The data in Figure 6 clearly demonstrate that the cytosolic ER depletion and nuclear ER retention patterns after estradiol injection were exactly as described by our laboratory and others for the rat uterine model system under a variety of experimental conditions (*29,35–41*) and the level of cytosolic ER did not return to control levels (0 hours) until 16–24 hr after treatment. It is this sustained cytosolic ER depletion and nuclear ER occupancy that correlates with estrogenic stimulation of cellular hyperplasia and DNA synthesis (*28,29*). The failure of coumestrol to mimic these ER dynamics is likely responsible for the inability of this phytoestrogen to stimulate DNA synthesis in these studies. However, again, in this second experiment, coumestrol treatment did not cause cytosolic depletion or nuclear ER accumulation/retention even though cytosolic ER levels were elevated two- to threefold above time zero controls by 24 hr.

The aforementioned experiments suggested that coumestrol was an atypical estrogen when administered subcutaneously. Therefore, we evaluated the sustained effects of this compound on uterine growth in the rat after oral administration in the drinking water. These studies demonstrated that 96 hr after treatment, orally administered coumestrol resulted in a dose-dependent increase in uterine wet and dry weight relative to controls at dose levels ranging from 5 to 100 µg/mL. Uterine wet and dry weights were essentially doubled by treatment with 100 µg of coumestrol/mL drinking water (~60 mg/kg body weight/day; Fig. 7). Time studies with the higher dose level of coumestrol (100 µg/mL drinking water) demonstrated that the uterotropic response peaked between 72 and 96 hr after treatment, and uterine wet and dry weights were increased five- to sixfold relative to the time-zero vehicle controls (Fig. 8). More important, however, was the observation that uterine DNA content 72 hr after coumestrol treatment (242 µg/uterus) was nearly equivalent to the control value (217 µg/uterus), suggesting that coumestrol failed to stimulate significant cellular hyperplasia and DNA synthesis even when administered in a sustained fashion under these experimental conditions.

To further characterize the uterotropic response patterns to orally administered coumestrol, we also assessed the effects of this phytoestrogen on cytosolic and nuclear ER and nuclear type II binding site levels 72 hr after oral administration (Fig. 9). These data essentially confirmed the injection studies (Figs. 5 and 6) in that oral administration of coumestrol also failed to cause accumulation of nuclear ER or deplete cytosolic ER. In fact, coumestrol treatment resulted in a three- to fourfold induction in the level of cytosolic ER, as was the case for the coumestrol injection studies. That nuclear type II sites were not stimulated by coumestrol treatment (Fig. 9) is consistent with the observation that uterine hyperplasia and DNA synthesis was not stimulated by coumestrol under these conditions (see legend to Figure 8). Estrogen stimulation of nuclear type II sites in the rat uterus is directly correlated with the induction of uterine cellular DNA synthesis and true uterine growth under a wide variety of experimental conditions (*28–30,35*). That cytosolic type II sites appeared to be slightly increased following coumestrol treatment (Fig. 8) is interesting; however, the relationship between this soluble [^{3}H]estradiol binding site and uterotropic

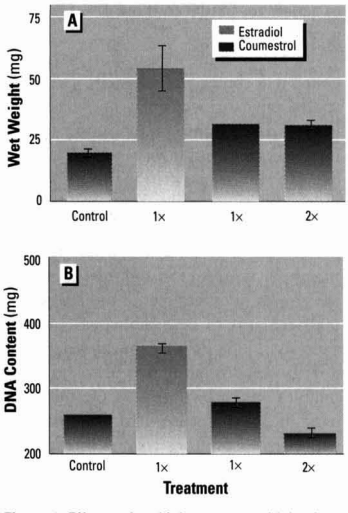

Figure 4. Effects of multiple coumestrol injections on uterine growth in the rat. Ovariectomized rats received one daily injection of 10 µg estradiol or one or two daily injections of 100 µg coumestrol as indicated. Controls were injected with 2% Tween 80 in 0.9% saline vehicle. (A) Uterine wet weights and (B) DNA content were determined 24 hr after the last injection. Data are means ± SEM.

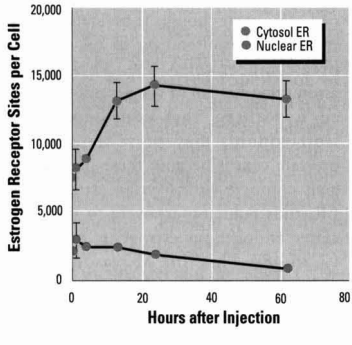

Figure 5. Effects of coumestrol on rat uterine estrogen receptor (ER) dynamics. Ovariectomized rats were injected subcutaneously with 100 µg coumestrol or vehicle (time 0 controls) and uterine cytosol and nuclear estrogen receptors were assayed by [^{3}H]estradiol exchange. Results are expressed as sites per cell (*23*). Data are means ± SEM.

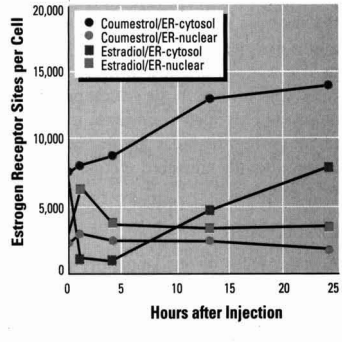

Figure 6. Effects of estradiol and coumestrol on estrogen receptor (ER) dynamics in the rat uterus. Ovariectomized rats received a single injection of estradiol (5 µg) or coumestrol (100 µg), and estrogen receptors (ER) were measured in cytosol and nuclear fractions by [^{3}H]estradiol exchange at the indicated times after injection. Results were based on DNA content and were expressed as sites/cell as described. In separate experiments essentially identical ER responses were observed following a 500 µg injection of coumestrol.

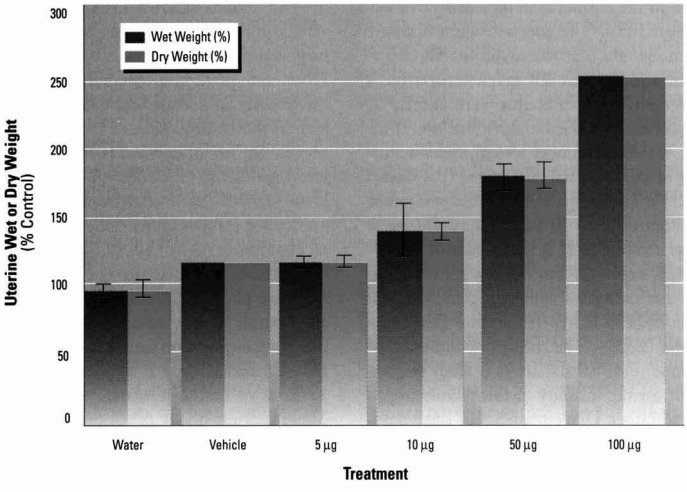

Figure 7. Dose response of rat uterus to coumestrol. Ovariectomized rats were given the indicated concentrations of coumestrol dissolved in drinking water containing 2% Tween 80. Controls received water or the 2% Tween-80 vehicle and uterine wet and dry weights were determined 96 hr after treatment. Data are means ± SEM.

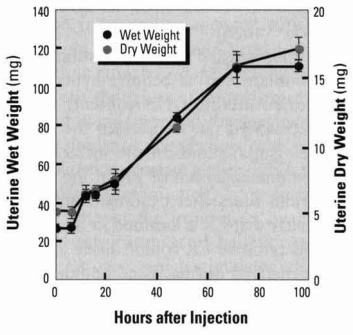

Figure 8. Effects of orally administered coumestrol on uterine growth in the rat. Ovariectomized rats were treated with 100 µg/mL coumestrol dissolved in drinking water containing 2% Tween 80 (vehicle). Controls received water-Tween 80 vehicle and uterine wet and dry weights were determined at the indicated times (0–96 hr) after treatment. The uterine DNA content (not shown in graph) after 72 hr of coumestrol treatment (242 µg/uterus) was not different from that measured in uteri from controls (217 µg/uterus). Data are means ± SEM.

response has not been evaluated.

On the basis of the aforementioned studies demonstrating that coumestrol treatment will stimulate cytosolic ER levels in the rat uterus (Figs. 5, 6, and 9), we suspected that subcutaneous or oral exposure to this phytoestrogen may alter the uterotropic response to estrogenic steroids. To evaluate this possibility, immature, ovariectomized rats were treated orally with vehicle (controls) or coumestrol (250 µg/mL) for 5 days before receiving three daily subcutaneous injections of various doses of estradiol (0.01-10 µg) and uterine weight was determined 24 hr after the last injection. Coumestrol pretreatment increased uterine sensitivity to estradiol as the response of the coumestrol pretreated uterus to doses of estradiol greater than 0.1 µg/day was significantly (p <0.05) greater than that observed in the vehicle pretreated controls. Therefore, coumestrol induction of ER during the 5-day pretreatment period significantly enhanced uterine sensitivity to estradiol.

Discussion

A major focus of our laboratory over the past decade has been estrogen regulation of normal and abnormal cell growth and proliferation. Our efforts have led to the identification of a bioflavonoid metabolite (MeHPLA) as an important cell growth regulating agent (*9,14*). MeHPLA is an endogenous ligand for nuclear type II sites (*14*), and occupancy of this site by MeHPLA and bioflavonoids such as luteolin and quercetin appears to inhibit estrogen stimulation of uterine growth in the rat and mammary tumor growth in mice (*8,9,14*). Subsequent studies demonstrate a direct correlation exists between the occupancy of type II sites by flavonoids such as luteolin, quercetin, and dihydroxybenzylidine acetophenone and the inhibition of breast (*8–10*), colorectal (*11*), pancreatic (*12*), and ovarian cancer (*13*) as well as the inhibition of leukemia (*42*) and lymphoblastoid cell proliferation (*43*). These bioflavonoids do not bind to the ER (*8*), suggesting that the antiestrogenic and/or inhibitory effects of these compounds on cellular proliferation are mediated through type II sites. Therefore, it is likely that dietarily derived bioflavonoids inhibit normal and abnormal cell growth through this mechanism as well.

Conversely, isoflavonoids such as coumestrol, genistein, and daidzein have been described as estrogens, antiestrogens, and anticarcinogens because of their abilities to bind to ER in estrogen target cells (*44–48*), even though these compounds inhibit the proliferation of ER negative breast cancer cells (*49*). This former assumption has led to the generally accepted hypothesis that consumption of isoflavonoids may prevent breast or prostate cancer because these phytoestrogens compete with ovarian estradiol for ER, thus reducing exposure to the more active endogenous estrogenic hormones (*7,46,48*). This line of reasoning was offered as an explanation as to why Japanese women, who have higher circulating levels of less potent, short-acting estrogens such as estriol and estrone, have a lower incidence of breast cancer than their American counterparts (*50*). Although these hypotheses regarding the protective effects of the so-called weak or impeded estrogens are logical, they are not necessarily supported by animal studies. It is well documented that estriol and estradiol-17α are very weak estrogens incapable of stimulating cellular hyperplasia, when exposure is acute (single injection). However, chronic exposure to estriol or estradiol-17α via multiple injection or subcutaneous implant causes uterine cellular hypertrophy, hyperplasia, and mammary cancer in rodents in a manner analogous to that achieved with estradiol (*28–31,51*). This is a likely paradigm for coumestrol, daidzein, and genistein if their pharmacology and mechanism of action are similar to those of other short acting estrogens (*28–30,51*).

Although some investigators have suggested that coumestrol, daidzein, and other related phytoestrogens may prevent cancer because these compounds inhibit malignant cell proliferation *in vitro* (*52,53*) and dimethylbenz[*a*]anthracene (DMBA) induction of mammary tumors in the rat (*54*), these phytoestrogens do not inhibit the growth of established DMBA-induced mammary tumors (*55*), and their effects on the growth of other types of tumors in animals remains to be established. In fact, there is a paucity of experimental data directly demonstrating that phytoestrogens inhibit tumor growth *in vivo*. On the other hand, it is well documented that sustained exposure to phytoestrogens during the neonatal period is associated with persistent vaginal cornification, cervico-vaginal pegs and downgrowths, and uterine squamous metaplasia, which mimics that observed after exposure to diethylstibestrol (*56,57*). These results demonstrate that phytoestrogens may hyperestrogenize target tissues under certain experimental conditions *in vivo*. Therefore, although it is

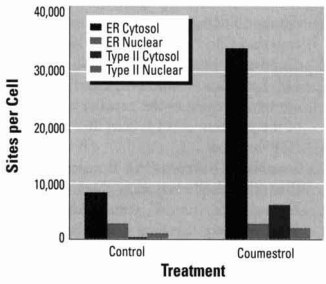

Figure 9. Effects of oral coumestrol administration on cytosolic and nuclear estrogen receptor (ER) and type II sites in the rat uterus. Uteri from ovariectomized rats treated with vehicle (controls) or coumestrol for 72 hr as described in Figure 8 were assayed for cytosolic and nuclear ER and type II sites by [^{3}H]estradiol exchange. Results are expressed as binding sites/cell.

certainly possible that acute exposure to phytoestrogens may block carcinogenic insult to prevent cancer (*46–48,54*), these compounds may also hyperestrogenize ER-containing target tissues after chronic exposure, and none of the parameters (dose and duration of exposure) involved in these response profiles have been adequately defined.

The present studies were performed to more completely define the interactions of isoflavonoids with ER and type II sites in the ovariectomized rat uterus and evaluate subsequent effects on uterine growth and DNA content after acute or sustained exposure to these phytoestrogens. The findings confirmed our hypothesis that isoflavonoids bind to the ER with low affinity and therefore should demonstrate very little estrogenic activity when administered acutely. In fact, the apparent dissociation constants of coumestrol and daidzein for rat uterine nuclear ER were approximately 180 nM and 1000 nM, respectively, whereas little competition was obtained with genistein (Fig. 1). Based on these binding affinities for ER, one has to wonder whether endogenous levels of either coumestrol or genistein will reach concentrations capable of occupying ER and eliciting estrogenic response under normal physiological conditions. Although we previously noted that some isoflavonoids and lignans failed to compete significantly for nuclear type II sites before becoming insoluble in the binding assays (*8*), we used dimethylsulfoxide to solubilize these compounds in the present studies to enhance their solubility in the aqueous binding assay buffer. Under these conditions coumestrol and daidzein competed for [^{3}H]estradiol binding to nuclear type II sites and displayed much higher affinities for this protein (Fig. 1). That coumestrol and daidzein displayed much higher affinities for nuclear type II sites (K_d 5–10 nM) than for the ER suggests that type II sites might be occupied *in vivo* at concentrations where these compounds will not bind to ER. This is currently being evaluated.

The ability of isoflavonoids to interact with both the ER and nuclear type II sites suggest that these compounds may display mixed agonist/antagonist activities. At lower concentrations (<100 nM) coumestrol may occupy nuclear type II sites (K_d 10 nM) and inhibit cell proliferation, as we have shown for luteolin and quercetin (*8*), whereas at higher concentrations (>100 nM) coumestrol may occupy ER (K_d ~180 nM), resulting in the stimulation of cellular proliferation. This concept is consistent with our observations that the binding of the ER complex in the nucleus results in the estrogen-induced dissociation of MeHPLA from nuclear type II sites, and similar events may occur after the binding of isoflavonoid-ER complexes in the nucleus as well (*14,58*). However, it is important to consider that although supraphysiological concentrations (μM) of coumestrol stimulate ER-dependent reporter gene transcription in MCF7 breast cancer cells or HeLa cervical cancer cells *in vitro* (*59,60*), whether endogenous levels of coumestrol reach concentrations required for ER (100–1000 nM) binding *in vivo* under physiological conditions remains to be resolved.

Although recent studies demonstrate that oral administration of coumestrol increased uterine wet and dry weight, nuclear ER levels, and uterine progesterone receptor content in intact, immature rats, uterine DNA content was not determined in these studies (*15–17*), and whether coumestrol stimulated cellular hyperplasia remains to be resolved. Therefore, even though coumestrol appeared to behave as a complete estrogen in these experiments, the animals were dosed with the phytoestrogen over a number of days, and it is possible that ovarian-derived estrogen contributed to the observed stimulation of uterine growth and progesterone receptor content as these animals reached puberty. The present studies using the immature, ovariectomized rat support this contention. Although coumestrol administration by single or multiple injection (Figs. 2–4) or orally in the drinking water (Figs. 7 and 8) increased uterine wet and dry weights relative to control, even sustained exposure to high doses of this phytoestrogen failed to increase uterine DNA content, suggesting that uterine hyperplasia was not observed. This is a significant finding demonstrating that increases in uterine wet and dry weight are not always indicative of uterine hyperplasia as reflected by a doubling in DNA content (*28,29*). It is more likely that the coumestrol-induced increase in uterine wet and dry weight in our studies in ovariectomized animals reflected increases in water and protein content. Studies by Yamazaki demonstrating that the isoflavonoid ipriflavone is estrogenic in intact, but not ovariectomized animals (*20*) support our findings with coumestrol and confirm the hypothesis that ovarian estrogens contribute to the net estrogenic response of the uterus to isoflavonoids.

Further evidence that coumestrol may be an atypical estrogen is provided by studies where the effects of this phytoestrogen on ER function were assessed. In two separate experiments designed to evaluate the temporal effects of coumestrol on ER dynamics and compartmentalization in the ovariectomized rat uterus, injection of 100 μg (or 500 μg; not shown) of coumestrol failed to deplete cytosolic ER or cause nuclear ER accumulation (Figs. 5 and 6) even though increases in uterine wet and dry weight were observed. These results are in sharp contrast to the data in Figure 6 where injection of immature, ovariectomized rats with estradiol resulted in the classical cytosolic ER depletion and nuclear ER accumulation and retention patterns which precede estradiol stimulation of uterine growth in immature (*28,29*) or adult, ovariectomized rats (*29–31,35*). Since sustained nuclear occupancy by the ER-estrogen complex is generally thought to be required for cellular hyperplasia and DNA synthesis (*28–31*), it is not surprising that coumestrol failed to stimulate uterine cellular hyperplasia (DNA content) under these experimental conditions in ovariectomized rats.

Even more surprising was the observation that subcutaneous injection (Figs. 5 and 6) or oral administration of coumestrol (Fig. 9) increased cytosolic ER levels two- to threefold without causing significant cytosolic depletion and nuclear accumulation of ER or induction of nuclear type II sites, which are characteristic responses to estrogenic hormone administration (*22,23,28*). These latter two nuclear events are typically correlated with estrogenic stimulation of cellular DNA synthesis and proliferation (*28–30,35*). These findings also imply that coumestrol may be an atypical estrogen which does not modulate ER or type II site function in a manner analogous to that of estradiol (Figs. 6 and 9), estriol, or estradiol-17α (*28–31*) in the ovariectomized rat uterus. Consequently, although recent studies suggest nuclear ER are elevated in coumestrol-treated, intact, immature rats (*15–17*), this ER could have been occupied by ovarian estrogen and not necessarily coumestrol.

Although it is certainly possible that much higher doses of coumestrol would

have significantly altered ER dynamics to stimulate true uterine growth in the present studies, injection of 500 µg of coumestrol under the conditions described in Figures 5 and 6 also failed to cause cytosolic ER depletion and/or nuclear ER accumulation and DNA synthesis (not shown). Nevertheless, 500 µg of coumestrol stimulated cytosolic ER in a manner similar to that shown obtained with 100 µg of this phytoestrogen (Fig 5). Therefore, increasing the dose level of coumestrol fivefold (~10 mg/kg body weight) failed to alter the response profiles at these short times (1–3 days) after injection. Whether this increase in cytosolic ER concentration after coumestrol treatment reflects phytoestrogen-induced ER activation, ER phosphorylation, and/or the stimulation of ER gene transcription remains to be resolved (*61,62*).

Regardless of the mechanism by which coumestrol increases ER concentration in the uterus, it appears that this isoflavonoid may enhance the sensitivity of this target tissue to estradiol. The data in Figure 10 demonstrate that coumestrol pretreatment significantly shifted the dose–response curve for estradiol, and it is likely that coumestrol induction of cytosolic ER as shown in Figures 5 and 6 was responsible for this increased sensitivity of the uterus to estradiol. Therefore, the mechanisms by which phytoestrogens such as coumestrol, daidzein, and genistein modulate estrogenic response and uterine growth may be much more complex than generally thought and may involve binding to ER, increasing the ER binding capacity of estrogen target tissues such as the uterus by causing ER activation or phosphorylation (*60,61*) or by enhancing ovarian release of estrogen. If this is the case, one might anticipate that the observed estrogenicity or antiestrogenicity of dietarily derived phytoestrogens such as coumestrol may be different in premenopausal and postmenopausal women. This being the case, it is difficult to speculate as to whether phytoestrogens such as coumestrol will prevent and/or protect against cancer by competing with ovarian estrogens for ER as suggested (*5,6*) or whether continuous consumption of antiestrogenic flavonoids such as luteolin and quercetin which inhibit the growth of a broad spectrum of rodent (*8,9*) and human cancers *in vitro* and *in vivo* (*10–13*) will reduce cancer incidence in humans by antagonism at the level of the type II site. Studies designed to accurately define the estrogenicity and antiestrogenicity of dietarily derived isoflavonoids and flavonoids and potential interactions with one another and endogenous estrogens and androgens in these experimental systems will be required to adequately address these issues.

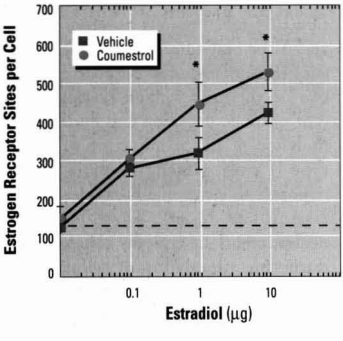

Figure 10. Coumestrol effects on estradiol stimulation of uterine growth in the rat. Adult, ovariectomized rats were treated orally with vehicle (2% Tween 80) or coumestrol (250 µg/mL) in the drinking water for 5 days before receiving three daily subcutaneous injections of the indicated doses (0.1–10 µg) of estradiol. Animals were sacrificed 24 hr after the last injection and uterine wet weights were determined. Results are expressed as the means ± SEM and the response to 1, 5, and 10 µg of estradiol in the coumestrol-treated animals was significantly different (*p <0.05) from the vehicle controls.

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A Variety of Environmentally Persistent Chemicals, Including Some Phthalate Plasticizers, Are Weakly Estrogenic

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Sewage, a complex mixture of organic and inorganic chemicals, is considered to be a major source of environmental pollution. A random screen of 20 organic man-made chemicals present in liquid effluents revealed that half appeared able to interact with the estradiol receptor. This was demonstrated by their ability to inhibit binding of 17ß-estradiol to the fish estrogen receptor. Further studies, using mammalian estrogen screens *in vitro*, revealed that the two phthalate esters butylbenzyl phthalate (BBP) and di-*n*-butylphthalate (DBP) and a food antioxidant, butylated hydroxyanisole (BHA) were estrogenic; however, they were all less estrogenic than the environmental estrogen octylphenol. Phthalate esters, used in the production of various plastics (including PVC), are among the most common industrial chemicals. Their ubiquity in the environment and tendency to bioconcentrate in animal fat are well known. Neither BBP nor DBP were able to act as antagonists, indicating that, in the presence of endogenous estrogens, their overall effect would be cumulative. Recently, it has been suggested that environmental estrogens may be etiological agents in several human diseases, including disorders of the male reproductive tract and breast and testicular cancers. The current finding that some phthalate compounds and some food additives are weakly estrogenic *in vitro*, needs to be supported by further studies on their effects *in vivo* before any conclusions can be made regarding their possible role in the development of these conditions. *Key words*: butylbenzyl phthalate, butylated hydroxyanisole, di-*n*-butylphthalate, phthalates, estrogenicity, sewage. *Environ Health Perspect* 103: 582–587 (1995)

Over the last 50 years, large amounts of some estrogenic man-made chemicals have been released into the environment (*1*). These chemicals include classical environmental estrogens, such as *o,p'*-DDT and its metabolites, methoxychlor, and many of the polychlorinated biphenyls (PCBs). More recently, chemicals originating from the plastics and detergent industries, such as alkylphenols (*2,3*) and bisphenol-A (*4*), have been discovered to be estrogenic. Evidence suggests that in many instances the presence of these chemicals has had deleterious effects on exposed wildlife populations (*5,6*). Estrogens influence many developmental and physiological responses in target cells by regulating the activity of specific genes. Their action is mediated by a soluble intracellular receptor that functions as a transcription factor (*7*) . Estrogens have been shown to have multiple sites of activity and exert biological actions on the reproductive tract and the mammary gland. They also influence the neuroendocrine system (*8*) and have skeletal effects (*9,10*). Untimely exposure to natural or synthetic estrogens can adversely affect human health, particularly with regard to the reproductive cycle and reproductive function. In addition to decreased sperm counts in men and increased incidence of disorders of the male reproductive tract (*11,12*), recent epidemiological studies suggest that cumulative exposure to estrogenic chemicals is related to the incidence of reproductive cancers (*13*).

As many of the estrogenic xenobiotics discovered to date have an anthropogenic source, the highest concentrations would be expected to occur near urbanized or industrial areas. Sewage is considered to be a major input source of organic contaminants into the environment. The release of liquid effluents into the rivers and oceans, the disposal of dry sludge onto the land, and the release of volatile organics into the atmosphere all contribute to this source of pollution. This fact, coupled with the report that sewage effluents are estrogenic (*14*), increases the possibility that there may be other estrogenic chemicals in the environment not yet discovered.

Extensive information exists on the occurrence and concentrations of organic micropollutants in raw, potable, and waste waters (*15,16*), yet only about 3,000 man-made organic compounds have been identified out of a probable 60,000 (*17*). The sources of these compounds range from domestic and industrial effluents and leachates from solid waste disposal sites to agricultural or urban run-off and atmospheric fall-out. The range of compounds found includes aliphatic and aromatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, organochlorine pesticides, PCBs, and phthalate esters (*18–21*), all of which are present in various environments at highly variable concentrations. For example, phthalates are present in waters at concentrations ranging from nanograms to milligrams per liter. The reasons for the reported variability in concentrations in the aquatic environment include the use of different methodologies for analysis, geographical variation, and variations in the source of the water sample (e.g., influent, effluent, river).

The estrogenic activity of environmental chemicals has nearly always been discovered because an estrogenic effect, either *in vivo* or *in vitro*, has occurred upon exposure to the chemical. With the exception of studies conducted by Soto et al. (*22*), no systematic screening of chemicals has been reported. Because the estrogenic activities of various widely used industrial chemicals continue to be discovered, it seems likely that additional chemicals also exhibit activity. Our interest in the aquatic environment led us to test some of the major chemicals present in sewage effluent to determine whether any of these chemicals are estrogenic.

Materials and Methods

Chemicals tested. We searched the scientific literature (using the Institute of Scientific Information database and also government reports, both published and unpublished) in order to discover what chemicals had been reported to be present in sewage effluents and at what concentrations. None of these reports quantified all of the chemicals present in effluent; many of them tended to focus on one group of chemicals rather than the whole range likely to be present. It is not known how many chemicals are present in effluent, although the number is probably high.

Based on this literature search, we made a list (Table 1) of selected man-made chemicals present in sewage effluent. We do not claim that this table is representative of all sewage effluents, but the chemicals listed are likely to be present at significant concentrations in most effluents (see Discussion for fuller explanation of this point).

Fish studies. Because of their documented presence in the aquatic environment, the initial examination for estrogenicity was carried out by measuring direct binding of the chemicals to the fish estrogen receptor. This initial screening process was both rapid and economical and was carried out using a cytosolic extract from the liver of rainbow trout; it is well documented that estradiol receptor-binding sites are present here in both male and female fish (*23*). Livers were removed from rainbow trout,

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A portion of this work was financially supported by the Department of the Environment, UK.

Received 23 March 1995; accepted 5 December 1994.

Table 1. Compounds tested for estrogenic activity and their primary uses

Compound	Primary uses/sources
Bis(2-ethylhexyl)phthalate (DEHP)	Plasticizer
Benzophenone	Manufacture of insecticides and antihistamines; fixative in strong perfumes (soaps, shampoo)
Butylated hydroxyanisole (BHA)	Antioxidant, especially in foods
Butyl benzyl phthalate (BBP)	Plasticizer, especially in the production of vinyl floor tiles, adhesives and synthetic leather
n-Butylbenzene	Petrochemical origin
p-tert-Butylbenzoic acid	Plastics industry; corrosion inhibitor; in polyester manufacture in dyeing
Caffeine	Drinks and pharmaceuticals
Cholesterol	Excreted steroid, emulsifying agent
p-Cresol (4-methylphenol)	To produce antioxidants; UV stabilizer
Butylated hydroxytoluene (BHT)	Antioxidant in food, petrol products, rubbers, plastics, and soaps
Di-*n*-butyl phthalate (DBP)	Plasticizer in food packaging, PVC, cellulosics and certain elastomers; insect repellant
2,4 Dichlorophenol	Fungicide and germicide products
3,4 Dimethylphenol	Disinfectant/microbicide
Bis-(2-ethylhexyl)adipate (DEHA)	Manufacture of plastics (PVC)
p-Hydroxybenzoic acid	Cosmetic, food, and pharmaceutical preservative
2-Methylphenol	Herbicide products, phenolic resins
Musk xylene	Scent
Musk ketone	Scent
4-Nitrotoluene	Manufacture of dyes
p-Toluene	Industrial solvent

frozen immediately in liquid nitrogen, and subsequently stored at -80°C until required. They were then thawed and homogenized on ice in 2.5 volumes of buffer (50 mM TrisHCl, 0.1 mM EDTA, 10 mM sodium molybdate, and 1 mM monothioglycerol, pH 7.4). The homogenate was centrifuged at 10,000*g* for 30 min at 2°C to yield a crude nuclear pellet and a crude cytosolic supernatant. The cytosol was then incubated on ice for 30 min in the presence of dextran-coated charcoal to remove any endogenous steroids and then spun at 50,000*g* for 1 hr at 2°C. The final supernatant was carefully aspirated, decanted, and a saturation analysis was carried out on this cytosolic extract to establish the concentration of [2,3,7,-^{3}H]17ß-estradiol (86 Ci/mmol) that saturated the receptor preparation (generally between 2 and 10 nM). Thereafter, cytosol samples with a protein content of 2–5 mg/ml were incubated in triplicate with a saturating concentration of 5 nM tritiated 17ß-estradiol, both alone and in the presence of competing ligands at a wide range of concentrations (up to 1 mM). We removed the unbound fraction by addition of charcoal and specific binding was quantified [as described by Pottinger (*23*)]. These experiments were repeated at least three times.

Mammalian studies. Apart from their presence in waters, many of the compounds identified as putative environmental estrogens originate either from the diet or from the human usage of plastics and cosmetics, and therefore humans could be exposed to them via many other routes. In view of this potential for human exposure, we tested several of the compounds further using mammalian-based assays employing two human breast cancer cell lines *in vitro*, ZR-75 and MCF7.

Human breast cancer ZR-75 cells were grown initially in phenol red-free Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) charcoal-stripped fetal calf serum containing no hormone for 7 days. They were then transferred into medium containing no hormone (NH), 10 nM 17ß-estradiol (E_2), 10^{-5} M octylphenol (OP), or 10^{-5} M of each of the environmental pollutants *n*-butylbenzene, di-*n*-butyl phthalate (DBP), butylbenzyl phthalate (BBP), 4-nitrotoluene, bis(2-ethylhexyl)adipate (DEHA), 2,4-dichlorophenol, benzophenone, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), or bis(2-ethylhexyl)phthalate (DEHP). Cells were cultured for a further 10 days and counted on days 0, 3, 6, 8, and 10. All experiments were carried out in duplicate and repeated twice.

To determine whether the estrogenic compounds stimulated transcriptional activity of the estrogen receptor directly, we examined their effects on transiently transfected MCF7 cells using the reporter plasmids pTKLUC and pERE-TKLUC. MCF7 cells were plated to 80% confluence in phenol red-free DMEM and 10% charcoal-stripped fetal calf serum and transfected using the calcium phosphate coprecipitation method, as previously described (*24*). The reporter plasmid pTKLUC contains the herpes simplex virus thymidine kinase (TK) promoter from -105 to +55 inserted in the Bgl II site of the luciferase reporter plasmid pGL2-Basic (Promega). pERE-TKLUC contains a single copy of the vitellogenin A2 estrogen response element (ERE) inserted upstream of the TK promoter in pTKLUC. The transfected DNA included the reporter (0.8 µg) and an internal control plasmid (pJ7LacZ; 0.2 µg). After transfection, cells were maintained with no hormone, E_2, OP, BBP, DBP, DEHP, BHA, or BHT at the concentrations indicated. After 24 hr, the cells were harvested, and extracts were assayed for luciferase (*25*) and ß-galactosidase (Galactolight, Tropix Inc, Bedford, Massachusetts) activities. We used ß-galactosidase to correct for differences in transfection efficiency. All experiments were carried out in duplicate and repeated at least twice.

We also examined the possibility that some of these chemicals might act as antagonists in the presence of 17ß-estradiol. In these experiments, MCF7 cells were transfected with pERE-TKLUC and pJ7LacZ, and then incubated with 10^{-11} M 17ß-estradiol alone, simultaneously with DPB or BBP, or simultaneously with the antiestrogens 4-hydroxytamoxifen (4-OHT) or ICI 182780. Both the phthalates and the antiestrogens were added at the concentrations indicated. The experiment was carried out in duplicate and repeated three times.

Results

Many of the compounds tested in this initial screen reduced the binding of the tritiated natural estrogen, 17ß-estradiol, to the receptor. BBP, DBP, DEHP, DEHA, benzophenone, *n*-butylbenzene, 4-nitrotoluene, BHA, and 2,4-dichlorophenol reduced the binding of tritiated 17ß-estradiol to the receptor, although whether this inhibitory effect was due to direct competition was not determined. Concentrations as high as 1 mM may have approached the limits of solubility of some chemicals in the solvent system used, as suggested by the observation that some of the curves appeared to flatten. In these cases, higher concentrations were not tested and hence full displacement curves were not obtained. No accurate estimations of the affinities of these chemicals for the receptor could be obtained because

in most cases the displacement curves were not parallel to that of 17ß-estradiol (Fig. 1).

Musk ketone, musk xylene, *p*-toluene, BHT, caffeine, cholesterol, *p*-hydroxybenzoic acid, *p*-tert butylbenzoic acid, 3,4-dimethylphenol, and 2-methylphenol did not impair binding of tritiated estradiol to the estradiol receptor (results not shown).

When the compounds were tested for their mitogenic effects on cell growth at 10^{-5} M, the three most potent were BBP, DBP, and BHA (Fig. 2). Many of the other compounds were either inactive or only weakly active at concentrations in excess of 10^{-4} M. The growth responses to these chemicals were all less than the maximal responses shown by the natural estrogen 17ß-estradiol and the environmental estrogen OP, which we have tested in this system previously (*26*).

When tested for their ability to stimulate the transcriptional activity of the estrogen receptor directly (Fig. 3), BBP stimulated transcription at concentrations in the range 10^{-6} to 10^{-4} M. DBP, and to a lesser extent BHA, also stimulated transcription at concentrations between 10^{-5} and 10^{-4} M (Fig. 3). Two closely related compounds, DEHP (a phthalate) and BHT (an antioxidant), did not stimulate transcription to any appreciable degree until concentrations in excess of 10^{-4} M were reached. At these high concentrations, the response to these latter two chemicals was less than 15% of the maximum response obtained with estradiol (results not shown).

OP stimulated transcription of the reporter gene (LUC) to a similar extent as 17ß-estradiol (albeit at a concentration 1000-fold greater) and was used for comparison because it is a recognized environmental estrogen (*26*). No ligand-dependent transactivation was detected with any of the compounds in transfections using the reporter plasmid pTKLUC, which lacks the consensus ERE (results not shown).

Of the 20 compounds initially tested (Table 1), the action of the two most potent compounds (the phthalates) was compared with the action of two antiestrogens (4-OHT and ICI 182780). The compounds were tested for their ability to inhibit transcription of the reporter caused by the presence of 17ß-estradiol at concentrations of 10^{-11} M (Fig. 4) and 10^{-8} M (data not shown). In view of the relative binding affinities of the phthalates for the receptor (Fig. 1), the lower concentration of 17ß-estradiol used would allow competition by the compounds in binding to the receptor. In contrast to the two antiestrogens, which inhibited the response in a dose-dependent manner, DBP and BBP increased the transcriptional activity of the receptor in the presence of 10^{-11} M 17ß-estradiol (Fig. 4).

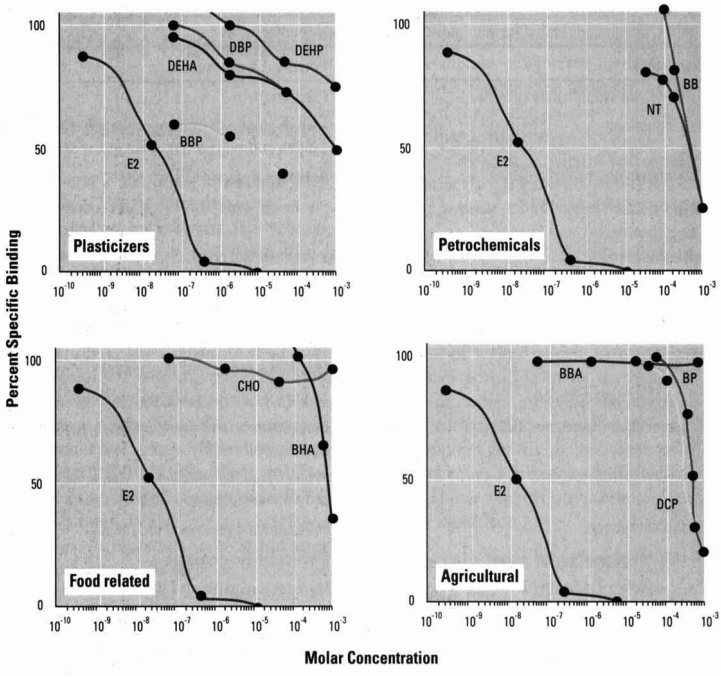

Figure 1. Inhibitory effects of organic chemicals present in sewage effluent on the binding of tritiated 17ß-estradiol to the rainbow trout estrogen receptor. Butylbenzyl phthalate (BBP), di-*n*-butylphthalate (DBP), bis(2-ethylhexyl)phthalate (DEHP), bis(2-ethylhexyl)adipate (DEHA), benzophenone (BP), *n*-butylbenzene (BB), 4-nitrotoluene (NT), butylated hydroxyanisole (BHA), and 2,4,dichlorophenol (DCP) reduced the binding of tritiated 17ß-estradiol to the receptor. Musk ketone, musk xylene, *p*-toluene, butylated hydroxytoluene (BHT), caffeine, cholesterol (CHO), *p*-hydroxybenzoic acid, *p*-tert butylbenzoic acid (BBA), 3,4-dimethylphenol, and 2-methylphenol did not impair binding of tritiated estradiol to the estradiol receptor (most results not shown). All experiments were repeated three times. The error bars are too small and are therefore not shown on the figure.

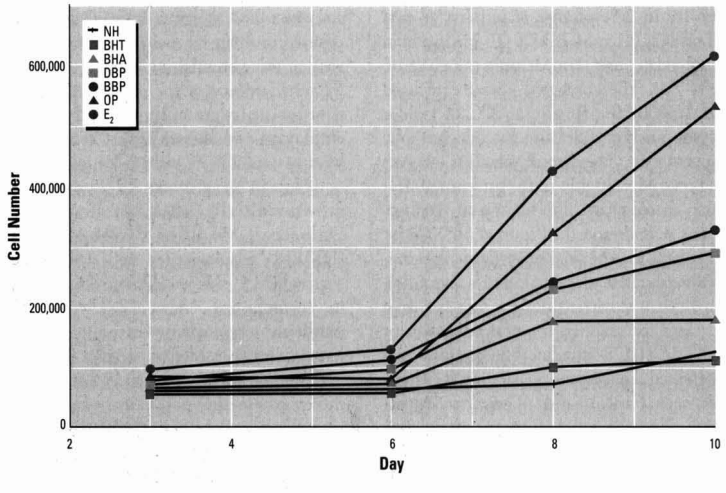

Figure 2. Mitogenic effects of various environmental chemicals on breast cancer cells. Cells were exposed to no hormone (NH), 10 nM 17ß-estradiol (E_2), 10^{-5} M octylphenol (OP), or 10^{-5} M of each of the environmental pollutants *n*-butylbenzene, di-*n*-butyl phthalate (DBP), butylbenzyl phthalate (BBP), 4-nitrotoluene, bis(2-ethylhexyl) adipate, 2,4-dichlorophenol, benzophenone, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), or bis(2-ethylhexyl)phthalate. Cells were cultured for 10 days and counted on days 0, 3, 6, 8, and 10. Only those compounds that enhanced cell growth are shown. All other compounds did not enhance breast cancer cell growth at this concentration to any significant degree. All experiments were carried out in duplicate and repeated twice. Similar results were observed in a replicate experiment, although the number of cells used per well at the beginning of the experiment differed. Mean values are presented from a single experiment.

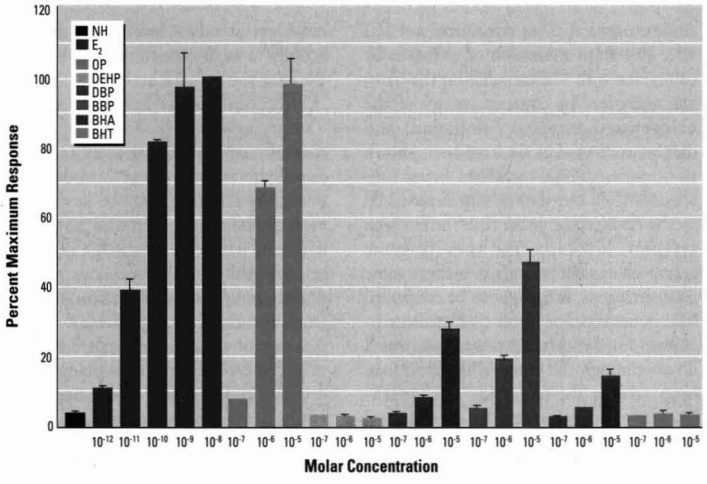

Figure 3. Stimulation of transcriptional activity of the estrogen receptor by environmental chemicals. Cells were maintained with no hormone (NH), 17ß-estradiol (E_2), octylphenol (OP), butylbenzyl phthalate (BBP), di-*n*-butylphthalate (DBP), bis(2-ethylhexyl)phthalate (DEHP), butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) at the concentrations indicated. Transcriptional activity of the estrogen receptor in the presence of environmental chemicals is expressed as a percentage of the maximum response induced by 17ß-estradiol, and is presented as mean +/- SEM.

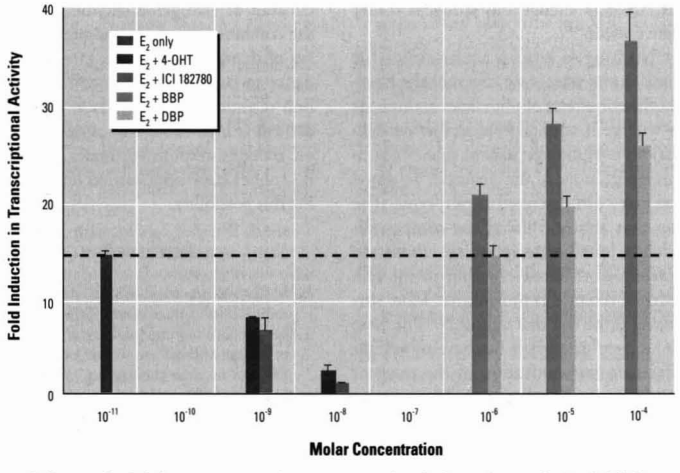

Figure 4. Estrogenic phthalates act as agonists, not antagonists, in the presence of estradiol. Cells were incubated with 10^{-11} M 17ß-estradiol alone, simultaneously with di-*n*-butylphthalate (DPB) or butylbenzyl phthalate (BBP), or simultaneously with the antiestrogens 4-hydroxytoluene (4-OHT) or ICI 182780. Both the phthalates and the antiestrogens were added at the concentrations indicated. Mean values are presented and the error bars represent the SEM.

Discussion

Microbial degradation of chemicals present in sewage results in a wide range of products, many of which are unidentified. Some of these products will be transient intermediates in the degradation process, while others will be more persistent. Thus, we do not know exactly what is in effluent, and we are left with the task of testing only the compounds that have been positively identified. This group of identified chemicals may represent only 20% of the total chemicals present. Of the 20 chemicals tested, 9 reduced the binding of tritiated 17ß-estradiol to the fish estrogen receptor. This initial screening process isolated a subset of chemicals that were likely to be able to bind to the estrogen receptor, but it was not possible to determine whether these chemicals were agonists or antagonists. Using more specific tests, designed to assess whether any of these chemicals were estrogenic , we showed that three of these compounds had significant effects on transactivation of the estrogen receptor and breast cancer cell growth.

BHA is commonly used as an antioxidant, particularly in foods. Therefore, its route of exposure to humans is likely to be mainly via ingestion. It has a low oral toxicity, and it has been estimated that the mean human intake of BHA averages 0.13 mg/kg body weight/day (*27*). Our studies indicate that BHA is six or more orders of magnitude less potent than 17ß-estradiol, and hence causes stimulatory effects on both the transcriptional activity of the human estrogen receptor and the growth of breast cancer cells *in vitro* only at concentrations of 10^{-5} M (2–3 ppm) and above. However, it is impossible to make predictions on its activity *in vivo* because no such studies have been carried out. BHA may bioconcentrate to a low degree in humans, although it is not certain whether the lack of full recovery of BHA from urine after ingestion is due to bioaccumulation of intact BHA or its metabolites or to unknown routes of biotransformation (*28*). Although it is reported to be present in some sewage effluents, BHA is not as ubiquitous as its chemical cousin BHT, which was found to be even less estrogenic than BHA.

In contrast, phthalates are the most abundant man-made chemicals in the environment (*29*). They are produced industrially in large quantities, mainly to impart flexibility into plastics, and can leach out of these materials into water, soil, or food over time. BBP is also used in the production of vinyl floor tiles, adhesives, and synthetic leather; DBP is more common as a plasticizer in food-packaging materials, PVC, the cellulosics, and certain types of elastomers (*30–32*). Thousands of tons of plastics are disposed of annually in landfill sites, thus enabling phthalate esters to migrate into groundwaters via the soil. The ubiquity of these compounds in the aqueous environment is well known, and their presence is reported in river, waste, and drinking waters as well as in fish and sediments (*33–39*). Commonly detected species include DBP, dimethyl phthalate, diethyl phthalate, DEHP, di-*n*-octylphthalate, BBP, and DEHA (*16*).

We have not tested many of these phthalates to determine whether any of them are estrogenic (we have tested only those phthalates listed in Table 1). However, our results indicate that a comprehensive survey of the estrogenic activities (if any) of all commonly used phthalates would be justified. The general population may be exposed to these compounds via their diet, either from food contamination, or from food or drinks directly contaminated by plastic wraps containing phthalates, or from polluted drinking water (*31,34,40*). In most cases, the greatest exposure is from food. Levels of DBP in foods range from 50 to 500 µg/kg in the United States (*41*). A 1987 study in the UK estimated that the average intake of DBP of food packaged in cellulose film

was 230 µg/day (*42*). Indeed, up to 14 mg DBP/kg was found in chocolate bars and potato snacks wrapped in printed polypropylene films (*43*).

In our studies, the phthalates DBP and BBP were estrogenic *in vitro* at concentrations between 10^{-6} and 10^{-4} M. However, these figures cannot be used to predict estrogenic activity *in vivo*. Because they are lipophilic, all phthalates have a tendency to accumulate in fatty tissues and can be absorbed through human skin very efficiently. However, once they are absorbed or ingested, they may be metabolically cleared from the body; little is known about the absorption and metabolism of phthalates. The oral toxicities of phthalate compounds in humans are generally low (*30*), although at high concentrations, they are testicular toxicants. It has been suggested that the concentration of these compounds (particularly DBP) in the cellular fraction of sperm from adult men is negatively correlated with either sperm density or the total numbers of sperm (*29*). Indeed, when administered to rats in high doses phthalates are embryofetal toxicants as well as testicular toxicants (*44–48*). In the female rat, the primary effect on reproduction is spontaneous abortion and decreased litter size. Recent studies on the embryolethality of BBP have shown that this effect is correlated with a lowering of plasma progesterone levels (*49*), and it is possible that this is a consequence of an estrogenic effect.

It is well established that, upon binding to 17ß-estradiol, the estrogen receptor binds to DNA as a homodimer and activates transcription of estrogen-responsive gene products by means of two distinct activational regions on the estrogen receptor, AF_1 in the N-terminal domain, which is estrogen independent, and AF_2 in the estrogen-binding domain, which is active only in the presence of estrogen (*50–53*). The environmental estrogen OP mimics this action exactly; it binds to the estrogen receptor in the same region as 17ß-estradiol and induces full activation (*26*). In contrast, the antiestrogen/partial agonist tamoxifen promotes DNA binding but fails to induce the activity of AF_2 and hence causes only a submaximal effect due to the constitutive activity of AF_1 (*54–56*).

Because none of the active compounds listed in Table 1 could induce full activation, at least at the concentrations used, the possibility that they may also be antiestrogenic was considered. Indeed, the potential for harmful effects of these chemicals on humans or animals will depend not only on their agonistic activity, but also on their potential to act as antagonists in the presence of other environmental estrogens and/or endogenous estrogens. Antiestrogens such as tamoxifen and ICI 182780 inhibit the action of estrogens by competing with 17ß-estradiol for the estrogen receptor. In contrast, many of the halogenated aromatic compounds and dioxins such as TCDD have been shown to be antiestrogenic in human breast cancer cells (*57*), but their action is mediated by the Ah receptor rather than the estrogen receptor. Similarly, the antiestrogenic action of dietary estrogens, such as some phytoestrogens, is thought to be controlled by a nonestrogen receptor-mediated mechanism (*58*). Synthetic antiestrogens, which do act through the estrogen receptor, have been used in the treatment of estrogen-responsive breast cancers for several years (*59*). Antiestrogenic activity may be deleterious if it blocks the action of estrogen during sexual differentiation or puberty. Our results demonstrate that *in vitro* the phthalate compounds are acting as agonists only and do not act as antiestrogens at any concentration throughout their active range. Therefore, we suggest that rather than being contra-active, they would enhance the effects of endogenous estrogens if they were present.

Nothing is known about either the acute *in vivo* estrogenic effects or the possible chronic effects of phthalates on humans or wildlife if administered at low concentrations over long periods of time. Prior to this report, none of the chemicals we tested had ever been described as estrogenic. The fact that almost 50% of the compounds initially tested were found to inhibit the binding of tritiated estradiol to the fish estrogen receptor is provocative. More surprising is the fact that almost 30% of these "inhibitory" chemicals can have significant effects on transactivation of the receptor and breast cancer cell growth.

The possible implications of this scenario to man and wildlife will depend entirely on the estrogenic potencies of these chemicals *in vivo*; to a large extent this will depend on the processes of metabolic transformation and bioaccumulation. In addition, the effects of simultaneous exposure to a variety of estrogenic chemicals should be investigated. Since all of the estrogenic chemicals discovered to date are lipophilic, they probably co-exist in fat and body fluids of exposed individuals. Much of the current literature suggests that environmental estrogens may act cumulatively and that measuring the total estrogenic burden due to environmental contaminants may have more relevance than assessing exposure by measuring levels of individual estrogens alone (*60,61*). Estrogen-responsive sites such as the reproductive tract or neuroendocrine centers are highly sensitive and hence it is possible that exposure to many weakly active compounds either persistently at low concentrations, or acutely in high concentrations, may alter the natural hormonal balance.

In conclusion, we have discovered that a surprisingly large proportion of environmentally persistent chemicals are weakly estrogenic and thus have introduced the possibility that there may be hundreds, or even thousands, of chemicals in the environment which possess some estrogenic activity. Although the chemicals we tested possess some common structural features (such as a benzene ring), there is no obvious part of their molecular structure that might be expected to enable binding to the estrogen receptor, and hence one cannot easily deduce which chemicals are and which are not estrogenic. Aquatic organisms are probably exposed to these weakly estrogenic chemicals largely, if not exclusively, via water. However, terrestrial animals (including humans) are probably exposed via many routes. The concentrations required to induce effects *in vivo* are essentially unknown, particularly when an organism is exposed simultaneously to a cocktail of estrogenic chemicals. Even if the combined effect of exposure to a number of chemicals is additive, there is no evidence to suggest that the total concentration of estrogenic chemicals in humans or animals is high enough to cause any effects on estrogen-responsive tissues. However, no studies have been carried out to examine this possibility.

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Fertility in Mice after Prenatal Exposure to Benzo[*a*]pyrene and Inorganic Lead

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Experimental evidence suggests that inorganic lead and benzo[*a*]pyrene (BaP) suppress the development of primordial oocytes during fetal life. We examined the single and combined effects of prenatal exposure to BaP and moderate doses of lead. The fertility and ovarian morphology of F_1 female NMRI mice in four treatment groups (nine mice per group) were investigated: control; lead (F_0 given 1 g $PbCl_2$/L in drinking water until mating); BaP (10 mg/kg body weight daily by oral intubation on days 7–16 of F_0 pregnancy); and combined lead and BaP. F_1 groups exposed prenatally to BaP either alone or in combination with inorganic lead showed markedly reduced fertility with few ovarian follicles compared to controls, whereas the group exposed to lead only had measures comparable to the controls. Mice exposed to both lead and BaP had a significantly longer gestation period (days to litter) compared to mice exposed only to BaP, lead, or controls. There is a nonsignificant indication that the compounds together further reduce number of offspring, number of litters, and litter size. These results suggest that lead and BaP have synergistic effects on impairment of fertility. The possibility of synergism may be of human relevance as inorganic lead and BaP are ubiquitous environmental pollutants. *Key words*: benzo[*a*]pyrene, fertility, inorganic lead, oogenesis, synergism. *Environ Health Perspect* 103:588–590 (1995)

The period of development of the female gonads in fetal life may be critical for fertility in adult life. Rodents show a reduced number of primordial oocytes or reduced fertility after prenatal exposure to inorganic lead (*1–3*), benzo[*a*]pyrene (BaP) (*4*), and other agents (*5,6*). Inorganic lead is also a suspected developmental neurotoxicant (*7*) and could possibly have adverse effects on the developing reproductive system through action on the hypothalamic-pituitary axis during fetal life. Studies exploring if similar mechanisms may be responsible for human subfecundity are scarce; in an epidemiologic study maternal smoking in the pregnancy was associated with reduced fecundability among their daughters in later life (*8*).

The reproductive effects of environmental pollutants are currently receiving attention, due in part to the possibility of human infertility (*9*). BaP and inorganic lead are of special interest. Both are ubiquitous environmental pollutants, and BaP is a component of cigarette smoke. We therefore conducted an experiment exposing mice prenatally to inorganic lead and BaP. The purpose of the study was to investigate lead and BaP for synergistic effects on female fertility. We chose a treatment dose of lead that was judged from the blood lead levels to be comparable to exposure levels found in many occupational settings. The treatment dose of BaP was similar to the lowest dose that induced subfertility in an earlier report (*4*).

Methods

Lead(II) chloride (CAS no. 7758-95-4) was supplied from Baker (Deventer, Holland). Benzo[*a*]pyrene (CAS no. 50-32-8) was a product of Sigma Chemical Company (St. Louis, Missouri).

Male and F_0 female Bom:NMRI mice were acclimatized to a 12/12 hr light/dark cycle (lights on at 0600 hr) at 24 ± 1°C. Laboratory chow (EWOS, R34) and tap water were provided *ad libitum*. Males were individually caged except during mating. We placed F_0 females in groups of three until the last week of pregnancy when they were caged alone.

At the age of 9 weeks, F_0 females were randomly assigned to one of four treatment groups, nine mice per group. Two groups received tap water with 1 g $PbCl_2$/L(0.75 g Pb) during the 6 weeks before mating, whereas the remaining two groups received tap water without any additions. We discontinued the lead treatment before mating to avoid exposure of the males. All F_0 females were caged with sexually active males. Females were inspected twice daily for a vaginal plug, which, in case of pregnancy, was counted as day 0 of gestation. During days 7–16 of pregnancy, one of the tap water groups and one of the lead–water groups received a daily treatment of 0.2 mL corn oil BaP (10 mg/kg body weight by oral intubation), and the remaining two groups received corn oil. Thus, the four treatment groups, each comprising nine F_0 females, were the control group, the lead group, the BaP group, and the lead plus BaP group. We recorded weights and sampled blood for lead measurement from tail veins on the day before caging with the males (Table 1). Assessed from a pilot trial on female mice of the same strain, the blood lead values in the lead-treated groups should give values of approximately 2 µmol/L 10 days later (during the second week of pregnancy). No F_0 females showed signs of general toxicity, and they all proved fertile. We kept F_0 females with their offspring until after weaning (21 days after delivery).

One F_1 female from each of the 36 litters was allocated in the experiment, each belonging to one of the four exposure groups they had been assigned to *in utero*. At the age of 6 weeks, each F_1 female was caged for 6 months with an untreated male proven to be sexually active. Males for four females that did not become pregnant after 30 days were replaced, but this procedure did not lead to any pregnancies. Dates and sizes of litters were recorded. F_2 offspring were inspected for gross deformities at birth, and their weight and sex were recorded at day 2 after birth when they were killed by cervical dislocation.

After 6 months of continuous breeding, the F_1 females were euthanized; the right ovary was excised, trimmed, and weighed. Ovaries were fixed in buffered formalin, embedded in paraffin, and 3-µm parasagittal sections were prepared. Three slides, made from tissue 90 µm apart, were stained with hematoxylin-azophloxine-saffron and reticulin and examinated by light microscopy.

The F_1 female was taken as the basic unit of experimentation. Several measures of fertility and ovarian morphology were recorded without knowledge of the treatment group. Measures of fertility for each F_1 female were number of offspring, number of litters during the breeding period, median litter size, and median days between deliveries (with the start of the experiment as the starting point for the first interval).

We recorded the weight of the right ovary for each F_1 female and counted follicles and corpora lutea in the three histological sections. Follicles were classified according to Pedersen and Peters (*10*).

Treatment effects on fertility, ovarian weights, and counts in the microscopic examination were evaluated by nonparametric tests (Wilcoxon rank sum test, Kruskall-Wallis test). Significance levels of <0.05 (two-tailed) were taken as criterion of treatment effects. The statistical analyses were performed with the BMDP software package (*11*).

Results

The 36 mated F_1 females gave birth to a total of 1985 offspring distributed over 182 litters. The distribution over the treat-

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We thank Uni Rykke and Aase Kindberg for skillful handling of the animals during the study.

Received 28 November 1994; accepted 27 February 1995.

Table 1. Descriptive values (median, range) for F_0 and F_1 female mice in the four treatment groups

	F_0 females (n = 9/group)				F_1 females (n = 9/group)	
	Body weight at time of mating (g)		Blood lead at time of mating (μmol/L)		Body weight after first pregnancy (g)	
Treatment group	Median	Range	Median	Range	Median	Range
Control	37.8	34.6–41.4	0.04	0.01–0.10	38.7	37.3–43.9
Lead	36.5	33.3–43.9	3.37	2.44–3.80	39.8	37.9–44.7
BaP	35.9	32.8–40.3	0.04	0.02–0.08	37.1	34.1–44.3
Lead plus BaP	37.9	32.7–46.3	3.71	2.70–5.26	38.0	32.9–44.7
Heterogeneity between groups, χ^2 (p)[a]	0.93 (0.82)		27.3 (<0.0001)		1.98 (0.58)	

BaP, benzo[*a*]pyrene.
[a]Chi-square with 3 degrees of freedom; two-tailed p-value.

Table 2. Values of different measures of fertility over the four treatment groups among F_1 female mice

	Treatment group				Heterogeneity between groups, χ^2 (p)[a]
Effect	Control (n = 9)	Lead (n = 9)	BaP (n = 9)	Lead plus BaP (n = 9)	
No. of litters	67	72	29	14	
No. of offspring (stillborn)	785 (4)	860 (8)	248 (8)	92 (9)	
Median no. offspring/F_1 (range)	92 (26–121)	95 (50–122)	22** (0–86)	4** (0–48)	24.5 (<0.0001)
Median no. litters/F_1 (range)	8 (3–8)	8 (8–8)	3** (0–8)	2**(0–5)	22.8 (<0.0001)
Median litter size/F_1 (range)	11.5 (6–15)	13 (6–18)	8** (3–11)	6.5** (1–12)	10.6 (0.01)
Median no. days between litters/F_1 (range)	20.5 (20–21)	20.5 (20–21)	21* (20–23)	22.5** (21–29)	15.5 (0.001)

BaP, benzo[*a*]pyrene.
[a]Chi-square with 3 degrees of freedom; two-tailed p-value.
*Significantly different from control group (two-tailed $p < 0.05$).
**Significantly different from control group (two-tailed $p < 0.005$).

ment groups is given in Table 2. The lead group had litters and offspring numbers that were slightly higher than the control group, whereas the BaP group and the lead plus BaP group both had considerably lower fertility as judged by these measures. The cumulative number of F_2 offspring over time and the distribution of number of litters among F_1 females in the four treatment groups are illustrated in Figures 1 and 2, respectively. Four F_1 females (one BaP treated, three lead plus BaP treated) were infertile in the study; only 1 out of 18 F_1 females producing more than 6 litters belonged to either of the 2 groups receiving BaP (Fig. 2).

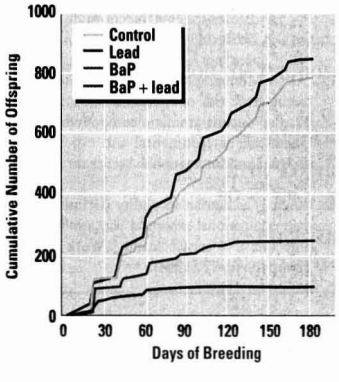

Figure 1. Cumulative number of F_2 offspring recorded over 6 months for the four F_1 treatment groups.

The Kruskall-Wallis test demonstrated highly significant heterogeneity over the treatment group for all measures of fertility: number of offspring, number of litters, median number of offspring per litter, and days between litters (Table 2). This was due to markedly lower fertility measures in the BaP and the lead plus BaP groups compared to the control group, whereas the lead group performed nonsignificantly better than the control group for all measures. In the comparison between the BaP group and the lead plus BaP group, the latter showed poorer fertility for all measures. Some of those differences were large but were only significant for median days between litters (Z-value = 1.98, two-tailed p = 0.05). For other effect measures, the significance levels ranged from 0.09 to 0.32.

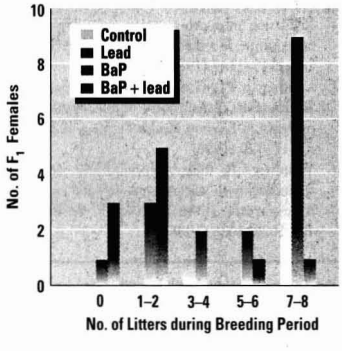

Figure 2. Frequency distribution of total litters recorded over 6 months among F_1 females in the four treatment groups.

A similar pattern was evident from examination of the ovaries (Table 3). Ovary weights, counts of follicles, and counts of corpora lutea were different in the treatment groups, with low weights and depletion of follicles and corpora lutea for most animals in the BaP and lead plus BaP groups. The F_1 females in the lead group had nonsignificantly higher weights and counts of follicles and corpora lutea compared to the control group. The lead plus BaP group had lower counts of follicles and corpora lutea and slightly higher median ovarian weight than the BaP group, but all differences were nonsignificant in the Wilcoxon rank sum test.

Discussion

We have shown that prenatal administration of 10 mg BaP/kg maternal body weight (with or without lead) leads to subfertility and a marked reduction in the number of ovarian follicles in F_1 females. These results are in agreement with a previous report (*4*) and are probably due to impaired development of primordial oocytes. This interpretation is further supported by an earlier report on toxic effects of BaP on primordial oocytes in weanling mice (*12*).

No treatment effects of lead were detected in the study. This is not surprising since we used moderate doses resulting in maternal blood lead levels about 2 μmol/L during the second week of pregnancy; this is considered to be the critical time of fetal folliculogenesis (*3*). This dose was far lower than that reported to have an independent effect on primordial germ cells (*3*) and fertility (*1–3*).

To our knowledge, the combined effect on fertility of inorganic lead and BaP has not been reported earlier. Our study was designed to investigate the hypothesis of an interaction between the two agents. We found a synergistic action of inorganic lead on the BaP effects for days between litters, and the manifestations of combined lead and BaP treatment were stronger, but not

Table 3. Values of different measures of ovarian effects over the four treatment groups among F_1 female mice[a]

Effect	Median (range) Control ($n = 9$)	Lead ($n = 7$)	BaP ($n = 8$)	Lead plus BaP ($n = 8$)	Heterogeneity between groups, χ^2 (p)[b]
Ovarian weight (mg)	13 (13–20)	14 (11–25)	9* (7–13)	10 (2–17)	12.4 (0.006)
No. of small follicles/F_1	44 (1–137)	60.5 (15–150)	0**(0–68)	0**(0–35)	18.8 (0.0003)
No. of medium follicles/F_1	9 (5–25)	12.5 (2–30)	0** (0–57)	0** (0–2)	19.3 (0.0002)
No. of large follicles/F_1	14 (6–23)	21.5 (12–29)	0** (0–19)	0** (0-0)	23.6 (0.0001)
No. of corpora lutea/F_1	16 (6–35)	22 (15–57)	0** (0–14)	0** (0–4)	23.4 (<0.0001)

BaP, benzo[*a*]pyrene.
[a]Histologic examinations were performed on three sections of the right ovary. Results were discarded for two animals in the lead group and one animal each in the BaP and the BaP plus lead-treated groups.
[b]Chi-square with 3 degrees of freedom; two-tailed p-value.
*Significantly different from control group (two-tailed $p < 0.05$).
**Significantly different from control group (two-tailed $p < 0.005$).

significantly so, for almost all indicators of impaired follicular development and fertility compared to the BaP treatment alone. It may be relevant that some lead compounds have synergistic effects on model carcinogens in rodents (*13–16*).

Mechanistic interpretations on the synergism between lead and BaP on the basis of our results can only be speculative. BaP seems to have a direct effect on primordial oocytes (*4,7*). Fetal treatment with high doses of inorganic lead also decreases the number of primordial follicles, possibly as an effect on the migration or multiplication of the developing germ cells (*2*). Lead is also a developmental neurotoxicant, and its synergistic action might be explained by a disturbance of the neuroendocrine balance that alone was not sufficient to impair fertility. However, Wide (*2*) found only small and nonsignificant alterations in the levels of ovarian steroid hormones for mice exposed prenatally to lead in doses that clearly reduced the number of primordial follicles.

The lead and BaP doses chosen in our study were probably not optimal. The lead dose was comparable to human exposures, and the daily BaP dose in our study was equal to the lowest effect level in an earlier study, but its effect on fertility was strong (*4*). The daily dose (10 mg/kg) is about 1 million times the main stream dose in 100 cigarettes (*17*). Fertility as outcome might have been more sensitive to the combined effects of lead and BaP if the BaP dose had been lower. BaP alone had a profound effect; even if the lead plus BaP group had almost total depletion of follicles and several animals were infertile, the differences were not significant.

There is currently much concern about human fertility (*9*). Biology indicates that the prenatal development of primordial germ cells may be crucial. Exposures to common environmental xenobiotics are under suspicion of interfering with gonadal development (*9*). Human exposures from environmental sources are considerably lower than exposures producing effects in laboratory animals but could be more relevant in case of synergistic actions. Human studies addressing effects of environmental agents are warranted, but the needed multigenerational designs restrict the possibilities (*18*). Animal models that have been developed should be further applied.

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Meeting Announcement

Engineering Solutions to Indoor Air Quality Problems

July 24–26, 1995
Sheraton Imperial Hotel and Conference Center
Research Triangle Park, NC

This international Symposium is cosponsored by the Air & Waste Management Association (A&WMA) and the U.S. Environmental Protection Agency's Air and Energy Engineering Research Laboratory. Participating organizations include the Consumer Product Safety Commission and the National Research Council Canada. The Symposium will provide a forum for the technical exchange of information on on-going research on characterizing sources of indoor air emissions and mitigating and preventing indoor air quality problems. Attendees will include researchers from the government, private sector, industry, and academia. The 2 1/2 day Symposium will include a general session, a poster session, continuing education courses, and an exhibition of related products and services.

Planned Program

Monday, July 24
a.m. - Source Characterization
p.m. - Source Management & Pollution Prevention Reception/
Exhibition/Poster Viewing

Tuesday, July 25
a.m. - Ventilation & Modeling
p.m. - Indoor Air Laboratory Tours

Wednesday, July 26
a.m. - Biocontaminant Control
p.m. - Indoor Air Laboratory Tours

For registration information or to receive a copy of the preliminary program, contact the A&WMA Registrar at 412-232-3444, extension 3142; fax 412-232-3450. Hotel reservations should be made directly with the Sheraton Imperial at 800-325-3535 or 919-941-5050. To ensure availability and rate ($70 single and double occupancy, plus applicable taxes), make your reservations by June 29. Be sure to mention the symposium to receive this rate.

For information on the technical program, contact:
Kelly Leovic
U.S. EPA, MD-54
Research Triangle Park, NC 27711
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For information on the exhibition contact:
Roy Neulicht at 919-677-0249, ext, 5126
fax 919-677-0065.

Drinking Water and Pregnancy Outcome in Central North Carolina: Source, Amount, and Trihalomethane Levels

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In spite of the recognition of potentially toxic chemicals in chlorinated drinking water, few studies have evaluated reproductive health consequences of such exposure. Using data from a case–control study of miscarriage, preterm delivery, and low birth weight in central North Carolina, we evaluated risk associated with water source, amount, and trihalomethane (THM) concentration. Water source was not related to any of those pregnancy outcomes, but an increasing amount of ingested water was associated with decreased risks of all three outcomes (odds ratios around 1.5 for 0 glasses per day relative to 1–3 glasses per day, falling to 0.8 for 4+ glasses per day). THM concentration and dose (concentration × amount) were not related to pregnancy outcome, with the possible exception of an increased risk of miscarriage in the highest sextile of THM concentration (adjusted odds ratio = 2.8, 95% confidence interval = 1.1–2.7), which was not part of an overall dose–response gradient. These data do not indicate a strong association between chlorination by-products and adverse pregnancy outcome, but given the limited quality of our exposure assessment and the increased miscarriage risk in the highest exposure group, more refined evaluation is warranted.

***Key words*: chlorination, low birth weight, preterm delivery, spontaneous abortion, trihalomethanes. *Environ Health Perspect* 103:592–596 (1995)**

It has been known for nearly 20 years that chlorination of surface waters produces small amounts of chloroform and other potentially toxic by-products (*1*). Ingestion of these agents by large numbers of people over extended periods of time has generated considerable concern with potential adverse health effects. Most of that concern has focused on carcinogenicity, with studies providing mixed support for an association between chlorination by-product concentrations and the risk of bladder and colon cancer (*2*).

Reproductive outcomes, known to be sensitive to environmental toxicants, have received much less attention. In addition to the obvious public health impact of congenital malformations and fetal and infant death, studies of reproductive consequences have the logistical advantage of a shorter interval between exposure and disease manifestation. This briefer period of interest facilitates more accurate recall of consumption over the relevant time period and improved estimation of contaminant concentration. The seasonal variation in chlorination by-product levels (higher in the summer) can be incorporated into reproductive health studies as a component of the exposure variability that is analyzed.

Laboratory research relevant to chlorination by-products and reproduction is limited (*3,4*) with most evaluations focused on single chemicals rather than the complex mixture encountered by humans in treated water. At exposure levels orders of magnitude higher than those encountered naturally, developmental toxicity in the form of reduced fetal weight, heart malformations, and reproductive toxicity related to adverse effects on sperm has been demonstrated for chloroform, bromoform, haloacetic acids, and related compounds (*3*). However, it is not clear whether they produce toxic effects at the low exposure levels of concern.

Prior research on the outcomes of interest, fetal loss, preterm delivery, and low birth weight, is limited in both quantity and quality (*4*). In the most thorough effort to evaluate preterm delivery, low birth weight, and small for gestational age (SGA), Kramer et al. (*5*) conducted a study in Iowa. Exposure was classified based on the community of residence in conjunction with a survey of chlorination by-products. Chloroform concentrations above 10 ppb in drinking water were associated with a small increase in risk of low birth weight [adjusted odds ratio (OR) = 1.3] and a somewhat greater risk of SGA (adjusted OR = 1.8). As noted by the investigators, there was no opportunity to consider fluctuations in the contaminant levels over time or individual variability in water consumption.

The only other study that considered trihalomethane (THM) levels in the community supply was conducted in northern New Jersey (*6*) using birth and fetal death certificates to identify birth weight, low birth weight (<2500 g), very low birth weight (<1500 g), term low birth weight, preterm delivery, SGA births, and fetal deaths. Mean birthweight was reduced slightly in relation to use of surface water supplies and in relation to use of water with THM concentrations above the federal standard of 100 ppb (*7*) as reflected in the nearest quarterly sample. The risk of adverse outcomes was generally elevated slightly in relation to both surface (versus ground) water use and elevated THM levels, with adjusted OR, in the range of 1.1–1.4. Except for a closer temporal relation between the pregnancy and the measurement, the same limitations noted for the Iowa study are applicable to the New Jersey study.

Other reports of less direct relevance found that stillbirth risk was associated with chlorinated versus chloraminated water supplies in Massachusetts (*8*) and that the use of bottled water rather than tap water may be associated with a reduced risk of spontaneous abortion in Northern California (*9*). However, the investigators suggested that a reporting bias may account for the latter association (*10*).

Given current knowledge, additional work is clearly needed to evaluate whether the previous suggestions of small adverse effects on pregnancy are likely to be causal. Data collected from interviews of women who experienced miscarriage, preterm delivery, and low birth weight births as well as term, normal birth weight controls allow for an examination of individual water consumption in relation to water source and measured community THM levels.

Methods

A population-based case–control study of miscarriage, preterm delivery, and low birth weight was conducted in Alamance, Durham, and Orange Counties in central North Carolina. The region was originally chosen for the concentration of textile industry employment in Alamance County and expanded to include the larger, more sociodemographically diverse populations of Durham and Orange counties.

All medically treated miscarriage cases among women in Alamance County during the period September 1988 through August 1991 were identified through medical care providers, including hospitals and private clinics, as described in detail elsewhere (*11*). Preterm deliveries (<37 weeks completed gestation) and low birth weight infants (<2,500 g) were identified at six area hospitals covering virtually all births to area residents during the period September 1988 to August 1989 in Orange and

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We thank Kate M. Brett, Laurie E. Evans, and Nancy Dole Runkle for work on data collection and analysis and acknowledge funding from the National Institutes of Health (HD23862, HD23862S), March of Dimes (15–157), Centers for Disease Control (R48/CCR402177), and U.S. EPA (CR 820076-02).

Received 5 January 1995; accepted 10 March 1995.

Durham Counties, and September 1988 to April 1991 in Alamance County. There was substantial overlap between the preterm and low birth weight groups, with 34% of eligible live birth cases preterm and not low birth weight, 17% low birth weight and not preterm, and 50% both preterm and low birth weight.

Controls were selected in a one-to-one ratio to live birth cases from the deliveries immediately following a preterm or low birth weight case of the same race and hospital as the case, but restricted to term, normal weight births. The controls selected for preterm and low birth weight cases in Alamance County also served as controls for the miscarriage cases. We considered the controls as a hospital- and race-stratified sample from the population. Therefore, we did not analyze the data as a pair-matched sample but controlled as needed for race and hospital in the analysis.

Ten to fifteen percent of cases and controls were lost due to subject refusal (higher for miscarriage cases than the other groups), and an additional 11 to 16% were lost due to being untraceable (Table 1). An abbreviated form of the questionnaire which did not include the questions pertaining to drinking water was used for subjects who would have otherwise refused (short questionnaire). Final response proportions ranged from 62 to 71%, lowest for miscarriage cases and highest for preterm delivery cases.

Telephone interviews were used to ascertain information on a wide range of potential risk factors for adverse pregnancy outcome, including sociodemographic attributes (age, race, education, marital status, income), pregnancy history, tobacco and alcohol use, prenatal care, physical exertion, psychological stress, and employment. Each woman was asked, What was your primary source of drinking water at home? Was it supplied by the community water company, from a private well, or bottled water? This was followed by the question, About how many glasses of water did you drink per day around the time of your pregnancy?

After analyzing water source and amount, we restricted the sample to women who were served by public supplies and who reported drinking one or more glasses of water daily (omitting approximately 30% of eligible subjects; see Table 1). A woman's address was used to assign her to one of the five public water supplies serving residences in this region. Although we did not have information on changes in water consumption during pregnancy, we were able to consider the changes in THM concentrations over time. The dates of pregnancy were used to assign the reported quarterly average THM value from the appropriate supplier as her THM score. For miscarriage cases and their controls, the fourth week of pregnancy was the time period used for making that assignment, and for preterm delivery cases, low birth weight cases, and their controls, the 28th week of pregnancy was used to assign the nearest THM value. These periods reflect the most likely intervals in which any adverse effects would occur.

With this information, we were able to analyze several indices of water exposure: 1) source: community supply, private well (referent), bottled water; 2) amount (glasses per day): 0, 1–3 (referent), 4+; 3) source × amount: private well, 1–3 glasses per day (referent); private well, 4+ glasses per day; community supply, 1–3 glasses per day; community supply, 4+ glasses per day; bottled (regardless of amount); 4) THM concentration: analyzed as a continuous measure and divided into tertiles based on distribution of controls, categorized separately for analyses of miscarriage and the live birth outcomes (as indicated in Tables 2–4); 5) THM dose (glasses per day × concentration): analyzed as continuous measure and divided into tertiles based on distribution of controls, categorized separately for analyses of miscarriage and the live birth outcomes as indicated in Tables 2–4.

The ORs were calculated comparing exposures of cases to that of controls, e.g., community versus private source or higher versus lower THM concentrations. The ORs provide an estimate of the relative risk, i.e., the magnitude of increased risk associated with the exposed versus the referent category. The confidence intervals (CIs) provide an indication of the statistical precision of those estimates.

Based on preliminary analyses to identify factors associated with adverse pregnancy outcomes, potential confounders included maternal age, race, hospital (for preterm delivery and low birth weight only) education, marital status, poverty level, smoking, alcohol consumption, employment, and nausea (for miscarriage only). Crude and adjusted ORs were compared, with adjustment for each of the above covariates one at a time. When the adjusted OR differed from the crude by 10% or more, the variable was considered as a confounder and incorporated into the adjusted ORs presented. When multiple confounders were identified, a logistic regression model was developed to simultaneously adjust for those confounders. Therefore, the adjusted ORs presented in the tables can be interpreted as free from confounding by all of the above variables, but only the subset that influenced the results (if any) were considered directly. Given the small numbers of subjects in some cells and the lack of a biological basis for postulating effect modification, we did

Table 1. Number of eligible and interviewed participants: Alamance, Durham, and Orange counties, North Carolina, 1988–1991

	Miscarriage analysis				Preterm and LBW analysis					
	Cases		Controls		Preterm cases		LBW cases		Controls	
	No.	%	No.	%	No.	%	No.	%	No.	%
Eligible	418	100.0	341	100.0	586	100.0	464	100.0	782	100.0
Physician refusal	9	2.2	1	0.3	3	0.5	4	0.9	5	0.6
Patient refusal	63	15.1	39	11.4	52	8.9	53	11.4	85	10.9
Untraceable	48	11.5	34	10.0	70	11.9	74	15.9	84	10.7
Other	18	4.3	4	1.2	6	1.0	7	1.5	4	0.5
Short questionnaire	12	2.9	22	6.5	39	6.7	25	5.4	51	6.5
Missing water data										
Source only	1	0.2	0	0.0	1	0.2	1	0.2	0	0.0
Amount only	0	0.0	0	0.0	1	0.2	1	0.2	3	0.4
Source and amount	6	1.4	4	1.2	2	0.3	3	0.6	7	0.9
Complete water data	261	62.4	237	69.5	412	70.3	296	63.8	543	69.4
Restriction for THM analysis										
No water consumed	40	9.6	22	6.5	44	7.5	35	7.5	40	5.1
Bottled or well water	77	18.4	70	20.5	104	17.7	67	14.4	141	18.0
Missing date/THM data	18	4.3	23	6.7	20	3.4	16	3.4	29	3.7
Complete THM data	126	30.1	122	35.8	244	41.6	178	38.4	333	42.6

Abbreviations: LBW, low birth weight; THM, trihalomethane.

not examine interactions between water exposures and other variables.

Results

Relative to women served by community supplies, women served by private wells were more likely to be white and somewhat more likely to be married, but were otherwise similar with respect to education, tobacco and alcohol use, and reproductive history. Bottled-water users were less likely to use tobacco and were more highly educated. The amount of water consumed was somewhat lower for women who were parous, white, and less educated. THM concentrations were somewhat higher for women who reported using tobacco or marijuana during pregnancy. In general, these potential confounders (which were controlled, as needed, in the analysis) were not strongly associated with water characteristics.

Risk of miscarriage was slightly increased among women who reported using bottled water compared to those who used private wells (Table 2), but this observation was based on few exposed cases. Also, bottled water users were not at increased risk relative to women served by community supplies (Table 2). Regardless of water source, women who reported not drinking any water were at highest risk and those drinking the largest amounts at slightly decreased risk. This pattern was also apparent in the analysis of source by amount and possibly reflected in the reduced risk in the highest tertile of THM dose (THM concentration × amount). THM concentration was not associated with miscarriage risk in the categorical analysis, yet the continuous measure predicted a rather substantial association, with an odds ratio of 1.7 per 50 ppb increment. This was attributable to a much higher risk associated in the highest sextile of exposure (adjusted OR = 2.8, 95% CI = 1.2–6.1) with an anomalously low risk in the second to highest sextile (adjusted OR = 0.2, 95% CI = 0.0–0.5) (not shown).

Preterm delivery showed virtually no association with water source, THM concentration, or THM dose; all adjusted ORs were between 0.8 and 1.2 (Table 3). The number of glasses of water consumed per day showed the same pattern as for miscarriage, with decreasing risk with increasing amount. The estimates were much more precise than for miscarriage, due to a case group nearly twice as large and a control group nearly three times as large.

Analysis of low birth weight (Table 4) indicated no association with water source and a decreased risk with increasing number of glasses per day. Categorical analysis of THM concentration indicated the lowest risk in the referent group but no trend of increasing risk across the middle and highest categories. Analysis using the continuous dose did not indicate a positive association.

Discussion

Overall, drinking water source was not related to the risk of adverse pregnancy outcome, with the possible exception of an increased risk of miscarriage among bottled water versus private well users. We considered only medically treated miscarriages and found some evidence of differential under-ascertainment related to social class (*11*). The association between miscarriage

Table 2. Miscarriage in relation to drinking water characteristics: Alamance County, North Carolina, 1988–1991

	Cases	Controls	Crude OR	Adjusted OR[a]	95% CI
Water source					
Private well[b]	78	68	1.0	1.0	—
Community	171	159	0.9	1.0	0.7–1.6
Bottled	12	10	1.0	1.6	0.6–4.3
Water amount (glasses per day)					
0	40	22	1.6	1.6	0.9–2.8
1–3[b]	137	120	1.0	1.0	—
4+	84	95	0.8	0.8	0.5–1.1
Water source × amount					
Private well/1–3[b]	36	29	1.0	1.0	—
Private well/4+	29	31	0.8	1.2	0.6–2.4
Community/1–3	95	85	0.9	0.8	0.4–1.4
Community/4+	49	60	0.7	0.6	0.3–1.2
Bottled/1+	12	10	1.0	1.0	0.3–3.1
THM concentration (ppb)					
40.8–59.9[b]	37	35	1.0	1.0	—
60.0–81.0	43	44	0.9	1.0	0.5–2.0
81.1–168.8	46	43	1.0	1.2	0.6–2.4
Per ppb change	126	122	1.5	1.7	1.1–2.7
THM dose (ppb × glasses/day)					
40.8–139.9[b]	50	47	1.0	1.0	—
140.0–275.0	45	40	1.1	1.0	0.6–1.9
275.1–1171.0	31	35	0.8	0.6	0.3–1.2
Per 250 unit change	126	122	1.0	1.0	0.7–1.2

Abbreviations: OR, odds ratio; THM, trihalomethane.
[a]Adjusted as needed for potential confounders listed in text; if no confounders identified, the crude OR is presented.
[b]Referent category.

Table 3. Preterm delivery in relation to drinking water characteristics: Alamance, Durham, and Orange counties, North Carolina, 1988–1991

	Cases	Controls	Crude OR	Adjusted OR[a]	95% CI
Water source					
Private well[b]	95	114	1.0	1.0	—
Community	294	388	0.9	0.9	0.7–1.2
Bottled	24	43	0.7	0.8	0.4–1.4
Water amount (glasses per day)					
0	44	40	1.4	1.4	0.9–2.2
1–3[b]	212	261	1.0	1.0	—
4+	157	244	0.8	0.8	0.6–1.0
Water source × amount					
Private well/1–3[b]	46	50	1.0	1.0	—
Private well/4+	34	48	0.8	0.7	0.4–1.3
Community/1–3	153	189	0.9	0.9	0.6–1.4
Community/4+	111	173	0.7	0.8	0.5–1.3
Bottled/1+	24	43	0.6	0.6	0.3–1.3
THM concentration (ppb)					
40.8–63.3[b]	80	110	1.0	1.0	—
63.4–82.7	102	118	1.2	1.2	0.8–1.8
82.8–168.8	62	105	0.8	0.9	0.6–1.5
Per 50 ppb change	244	333	0.8	0.8	0.6–1.2
THM dose (ppb × glasses/day)					
44.0–169.9[b]	78	108	1.0	1.0	—
170.0–330.8	97	115	1.2	1.2	0.8–1.7
330.9–1171.0	69	110	0.9	0.9	0.6–1.3
Per 250 unit change	244	333	0.9	0.9	0.8–1.1

Abbreviations: OR, odds ratio; THM, trihalomethane.
[a]Adjusted as needed for potential confounders listed in text; if no confounders identified, the crude OR is presented.
[b]Referent category.

and bottled water use may reflect a systematic tendency for bottled water users to more comprehensively seek medical care for miscarriages or for women with heightened health concerns to drink bottled water. Socioeconomic status may influence both miscarriage identification and bottled water use, but the associations we reported were adjusted for mother's education and family income.

A consistent pattern of decreasing risk with increasing consumption of water suggests either a genuine beneficial effect of such consumption or a reporting artifact. There are potential benefits of increased fluid consumption during pregnancy since the plasma volume must expand markedly in that period (*12*), but it is not clear that restricted fluid intake would influence the outcomes we addressed. With the available data, we could not examine total fluid consumption or consider beverages prepared from tap water. Analysis of water source by amount added little additional insight to this pattern.

Analysis of THM concentrations yielded some indication of an association with miscarriage, with a notably increased risk in the most highly exposed subset driving a linear dose–response pattern. Analysis by tertiles yielded little evidence of increased risk, whereas isolation of the most highly exposed sextile generated a pronounced association, with an aberrantly low risk in the next to highest sextile. Although limited by imprecision, these data encourage further examination of women who drink water with THM levels in the range of 100 ppb and above, which is the federal standard. Preterm delivery was unrelated to THM concentration but low birth weight risk was reduced among women in the lowest tertile of exposure with no increase in risk above that exposure level. Total dose of THM (incorporating THM concentration and amount consumed) yielded little association with any of the outcomes.

The miscarriage results have few prior studies to which they can be compared, but appear not to support the previous observation of decreased risk among bottled water users (*9*). The absence of association with water source is consistent with the report of Aschengrau et al. (*8*), that risk was similar in Massachusetts communities served by chlorinated versus chloraminated supplies. To our knowledge, no previous study has explicitly evaluated THM concentration in relation to miscarriage.

Preterm delivery and low birth weight results may be compared to those from Iowa (*5*) and New Jersey (*6*). The absence of association with preterm delivery in our study is consistent with the lack of association found in Iowa (*5*), but is not notably discrepant with the small associations (ORs <1.5) found in New Jersey (*6*). The small increase in risk of low birth weight for the upper two tertiles in the present study is likewise compatible with small increases reported in each of the other two studies (*5,6*). We were not able to examine risk of SGA births for comparison to the strongest findings of Kramer et al. (*5*) due to our method of selecting cases. Term births who weighed >2500 g, a large proportion of all SGA deliveries, were not selected as cases in our study.

The limitations in our study should be noted. A sizable fraction of nonrespondents (due to refusal, being untraceable, or having key water information unavailable) raises the question of whether the participants differed from nonparticipants in a manner that would distort exposure–disease associations. The interview itself may generate erroneous reports, particularly of the amount of water consumed. We did not ask about preparation of cold beverages from tap water, such as frozen orange juice. Furthermore, we did not ask where the water was consumed (home, work, or elsewhere), or whether home filters were used. Thus, inferences about chlorination by-product exposure based on available data are subject to error.

The link to water suppliers is likely to be accurate, but the assignment of a particular THM score based on the nearest measurement day is certain to contain error relative to the true THM values over the etiologic period of interest. The THM sample is taken from an approximately appropriate point in time at locations other than the occupant's home. Furthermore, the pattern of home water use and ventilation could produce rather different exposures even for a given tap water THM concentration. Changes in water consumption during the course of pregnancy were not ascertained, requiring respondents to provide an average value for the entire pregnancy that may differ from the consumption in the etiologically relevant time period. Most of these sources of error are likely to be similar for cases and controls, yielding relative risk estimates that are biased toward the null value (*13*). Given the seasonal patterns in THM levels, matching controls on time of birth could have further biased the results towards the null value (*13*), but analyses adjusted for season (summer versus other) did not differ substantially from those reported.

On the other hand, along with Bove et al. (*6*), our study was among the first to try to link THM measurements in both time and space to study subjects. Availability of data on water ingestion and a wide array of potential confounders distinguishes this study from previous record-based investigations (*5,6*). Accuracy of identifying pregnancy outcomes is also certain to be improved using hospital data as opposed to birth certificate information. Finally, because of the extensive array of information we obtained through the interview, we were able to examine

Table 4. Low birth weight in relation to drinking water characteristics: Alamance, Durham, and Orange counties, North Carolina, 1988–1991

	Cases	Controls	Crude OR	Adjusted OR[a]	95% CI
Water source					
Private well[b]	63	114	1.0	1.0	—
Community	225	388	1.0	1.0	0.7–1.4
Bottled	13	43	0.5	0.8	0.4–1.6
Water amount (glasses per day)					
0	35	40	1.5	1.5	0.9–2.5
1–3[b]	150	261	1.0	1.0	—
4+	116	244	0.8	0.6	0.6–1.1
Water source × amount					
Private well/1–3[b]	32	50	1.0	1.0	—
Private well/4+	22	48	0.7	0.8	0.4–1.6
Community/1–3	12	189	0.9	0.8	0.4–1.3
Community/4+	86	173	0.8	0.8	0.5–1.4
Bottled/1+	13	43	0.5	0.6	0.3–1.6
THM concentration (ppb)					
40.8–63.3[b]	48	110	1.0	1.0	—
63.4–82.7	74	118	1.5	1.5	1.0–2.3
82.8–168.8	57	105	1.3	1.3	0.8–2.1
Per 50 ppb change	178	333	1.1	0.9	0.6–1.4
THM doses (ppb × glasses/day)					
44.0–169.9[b]	60	108	1.0	1.0	—
170.0–330.8	63	115	1.0	1.0	0.6–1.5
330.9–1171.0	55	110	0.9	0.8	0.5–1.3
Per 250 unit change	178	333	1.0	1.0	0.8–1.2

Abbreviations: OR, odds ratio; THM, trihalomethane.
[a]Adjusted as needed for potential confounders listed in text; if no confounders identified, the crude OR is presented.
[b]Referent category.

and control for confounding much more effectively than studies based on birth certificates.

The challenge in interpreting our results and the literature as a whole is that we would like to distinguish between the absence of association and the presence of a modest association. To do so with confidence requires large studies with refined exposure assessment. Subject to some uncertainty, literature suggests that there is not a strong association between THM exposure and adverse pregnancy outcome but provides some tentative suggestions that risk of miscarriage (based on the present study) and low birth weight or small-for-gestational-age births (based on previous studies) may be affected. More sophisticated approaches to exposure assessment through water quality models in the context of rigorous epidemiologic study designs can be expected to help reduce the uncertainty.

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Mortality Study of Workers in 1,3-Butadiene Production Units Identified from a Chemical Workers Cohort

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The International Agency for Research on Cancer has given the designations of "sufficient evidence" of carcinogenicity of 1,3-butadiene in experimental animals and "limited evidence" of carcinogenicity in humans. To investigate the carcinogenic effect in humans, we conducted a cohort mortality study among 364 men who were assigned to any of three 1,3-butadiene production units located within several chemical plants in the Kanawha Valley of West Virginia, including 277 men employed in a U.S. Rubber Reserve Plant which operated during World War II. The butadiene production units included in this study were selected from an index developed by the Union Carbide Corporation, which listed for each chemical production unit within their South Charleston, West Virginia and Institute, West Virginia, plants all products, by-products, and reactants. Departments included in the study were those where butadiene was a primary product and neither benzene nor ethylene oxide was present. A total of 185 deaths were observed; the standardized mortality ratio (SMR) for all causes of death was 91, reflecting lower mortality among the study population than the U.S. population. The study found a significantly elevated standardized mortality ratio (SMR) for lymphosarcoma and reticulosarcoma based on four observed cases (SMR = 577; 95% CI = 157–1480), which persisted in an analysis using county referent rates. An excess of lymphosarcoma and reticulosarcoma among all workers and among workers with routine exposure to 1,3-butadiene was also observed in the only other cohort of 1,3-butadiene production workers previously studied. A statistically nonsignificant excess of stomach cancer was observed in the overall cohort (n = 5; SMR = 243; 95% CI = 79–568) that was most pronounced among workers employed in the rubber reserve plant for 2 or more years (n = 5; SMR = 657; CI = 213–1530). We conclude that the results of this study add to the weight of evidence suggesting that butadiene is carcinogenic in humans. *Key words*: butadiene, cancer, lymphosarcoma, mortality, reticulosarcoma. *Environ Health Perspect* 103:598–603(1995)

1,3-Butadiene is used in the manufacture of synthetic rubbers (such as styrene-butadiene rubber or poly-butadiene rubber) and thermoplastic resins (*1*). Approximately 3000 million pounds of butadiene are produced in the United States each year (*2*). NIOSH estimates that approximately 9500 workers in the United States are occupationally exposed to 1,3-butadiene (*1*).

The International Agency for Research on Cancer (IARC) reviewed the literature on butadiene in 1992 and concluded that there is "limited evidence" of the carcinogenicity of butadiene in humans and "sufficient evidence" of the carcinogenicity of butadiene in experimental animals based on three long-term animal bioassays (*3*). Epidemiologic studies have been conducted among workers exposed to butadiene in the manufacture of styrene-butadiene rubber (*4–7*) and among workers involved in the production of butadiene monomer (*8–10*). Meinhardt et al. (*4,5*) examined mortality among 2756 white males employed in two styrene-butadiene rubber production facilities in Port Neches, Texas. Elevated, although not statistically significant, increased mortality was observed for lymphatic and hematopoietic neoplasms at one of the two plants [standardized mortality ratio (SMR) = 155], in particular lymphosarcoma and reticulosarcoma (SMR = 181) and leukemia (SMR = 203). These excesses were most pronounced among workers hired during World War II. Matanoski et al. (*6*) analyzed the mortality experience of 13,920 rubber production workers in the United States and Canada and found no significant increase in mortality from lymphatic or hematopoietic cancer, or any other cancer site. However, a nonsignificant excess risk of "other lymphatic cancer" (SMR = 202) was noted among production workers. An update of this study was reported by Matanoski et al. in 1990 (*7*). As in the earlier study, there was no significant increase in lymphatic or hematopoietic cancer, or any other cancer site. Production workers had a significant excess of "other lymphatic cancer" (SMR = 260). A significant excess of all lymphopoietic cancers was noted for blacks (SMR = 507). A nested case–control study of lymphopoietic cancer was conducted within this cohort. This study examined lymphopoietic cancer risk in relation to indices of cumulative butadiene and styrene exposure, and found a significant excess risk of leukemia associated with butadiene exposure (*11*).

Only one previous study has examined the mortality experience of butadiene production workers (*8–10*). This study included 2586 male workers employed at a facility located in Port Neches, Texas, for at least 6 months between 1943 and 1979. Butadiene was produced by the catalytic dehydrogenation of n-butane. The first analysis of the cohort by Downs et al. found eight deaths from lymphosarcoma and reticulosarcoma, yielding a significant SMR of 235 compared to national rates; the SMR was 185 and nonsignificant compared to county rates. In the update by Divine et al. (*9*), there was one additional lymphosarcoma and reticulosarcoma death yielding a significant SMR of 229 (CI = 104–435; county-based SMR's were not reported). In a third update (*10*), the SMR for lymphosarcoma and reticulosarcoma in the total cohort was not significant (n = 9; SMR = 209; 95% CI = 95–396). However, among the subset of 1056 workers with routine exposure to 1,3-butadiene, there were 6 deaths from lymphosarcoma and reticulosarcoma (SMR = 452; 95% CI = 165–984).

To investigate the carcinogenic effects of exposure to butadiene in humans, a cohort of workers employed in butadiene units was identified from within a large cohort (29,139 workers) of chemical workers whose mortality experience has previously been reported (*12*). Rinsky et al. analyzed the mortality experience of the overall cohort without regard to particular exposures (*12*). The primary hypothesis of the current study was that exposure to butadiene is associated with excess mortality from malignant neoplasms of the lymphatic and hematopoietic tissue. A secondary hypothesis was that butadiene would cause excess mortality from neoplasms of other sites.

Background

The study population was identified from records of 29,139 workers at three Union Carbide Corporation facilities in the Kanawha Valley, West Virginia: the South

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We thank David A. Dankovic for assistance in interpreting the toxicologic data and Bernice Vehr and the support group of the Industrywide Studies Branch at NIOSH for meticulously coding study information.

Received 16 December 1994; accepted 24 February 1995.

Charleston plant, the Institute plant, and the Technical Center. The first two of these facilities produced butadiene and are included in this study. The South Charleston plant began operations in 1925, around the process of stripping ethylene from natural gas. It evolved into a chemical and plastics facility, producing a wide variety of chemical substances, including butadiene, ethylene oxide, polyethylene, vinyl chloride resins, and polyols. The Institute plant was originally built by the U.S. government (U.S. Rubber Reserve Corporation) to produce styrene-butadiene rubber to replace the supply of natural rubber that was cut off during World War II. This facility was bought by Union Carbide in 1947. The plant was then used as a larger production facility for materials developed at the South Charleston plant, such as acetone, isopropanol, butanol, and acetaldehyde. As of 1986, when the Institute plant was sold to Rhone-Poulenc, it had expanded to include the manufacture of agricultural chemicals and a wide range of ethylene oxide and propylene oxide-based products.

Butadiene production units were identified from a chemical and department index developed by Union Carbide. The index listed all products, by-products, and reactants for each chemical production unit within the South Charleston and Institute plants. Departments were identified where butadiene was a primary product and neither benzene nor ethylene oxide was present.

South Charleston (1941–1965). The butadiene production process at South Charleston involved the recovery of butadiene monomer from olefin cracking (see appendix for process description). Among the chemicals used in the process was bis(2-chloroethyl)ether, which also has been evaluated by IARC for carcinogenicity. The IARC regards bis(2-chloroethyl)ether as having limited evidence of carcinogenicity in animals (*13,14*), based on a bioassay in which an excess of hepatomas was observed in mice (*15*). This chemical showed negative results in a bioassay measuring pulmonary tumor response in mice (*16*) and in a long-term bioassay in rats (*17*).

Rubber Reserve Unit, Institute plant (1943–1946). The U.S. Rubber Reserve Corporation butadiene production unit operated from 1943 to 1946 at the Institute plant. The unit produced butadiene monomer indirectly from ethanol (see appendix for process description). As in the South Charleston unit, bis(2-chloroethyl)ether was used in this process. In addition, large quantities of acetaldehyde were present. Acetaldehyde is considered by IARC to have "sufficient evidence" of carcinogenicity in animals (*18*) based on its ability to induce tumors of the larynx and nasal epithelium in hamsters (*19*) and rats (*20*).

Institute plant (1959–1971). The Institute plant also produced butadiene monomer as a by-product of olefin cracking from 1959 to 1971. The process was the same as described for the butadiene production units at the South Charleston plant; however, in 1965 dimethyl acetamide was substituted for bis(2-chloroethyl)ether.

Methods

The study population was identified by searching a computer file of work history records of 29,139 workers included in a mortality study of males employed from 1940 to 1979 at any of the Union Carbide chemical plants operating in the Kanawha Valley (*12*). The computer file of work history records contained a code representing each department in which an individual worked, but not the starting or ending dates. A total of 527 individuals were identified as having ever worked in the department codes relating to the butadiene units in the South Charleston or Institute plants identified for study. Copies of personnel records were collected for these individuals, and the starting and ending dates of their employment in each department were coded. Only 364 individuals who worked in the departments during the years when butadiene was produced were retained in the study (n = 364). As in the overall Kanawha Valley chemical workers cohort, there was a high proportion of individuals whose race was unknown (28%). Among those whose race was known, 94% were white. Therefore, individuals whose race was unknown were assumed to be white.

The Rinsky et al. (*12*) Kanawha Valley study determined vital status through 31 December 1978. For individuals not known to be deceased as of that date, vital status through 31 December 1990 was determined by matching with records of the National Death Index (NDI). Individuals known to be alive as of 31 December 1978 who were not identified as deceased from National Death Index records 1979–1990 were assumed alive as of 31 December 1990. For workers who were deceased, death certificates were obtained from state vital statistics offices and were coded according to the International Classification of Diseases (ICD) revision in effect at the time of death. The mortality experience of the cohort was compared to United States and to Kanawha County mortality rates using a modified life-table analysis system (LTAS) developed by NIOSH (*21,22*). The county rate analysis was restricted to the time period 1960 through 1990 for which county referent rates are available in the NIOSH lifetable. Standardized mortality ratios, 95% confidence intervals, and two-sided p-values were calculated. Confidence intervals and p-values were calculated using an exact method (if either the observed or expected was less than 6) or an approximate method (if observed or expected frequencies were 6 or more). Because of the small size of the cohort and therefore the small numbers of deaths, latency and duration analyses were performed by simply dichotomizing both latency and duration categories so that approximately equal numbers of expected deaths were below and above the cutpoints.

For specific cancer site categories in which a statistically significant elevated SMR was observed, concomitant chemical exposures of the deceased workers were identified. This was accomplished by listing all departments other than butadiene in which their personnel records indicated they worked and then identifying the chemicals used and produced in each department from the index assembled by Union Carbide.

Results

Among the 364 persons who were identified as working in any of the butadiene production units, 277 individuals worked in the Rubber Reserve Unit which produced butadiene from ethanol, and 87 worked in the units at the South Charleston and Institute plants which produced butadiene from olefin cracking. Among these 364 persons, 176 (48.3%) were alive, 185(50.8%) were deceased, and 3 (0.8%) had unknown vital status as of the study end date of 31 December 1990. Table 1 shows the mortality pattern of the total cohort through 1 January 1990 based on U.S. referent rates. As in the previous study of the mortality experience of 29,139 workers in the three Kanawha Valley plants through 1978 (*3*), the SMR for deaths from all causes in the butadiene production cohort was <1.00. The SMR for deaths from all malignant neoplasms was 1.05 (CI = 0.78–1.40), which was higher than the SMR for deaths from all malignant neoplasms in the larger chemical workers cohort (SMR = 0.93; CI = 0.88–0.99) (*12*). Among the 92 specific causes of death and 26 major categories examined in the NIOSH lifetable, there was only one significantly elevated SMR, which was for the category "lymphosarcoma and reticulosarcoma" (n = 4; SMR = 5.77; CI = 1.57–14.8). County-based analyses, which covered only the time period 1960–1990, resulted in a similar SMR (SMR = 5.78; CI = 1.57–14.8).

Table 2 provides the SMRs for lymphosarcoma and reticulosarcoma by dura-

Table 1. Mortality (through 31 December 1990) from specific causes for butadiene production workers

Cause	Observed	Expected	SMR	95% CI
Tuberculosis	0	1.72	—	
Malignant neoplasms				
Buccal and pharynx	1	1.29	0.77	0.02–4.29
Digestive organs	11	12.2	0.90	0.45–1.61
Stomach	5	2.06	2.41	0.79–5.68
Respiratory system	19	16.6	1.14	0.69–1.79
Trachea, bronchus, and lung	19	15.8	1.20	0.72–1.88
Male genital organs	3	3.70	0.81	0.17–2.37
Urinary organs	1	2.32	0.43	0.01–2.39
Lymphatic and hematopoietic	7	3.99	1.75	0.70–3.61
Lymphosarcoma and reticulosarcoma	4	0.69	5.77	1.57–14.8
Hodgkin's disease	0	0.34	—	
Leukemia and aleukemia	2	1.62	1.23	0.15–4.44
Other lymphatic or hematopoietic	1	1.33	0.75	0.02–4.17
Other sites	6	5.36	1.12	0.41–2.44
Neoplasms of benign and unspecified nature	0	0.62	—	
Diabetes melitis	2	2.90	0.69	0.08–2.49
Blood and blood-forming diseases	0	0.54	—	
Alcoholism and mental disorders	0	1.21	—	
Nervous system diseases	1	2.14	0.47	0.01–2.59
Diseases of the heart	75	82.2	0.91	0.72–1.14
Diseases of the circulatory system	21	18.2	1.15	0.71–1.76
Respiratory system diseases	7	13.9	0.50	0.20–1.03
Digestive system diseases	5	9.36	0.53	0.17–1.25
Diseases of genitourinary system	2	2.96	0.68	0.08–2.44
Diseases of the skin and subcutaneous tissue	0	0.17	—	
Musculoskeletal diseases	0	0.35	—	
Symptoms and ill-defined conditions	2	2.48	0.81	0.09–2.91
Accidents	10	10.3	0.97	0.46–1.78
Suicide and homicide	3	4.62	0.65	0.13–1.90
All other causes	3	2.88	1.04	0.21–3.04
Certificates not obtained	6			
All cancers	48	45.5	1.05	0.78–1.40
All causes	185	202.2	0.91	0.79–1.06

SMR, standarized mortality ratio.

Table 2. Standardized mortality ratios (SMRs) for lymphosarcoma and reticulosarcoma by duration of employment and time since first employment in butadiene production processes

	Duration of employment[b]					
	< 2 years		≥ 2 years		Total	
Latency[a]	Observed	SMR	Observed	SMR	Observed	SMR
< 30 years	1	4.92	0	—	1	2.41
≥ 30 years	0	—	3	19.8**	3	10.8**
Total	1	3.03	3	8.27*	4	5.77*

[a]Latency categories were selected to divide expected deaths from all causes into two approximately equal categories. There were 95 expected deaths in the < 30 years latency category and 107 in the ≥ 30 years latency category.
[b]Duration of employment categories were selected to divide expected deaths from all causes into two approximately equal categories. There were 100 expected deaths in the < 2 years duration category and 102 in the ≥ 2 years duration category.
*$p < 0.05$; **$p < 0.01$.

tion of employment in butadiene production processes and latency (defined as time since first employment in butadiene production processes). Three of the four deaths from lymphosarcoma and reticulosarcoma occurred in the >2 years' duration and >30 years' latency categories (SMR = 19.8; CI = 4.08–57.8). Table 3 provides additional information about the work histories of the four individuals, three of whom worked in the Rubber Reserve unit at the Institute plant. Aside from their assignments to butadiene production units, there were no commonalities among the four cases except that two had been assigned to an acetaldehyde unit, one for 8 years and one for 29 years.

There was a statistically nonsignificant excess of stomach cancer in the overall cohort ($n = 5$; SMR = 2.43; CI = 0.79–5.68) that was most pronounced among workers employed in the Rubber Reserve plant for over 2 years ($n = 5$; SMR = 6.57; CI = 2.13–15.3; Table 4). County-based analyses for the overall cohort showed identical SMRs of 2.93 for the county and U.S. referent rates 1960–1990. Table 5 provides information about the work histories of the five individuals who died of stomach cancer. Aside from their assignments to the butadiene unit, the only commonality among the work histories of the cases was that two had been assigned to "maintenance of grounds."

Discussion

The major finding of this study is excess mortality from lymphosarcoma and reticulosarcoma among workers employed in butadiene production processes located within two large chemical plants. An excess of lymphosarcoma and reticulosarcoma (SMR = 239) was observed in the only other butadiene production cohort previously studied (*8–10*). The latter plant used a different process (the catalytic and oxidative dehydrogenation of *n*-butane) from either of the two processes used by Union Carbide. A nonsignificant excess in lymphosarcoma and reticulosarcoma deaths was also found at one of two plants producing styrene-butadiene rubber (*4*). Elevated lymphoma incidence has also been observed in mouse bioassays (*23–26*).

A prior mortality study of workers at the Union Carbide's Kanawha Valley plants found a significant excess of lymphosarcoma and reticulosarcoma (SMR = 1.40; CI = 104–187) (*12*). An excess of deaths from this cause in the county where the plant is located (Kanawha County, West Virginia) has been noted previously (*27*). The county rate analyses in the current paper show that the expected number of deaths for lymphosarcoma and reticulosarcoma are approximately 15% higher in Kanawha County than in the U.S. population. Thus, geographical variation does not explain a substantial proportion of the increased risk among workers in butadiene units. A previous study which evaluated occupational risk factors for lymphopoietic cancers within Union Carbide's Kanawha Valley plants did not specifically evaluate the risks for lymphosarcoma and reticulosarcoma, but instead included these tumors in the broader grouping, "non-Hodgkin's lymphoma" (*28*). That study did not find an association between butadiene exposure and non-Hodgkin's lymphoma. Those results cannot be directly compared to findings of the current study because the periods of case ascertainment, disease groupings, and classification of butadiene exposure were different.

Our study also found an excess of stomach cancer among workers employed in the Rubber Reserve Unit for over 2 years. Review of the work histories of all the individuals who died of stomach cancer did not reveal any likely confounding exposures. Stomach cancer was in deficit in the overall Kanawha Valley chemical worker cohort followed through 1978 (SMR = 79; CI =

Table 3. Work histories of individuals who died of lymphosarcoma and reticulosarcoma

Age at death (years)	Beginning year of employment in butadiene unit	Approximate length of employment in butadiene (months)	Time from initial exposure to death (years)	Year of death	Butadiene department worked in	Other departments/ exposures
65	1942	39	33	1975	Rubber reserve	Acetaldehyde unit,[a] outside work
64	1943	35	36	1979	Rubber reserve	Maintenance of grounds, acetaldehyde unit,[a] upper island chemicals[b]
52	1946	9	25	1971	Rubber reserve	Isopropanol-acetone,[c] laborer, maintenance, general stores and purchasing
63	1952	96	32	1984	Olefin unit, S. Charleston	Maintenance labor, gas plants/ olefins,[d] outside work, acetylene unit,[e] weighmaster, chemicals and resins—packaging and shipping

[a]Acetaldehyde unit: A variety of chemicals, many requiring acetaldehyde as a reaction material, were produced in this department. These included vinyl isobutyl ether, vinyl ethyl ether, vinyl butyl ether, vinyl methyl ether, hexaldehyde, 2-ethyl butyraldehyde, propionaldehyde, 2-methyl pentaldehyde, methyl isoamyl ketone, 2,3-dimethyl pentaldehyde, ethyl propenal ether, ethyl acetate, and 2-methyl pentaldehyde. Refined acetaldehyde was also produced.
[b]Upper island chemicals area units: A large variety of chemicals, including ethers, ketones, alcohols, and acids were produced in these units.
[c]Isopropanol-acetone department: Materials handled in this department included propylene, diisopropanol sulfate, fuel gas, water, sulfuric acid, and "merrill oil." Products included isopropanol, diisopropyl ether, isopropyl oils, and weak sulfuric acid.
[d]Gas plants/olefins: Materials handled in this department included butane, propane, acetone, gasoline, butadiene vent gas, methanol, blowbacks from ethylene absorbers at "MB," polyethylene, chlorohydrin, propylene absorbers from isopropanol, caustic 20%, anti-oxidant, "DuPont No. 5," wood chips, and alumina pellets. Products included ethylene, propylene, and acetylene. By-products included crude butadiene, propane, "pyrofax," benzene, hydrogen-methane, sulfur saturated wood chips, hydrogen, and residues.
[e]Acetylene unit: Process materials included calcium carbide, sodium hydroxide, and sulfuric acid. Products included acetylene and calcium hydroxide.

59–104) (*12*). Prior epidemiologic studies of butadiene-exposed workers have reported a decreased SMR for digestive cancers overall (*4*), a decreased SMR for stomach cancer (*8–10*) and a slightly elevated SMR (1.05) for stomach cancer which was higher among black workers (SMR = 1.45) and maintenance workers (SMR = 1.51) (*7*). Carcinomas of the forestomach have been found to be elevated in two mouse bioassays of 1,3-butadiene (*23–26*).

The current study has several limitations. One limitation is that cancer mortality, rather than incidence, was considered, and thus any increased risk at cancer sites with high survival rates might not be detected. This limitation could not be readily overcome because there is no population-based cancer registry in the Kanawha Valley area. It is not known whether there are living individuals in the cohort who have been diagnosed with lymphosarcoma or reticulosarcoma. An important limitation is the potential for confounding exposure both within the butadiene production units and outside the units. We attempted to address the issue of confounding in the design of the study by selecting *a priori* only departments where butadiene was a primary product and benzene and ethylene oxide were not present. We also identified potential confounding exposures outside the butadiene units by examining the work histories of cases to determine whether there were common exposures. Among the potential confounding exposures which could not be controlled for in the study design, acetaldehyde was of the greatest concern both because it was present in the rubber reserve process and because two of the four individuals who died of lymphosarcoma and reticulosarcoma worked in the acetaldehyde unit. In addition, a case–control study of risk factors for lymphopoietic cancer within Union Carbide's Kanawha Valley chemical plant (*28*) found an elevated odds ratio for non-Hodgkin's lymphoma associated with exposure to acetaldehyde, but noted that odds ratios and duration trends were similar for acetaldehyde and acrylonitrile because of concomitant use of the two chemicals.

Table 4. Standardized mortality ratios (SMRs) for malignant neoplasms of the stomach by duration of employment and time since first employment in the rubber reserve plant

	Duration of employment[b]					
	< 2 years		≥ 2 years		Total	
Latency[a]	Observed	SMR	Observed	SMR	Observed	SMR
< 30 years	0	—	2	5.63	2	2.25
≥ 30 years	0	—	3	7.40	3	3.69
Total	0	—	5	6.57*	5	2.94*

[a]Latency categories were selected to divide expected deaths from all causes into two approximately equal categories. There were 73 expected deaths in the < 30 years latency category and 91 in the ≥ 30 years latency category.
[b]Duration of employment categories were selected to divide expected deaths from all causes into two approximately equal categories. There were 87 expected deaths in the < 2 years duration category and 77 in the ≥ 2 years duration category.
*$p < 0.01$.

To examine further whether the risk of lymphosarcoma and reticulosarcoma might be attributable to an elevated risk associated with the acetaldehyde unit, to which two of the four cases had been assigned, we identified 233 workers from the large cohort of 29,139 who had ever been assigned to this unit and followed their mortality through 1991. A total of 48 deaths were identified. Aside from the two deaths from lymphosarcoma and reticulosarcoma previously identified among workers who had been included in the butadiene production study, there were no other deaths from this cause.

The toxicologic data are consistent with the conclusion that the excess of lymphosarcoma and reticulosarcoma found in the study are likely to be related to butadiene rather than confounding exposure to acetaldehyde. Acetaldehyde has only been demonstrated to induce upper respiratory tumors in rodents at levels (1000 ppm and above) substantially above the levels at which acetaldehyde has been demonstrated to cause eye irritation in humans (29). In contrast, butadiene has been shown to cause an increase in incidence of lymphocytic lymphomas and histiocytic sarcomas in mice (formerly known as type A reticulum cell sarcomas) at concentrations as low as 200 ppm, with marginally signifi-

Table 5. Work histories of individuals who died of stomach cancer

Age at death (years)	Beginning year of employment in butadiene unit	Approximate length of employment in butadiene (months)	Time from initial exposure to death (years)	Year of death	Butadiene department worked in	Other departments/exposures
75	1943	37	21	1964	Rubber reserve	None
67	1944	30	18	1962	Rubber reserve	None
65	1943	42	33	1976	Rubber reserve	Maintenance of grounds, polyethylene department[a]
65	1943	54	33	1976	Rubber reserve and S. Charleston	Gas plants/olefins[b]
74	1943	29	32	1975	Rubber Reserve	Maintenance of grounds, janitors and watchmen, general maintenance, building services, and guards

[a]"Dowtherm," "Ucon," "Heat transfer fluid 500," butyl hydroxy toluene, "Super floss" and "Vazo." Products were various grades of homopolymers and copolymers, recovered vinyl acetate, unreacted ethylenes, and oils.
[b]Gas plants/olefins: Materials handled in this department included butane, propane, acetone, gasoline, butadiene vent gas, methanol, blowbacks from ethylene absorbers at "MB," polyethylene, chlorohydrin, propylene absorbers from isopropanol, caustic 20%, anti-oxidant, "DuPont No. 5," wood chips, and alumina pellets. Products included ethylene, propylene, and acetylene. By-products included crude butadiene, propane, "pyrofax," benzene, hydrogen methane, sulfur saturated wood chips, hydrogen, and residues.

cant increases in histiocytic sarcomas down to 20 ppm (*24,25*).

When studying workers employed at large chemical production complexes, it is impossible to rule out the potential importance of confounding exposures. Ott et al. (*28*) noted that the average production worker at these plants was exposed to 58 different chemicals, many of which were correlated. Other studies conducted in the same chemical worker population have identified associations of lymphosarcoma and reticulosarcoma with maintenance (Teta J, unpublished data) and strong acid ethanol production (*30*), and the broader category non-Hodgkin's lymphoma with units using acetaldehyde (*28*). These observations were made in efforts to investigate the observed excess of death for the original cohort study of 29,139 workers or in surveillance studies. The current study, on the other hand, was initiated in 1986 to examine the hypothesis that exposure to butadiene might be related to hematopoietic cancer. If exposure data were available, the association between mortality from lymphosarcoma and reticulosarcoma might be evaluated by determining whether there is evidence for an exposure–response relationship. However, there are no air monitoring data for any of the butadiene production processes, and thus air concentrations of butadiene (and other chemicals) cannot be estimated. Personnel records do not indicate the types of tasks or job assignments an individual worker had; thus, there is no way to know whether the four individuals who developed lymphosarcoma or reticulosarcoma were exposed to higher concentrations of butadiene than other members of the cohort. Finally, the U.S. Rubber Reserve plant operated for only 4 years, and thus we could not evaluate the effects of prolonged exposure to this process.

Despite the limitations outlined above, the study has demonstrated an excess of mortality from lymphosarcoma and reticulosarcoma, which is consistent with the only other butadiene production cohort previously studied. Finding an excess of neoplasms of the lymphatic system in relation to butadiene exposure is consistent with the mouse bioassay data as well (*18,19*). We conclude that the results of this study add to the weight of the evidence suggesting that butadiene is carcinogenic in humans.

Appendix. Process Description

Rubber Reserve Process: Production of Butadiene from Ethanol (Institute Plant, Early 1940s)

The rubber reserve plant was operated by the Union Carbide Corporation during World War II under contract with the U.S. government. The rubber reserve process produced butadiene indirectly from ethanol. To better understand the process, the plant may be considered as being composed of four major divisions: 1) an acetaldehyde conversion system, 2) a butadiene conversion system, 3) a butadiene purification system, and 4) a recovery distillation system.

In the acetaldehyde conversion system, the ethanol was introduced into a catalytic converter containing a copper-chromium catalyst and was partially converted to acetaldehyde. The converters were heated by circulating liquid Dowtherm. The reaction involved was

$$\text{Ethanol} \xrightarrow[\text{Cu - Cr}]{} \text{Acetaldehyde} + \text{Hydrogen}$$

In this step, side reactions occurred that resulted in the formation of acetic acid, ethyl acetate, butylaldehyde, and butanol.

The equipment in the butadiene converter system is similar to that used in the acetaldehyde conversion system. The process was as follows:

$$\text{Ethanol} + \text{Acetaldehyde} \xrightarrow[\text{Titanium Oxide}]{} \text{Butadiene} + \text{Water}$$

In passing through the titanium oxide, the combined ethanol and acetaldehyde are 15–20% converted to butadiene at an efficiency of 60–65%. Conversion of this mixture resulted in the formation of a large number of by-products. Among these were ethylene, ethane, propylene, propane, butylene, butane, carbon dioxide, carbon monoxide, diethyl ether, butyraldehyde, ethyl acetate, methyl ethyl ketone, carbon, acetic acid, butanol, and other unidentified hydrocarbons.

The major process steps in the purification phase were as follows: 1) stripping the crude butadiene from the condensed liquids of the butadiene converters, 2) removal of acetaldehyde from the butadiene, and 3) removal of butane and butylene from the butadiene.

The crude butadiene was removed from the condensate collected from the catalytic converter by distillation in a heated column. The crude butadiene at this point is a mixture of butenes, butane, and the binary azeotrope of butadiene and acetaldehyde. The crude mixture, including the azeotrope with acetaldehyde, was passed through a water scrubber to absorb the acetaldehyde present in the vapor. The acetaldehyde-free vapor from the scrubber was then fed to another column and the butadiene was absorbed by Chlorex (di-2-chloroethyl ether). The Chlorex preferentially absorbed the butadiene and allowed the butylenes and butane to pass out of the column. The remaining vapors consisted largely of butadiene, water, and some Chlorex. Chlorex, when heated, breaks down and forms dilute hydrochloric acid. Caustic soda was used in the solvent system to neutralize the hydrochloric acid. The final step in the purification process required the water and the Chlorex to be condensed and separated. The resulting product is butadiene at a purity of 99.5%.

The function of the recovery distillation system was to reconcentrate the unre-

acted acetaldehyde and ethanol from the conversion system, remove the by-products from the material cycle and to feed the converters.

Union Carbide Process: Recovery of Butadiene from Olefin Cracking (Institute Plant, 1959–1971 and South Charleston Plant, 1941–1965)

The Union Carbide olefin unit used a high-temperature cracking process to produce ethylene from hydrocarbons. The process was developed to recover butadiene as a by-product of the normal ethylene process. The relatively pure mixture of the four carbon molecules (less than 50% butadiene) was supplied from three sources in 10,000-gallon capacity feed tanks.

Crude feed from the feed tanks was fed to an absorber column, and Chlorex was the solvent used to desorb the butadiene. The Chlorex, after absorption of the majority of the butadiene, was routed to a stripping column. The crude butadiene at this phase of the process was 88–90% pure. The material was then scrubbed with water to remove any aldehydes which might have been present. The condensed, partially refined butadiene was then compressed and piped to the 10,000-gallon capacity intermediate storage tanks prior to recracking. To control the formation of popcorn-type polymers, sodium nitrite was added to the process stream to remove the oxygen.

Final refining of butadiene was a distillation process carried out in a two-column system. The 88–90% butadiene stream was introduced to a fore column where vinyl acetylene was removed from the process stream. The butadiene was now 97% pure and final purity of 99.5% was obtained from the refined butadiene condenser and then pumped to the refined storage tanks. Before loading or shipping the final product, Catechol (*p*-tertiary butyl catechol) was injected into the product to inhibit polymerization.

Subsequent process changes, 1965 and later, used dimethyl acetamide as the absorbent solvent in place of Chlorex.

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Imported Seabass as a Source of Mercury Exposure: A Wisconsin Case Study

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The Wisconsin Division of Health investigated mercury exposure in a 40-year-old man, his 42-year-old wife, and their 2.5-year-old son. At the time of our investigation, these individuals had blood mercury levels ranging from 37 to 58 µg/L (normal <5 µg/L) and hair samples from the adults contained 10–12 µg mercury/g dry weight. A personal interview and home inspection failed to identify any occupational or household sources of mercury exposure. The family's diet included three to four fish meals per week. The fish was purchased from a local market and included Lake Superior whitefish, Lake Superior trout, farm-raised trout and salmon, and imported seabass. Analysis of these fish found that only one species, the imported seabass, contained significant mercury levels. Two samples of the seabass obtained from the vendor on different days contained mercury concentrations of 0.5 and 0.7 mg/kg. Based on consumption estimates, the average daily mercury intakes for these individuals ranged from 0.5 to 0.8 µg/kg body weight. Six months after the family stopped consuming the seabass, blood mercury levels in this man and woman were 5 and 3 µg/L, respectively. Analysis of sequential blood samples confirmed that mercury elimination followed first-order kinetics with a half-life of approximately 60 days. *Key words*: dietary exposures, half-life, mercury, seabass. *Environ Health Perspect* 103:604–606 (1995)

The hazards posed by methylmercury-contaminated seafood were first recognized in 1955 when a poisoning outbreak occurred in Minamata, Japan. In that incident, severe brain damage was described in 22 infants whose mothers had ingested contaminated fish during pregnancy (*1*). Similar prenatal effects were observed in the aftermath of two poisoning outbreaks that occurred in Iraq (*2*), confirming the sensitivity of the developing central nervous system to the toxic effects of organic mercury. The symptoms of methylmercury poisoning in adults include paresthesia; impaired peripheral vision, hearing, taste, and smell; slurred speech; unsteadiness of gait and limbs movements; muscle weakness; irritability; memory loss; depression; and sleep disturbances (*3*). Prenatal and infantile exposures can cause permanent brain damage resulting in mental retardation, blindness, inability to walk, retention of primitive reflexes, and lack of coordination (*1*).

Current regulations administered by the U.S. Food and Drug Administration limit the methylmercury content in commercially marketed fish to 1.0 mg/kg (1.0 ppm) (*4*). This guideline is based on an acceptable daily intake (ADI) for methylmercury (as mercury) of 30 µg and assumes a maximum fish ingestion rate of 30 g/day—the equivalent of one 7–8 oz serving per week—by a 70-kg adult. Higher ingestion rates, lower body weights, higher mercury levels in the fish, or a combination of these factors would result in exceeding the acceptable daily intake for methylmercury and could pose a health risk, especially in cases involving prenatal exposure.

During 1994, the Wisconsin Bureau of Public Health investigated mercury exposure in a 40-year-old man, his 42-year-old wife, and their 2.5-year-old son. These individuals were found to have elevated blood and hair mercury levels after they consumed three to four fish meals per week over a period of 8 to 9 months. Our investigation included a home inspection, personal interviews, assessment of dietary and occupational exposures to mercury, blood and hair analyses, and analysis of mercury levels in five species of fish that were regularly consumed by this family.

Methods

The personal, dietary, and residential information included in this report were obtained by an interview and home inspection. Analysis of mercury levels in blood and unskinned fish fillets was performed by the Wisconsin State Laboratory of Hygiene according to an adaptation of the cold vapor atomic absorption method developed by Chang et al. (*5,6*). The laboratory's limit of quantitation was 5 µg/L for blood analyses and 0.02 mg/kg for fish tissue. A 2.5-inch-long scalp segment of the woman's hair was analyzed by the Wisconsin Occupational Health Laboratory using nitric acid digestion followed by a flow-injection atomic absorption method adapted from NIOSH S199 and S342 (*7*). A scalp segment of the man's hair was analyzed by a private laboratory according to a similar protocol (method reference unavailable).

Case Findings

During March 1994, the Wisconsin Bureau of Public Health was contacted by a 40-year-old man who expressed concern about his family's exposure to mercury. He stated that a sample of his hair had been analyzed for toxic metals and found to have a high mercury content. He also said that he was experiencing sleep disturbances and had difficulty concentrating, and asked whether these symptoms might be due to mercury exposure. The caller was especially concerned about his 2.5-year-old son's exposure to mercury.

When asked about possible mercury exposure, the caller stated that his family regularly consumed three to four fish meals per week and asked whether the fish might contain unsafe mercury levels. He also wondered whether he should consider having his mercury-amalgam dental fillings removed. One week earlier, each member of this family had been seen by their physician. At that time, blood samples were collected for mercury analysis. It was recommended that the caller delay replacement of his dental amalgams until the results of the blood tests were available and an assessment of the family's mercury exposure was completed. A summary of the medical test results and personal information is shown in Table 1.

Other than the sleep and concentration difficulties reported by the caller, none of the family members exhibited clinical symptoms of mercury toxicity. Venous blood samples contained mercury levels that ranged from 37 to 58 µg/L (normal, <5 µg/L), confirming recent exposure to this metal. Based on these results, the family was advised to stop eating fish and other seafood products until the source of their mercury exposure could be determined.

A personal interview and home inspection failed to identify any significant occupational or household sources of mercury exposure. Both adults were attorneys and had offices in separate buildings. Their single-family home was located in a residential neighborhood of a medium-sized city. The home was constructed in 1976, was in good condition, and had not been repainted or remodeled since the family purchased it 3 years earlier. Neither adult could recall using elemental mercury or mercury-containing products in the home.

A 25-year-old woman who was employed by the family as a daycare provider spent approximately 40 hours per week in their home. She consumed a vegetarian diet which did not include fish or other seafood. A venous blood sample collected from her at the time of our investigation contained no detectable mercury (<5 µg/L).

All of the fish consumed by this family

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Received 17 January 1995; accepted 10 March 1995.

Table 1. Medical test results and personal data

	Man	Woman	Son
Age	40	42	2.5
Body weight kg (lbs)	57 (126)	52 (115)	13 (30)
Fish meals/week	3-4	3-4	3-4
Fish/meal (g)	227	150	75
Hair mercury (μg/g)	12	10	NA
Blood mercury (μg/L)			
Day 0	58	37	37
Day 15	45	24	NA
Day 70	24	14	NA
Day 200	5	3	NA
Hair Hg/blood Hg ratio	207	270	NA

NA, not available.

Table 2. Mercury content of fish

Type of fish	Mercury content (μg/g)
Lake Superior whitefish	< 0.02
Lake Superior trout	< 0.02
Farm-raised salmon	0.05
Farm-raised trout	0.05
Seabass	
Filet 1	0.5
Filet 2	0.7

was purchased from a local seafood market. The most frequently eaten fish were imported seabass (two meals/week), Lake Superior whitefish (one to two meals/month), Lake Superior trout (one to two meals/month), farm-raised trout (one to two meals/month) and farm-raised salmon (one to two meals/month). At each fish meal, about 454 g (1 lb) of fish was divided among the family members with the man, woman, and son consuming about 227, 150, and 75 g, respectively. One sample of each type of fish was obtained from the seafood market and tested for mercury content. In addition, a filet of the imported seabass was submitted by the family for analysis. Mercury levels in these fish samples are shown in Table 2.

Correlation of Mercury Intake with Blood Mercury Levels

According to studies conducted by Clarkson (*8*), the steady-state blood mercury level of a 70-kg adult expressed in micrograms per liter is approximately equal to the daily methylmercury intake expressed in micrograms mercury per day. This estimate is consistent with our case findings since the man in our study had an estimated mercury intake of 45 μg/day and a steady-state blood mercury level of 58 μg/L. The woman in our study had an estimated intake of 30 μg/day and a steady-state blood mercury level of 37 μg/L.

To further test the relationship of the mercury content of the seabass and blood mercury levels in these individuals, the mathematical model developed by Kershaw et al. (*9*) and the fish consumption history provided by this family were used to estimate mercury levels in the seabass. These estimated values were compared to the actual mercury levels detected in the filets. Kershaw's study of the deposition and clearance of methylmercury identified a biological half-life of 52 days and determined that the ratio of hair and blood mercury levels ranged from 265 to 280. It also defined the relationship between the steady-state concentration of methylmercury in blood (B, μg/L) and the daily mercury intake (d, μg) as:

$$B = d(f/\text{blood volume})(t_{1/2}/\ln 2)$$

where f is the fraction of daily intake deposited in the tissue compartment (5.9%), blood volume is 7% of body weight for adults (8% for children), $t_{1/2}$ is the average biological half-life (52 days), and ln2 is the natural logarithm of 2 (0.693). By applying this formula to the fish consumption and clinical test data for this family, the concentration of mercury in the seabass was estimated to be between 0.4 and 0.8 mg/kg (Table 3), which was similar to the levels detected in two samples of this fish (0.5 and 0.7 mg/kg) providing additional evidence to support the conclusion that the seabass was this family's principal source of mercury exposure.

Table 3. Estimated mercury content of seabass

	Blood mercury (μg/L)	Calculated mercury intake[a] (μg) Daily	Calculated mercury intake[a] (μg) Weekly	Seabass intake (g) Weekly	Estimated mercury content in seabass (μg/g)
Father	58	52	367	454	0.8
Mother	37	31	214	300	0.7
Child	37	9	63.7	150	0.4

[a]Calculated from blood mercury level as follows: 58 μg/L = daily mercury intake(0.059/4.0 L)(51.9 days/0.693); mercury intake = 52.4 μg/day or 367 μg/week.

Biological Half-Life Calculation

The kinetics of mercury excretion and the elimination rate constant k were obtained by plotting the natural logarithm of the ratio of blood mercury levels at days 0, 15, 70, and 200 and steady-state blood mercury concentrations $\{\ln[Hg]/[Hg_0]\}$ versus the time in days (Fig. 1) (*10*). The data points in this figure fall on a straight line indicating that excretion followed first-order kinetics. Elimination rate constants of $1.1–1.2 \times 10^{-2}$ per day were derived for the adults. Using the standard formula $t_{1/2} = \ln 2/k$ (*11*) these rate constants yielded biological half-lives of 58 and 63 days. These half-lives fall within the range of 39 to 67 days that was described by Kershaw et al. (*9*).

Discussion

The family described in this case study was found to have elevated blood and hair mercury levels after they had consumed imported seabass approximately twice a week over a period of several months. The seabass was purchased from a local seafood market and according to the vendor originated in the South American nation of Chile. Two samples of this fish contained mercury levels of 0.5 and 0.7 mg/kg. Most of the mercury is presumed to have been methylated although the analytical methods used in our study did not speciate the mercury. Steady-state blood mercury levels in the family members ranged from 37 to 58 μg/L. Normal blood mercury values range from below detection to 5 μg/L, and levels as low as 10 to 20 μg/L have been associated with memory disturbances, tremors, and impaired hand–eye coordination (*12*).

Current food safety guidelines developed by the FDA allow seafood to contain up to 1 mg/kg mercury as methylmercury (*4*). This guideline was based on a review of data from poisoning incidents that occurred in Minimata and Niigata, Japan, in which no symptoms of toxicity were observed in adults whose blood mercury levels were below 200 μg/L, and also based on a Swedish study by Skerfving (*13*). The Skerfving study associated a steady daily intake of approximately 300 μg mercury as methylmercury by a 70-kg adult with a blood mercury level of approximately 200

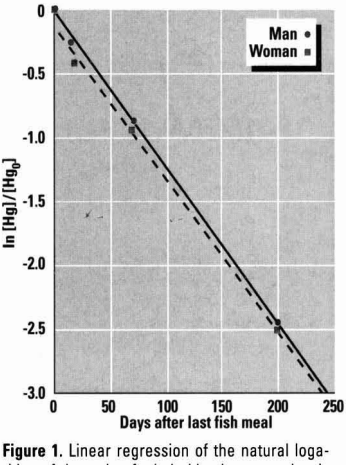

Figure 1. Linear regression of the natural logarithm of the ratio of whole-blood mercury levels at days 0, 15, 70, 200 versus the time in days. For the woman, -slope = k = 0.011, R^2 = 0.99; for the man, -slope = k = 0.012, R^2 = 0.99

µg/L. The agency used an uncertainty factor of 10 to derive an ADI of 30 µg. The FDA assumed that daily ingestion of 30 µg mercury was unlikely since, according to the Tuna Research Foundation (TRF) survey (*14*), the average consumption of all fish among the fish-eating population of the United States was only 18 g/day; and daily consumption of species that contained high methylmercury levels was considerably lower. In their 1986 article, FDA scientists Tollefson and Cordle wrote, "If 11.53 g of swordfish with a mercury level of 1.5 ppm were consumed each day, and this would include over 95% of all swordfish eaters, the daily mercury intake would be 17.3 µg, still below the ADI of 30 µg" (*15*: p. 206).

All three members of the family described in this case study exceeded the average fish consumption rate of participants in the TRF survey. The father consumed an average of 113 g of fish per day—more than 6 times the TRF survey average. His wife and son consumed approximately 75 and 37 g of fish per day. Their estimated mercury intakes ranged from 9 µg/day for the child, to 31 and 52 µg/day for the mother and father, respectively. While no overt clinical symptoms were observed in these individuals, one adult complained of sleep disturbances and concentration difficulties. These symptoms are consistent with the neurotoxic effects of methylmercury exposure. The family was not evaluated for subclinical health effects such as memory and visual disturbances that have been associated with chronic exposure to low levels of methylmercury.

This case study provides support for the methylmercury uptake and excretion models developed by Kershaw et al. (*9*) and Skerfving (*13*). In addition, this case demonstrates the failure of existing food safety regulations that were based on average fish consumption rates and body weights to protect individuals whose dietary habits and body weights fall outside of the normal range. The daily mercury intakes of both adults in this case study exceeded the FDA's ADI for mercury even though none of the fish in their diet exceeded the 1 mg/kg guideline set by that agency. The highest mercury level detected in any of the fish they consumed was 0.7 mg/kg, a level that is not uncommon in sport fish from many Wisconsin lakes or in predatory marine species such as tuna and swordfish. The "1994 Health Guide for People Who Eat Sport Fish from Wisconsin Waters" (*16*) recommends that women of childbearing age not eat fish that contain mercury levels above 0.5 mg/kg. Species listed in this advisory include walleye, northern pike, largemouth bass, and catfish from several inland lakes.

To prevent the public health risks that are posed by methylmercury-contaminated seafood and fish, federal and state agencies may be need to revise existing food safety guidelines. Based on our findings, it may be necessary to lower the concentration of mercury that is permitted in commercial fish to at least 0.5 mg/kg, the level advised for sport fish. It may also be prudent for the FDA to provide consumption frequency advice to commercial fish consumers.

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Xenoestrogens Released from Lacquer Coatings in Food Cans

José Antonio Brotons, María Fátima Olea-Serrano, Mercedes Villalobos, Vicente Pedraza, and Nicolás Olea

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We present data showing that some foods preserved in lacquer-coated cans and the liquid in them may acquire estrogenic activity. Hormonal activity was measured using the E-screen bioassay. The biological activity of vegetables packed in cans was a result of plastic monomers used in manufacturing the containers. The plastic monomer bisphenol-A, identified by mass spectrometry, was found as a contaminant not only in the liquid of the preserved vegetables but also in water autoclaved in the cans. The amount of bisphenol-A in the extracts accounted for all the hormonal activity measured. Although the presence of other xenoestrogens cannot be ruled out, it is apparent that all estrogenic activity in these cans was due to bisphenol-A leached from the lacquer coating. The use of plastic in food-packaging materials may require closer scrutiny to determine whether epoxy resins and polycarbonates contribute to human exposure to xenoestrogens. *Key words*: bisphenol-A, food containers, lacquer coating, xenoestrogens. *Environ Health Perspect* 103:608–612 (1995)

Epoxy resins are used as plastic coatings in the food-packing industry. It has been well documented that polymerization of epoxy resin reactions may not be fully complete, and that a significant proportion of unreacted epoxy compounds can be recovered from food packed in containers lined with these plastics (*1–3*). The migration of cured resin components into foods has also been reported. Unreacted epoxy compounds are thought to be toxic due to their alkylating properties (*4*).

A directive of the European Union (EU) has established a specific migration limit in food of 0.02 mg/kg for diglycidyl ether bisphenol-A (BADGE; CAS no. 1675-54-3). The presence of the monomer bisphenol A (4,4′-isopropopylidenediphenol, CAS no. 80-05-07) in these coatings was considered of lesser importance, and a higher tolerance limit (3 mg/kg) for its specific migration was therefore established by the EU Commission; however concerns about the toxicity of this compound were heightened recently when it was shown that bisphenol-A was estrogenic (*5*).

Europe Union directives 76/893, 80/590, 82/711, 85/572, and 90/128 summarize the European regulations of polymers in contact with foods. Specific migration can be assessed either in foods in contact with polymeric materials, or in substitutive simulants. Before testing, simulants should remain in contact with the interior of the can for similar periods and under similar conditions to those that characterize the product's normal shelf-life. For canned vegetables, the recommended simulants are distilled water or 3% acetic acid in water, depending on the pH of the preserved vegetables (water for pH >4.5, 3% acetic acid for pH <4.5). Moreover, for vegetables sterilized inside cans, testing polymer migration into simulants by heating cans at 121°C for 30–60 min was recommended.

The present study was designed to determine whether estrogenic activity due to plastic components was present in foods packed in lacquer-coated cans. Here we demonstrate the presence of estrogenic activity in foodstuffs inside cans and identify the estrogenic component as a chemical leached from the inner plastic coating.

Methods

Cell line and cell culture conditions. MCF7 human breast cancer cells originally established by Soule and colleagues (*6*) were a gift from C. Sonnenschein (Tufts University, Boston); they were at passages 70–103 after cloning at the time of study. For routine maintenance, cells were grown in our laboratory in Dulbecco's modification of Eagle's Medium (DME) supplemented with 5% fetal bovine serum (FBS; PAA Labor und Forschungs Ges, MBH, Linz, Austria) in an atmosphere of 5% $C0_2$/95% air under saturating humidity at 37°C.

Plasma-derived human serum and removal of sex steroids. Plasma-derived human serum was prepared from outdated plasma by adding calcium chloride to a final concentration of 30 mM to facilitate clot formation. Sex steroids were removed from serum by charcoal-dextran stripping (*7*). Briefly, a suspension of 5% charcoal (Norit A; Sigma, St. Louis, Missouri) with 0.5% dextran T-70 (Pharmacia-LKB, Uppsala, Sweden) was prepared. Aliquots of the charcoal-dextran suspension of a volume similar to the serum aliquot to be processed were centrifuged at 1000*g* for 10 min. Supernatants were aspirated and serum aliquots were mixed with the charcoal pellets. This charcoal–serum mixture was maintained in suspension by rolling at 4 cycles/min at 37°C for 1 hr. The suspension was centrifuged at 1000*g* for 20 min, and the supernatant was then filtered through a 0.20-µm filter (Gelman Sciences, Ann Arbor, Michigan). Charcoal dextran-treated human serum (CDHS) was stored at -20°C until needed.

Cell proliferation experiments in culture: E-screen test. MCF7 cells were used in the E-screen test according to a technique slightly modified from that originally described by Soto et al. (*8*). Briefly, cells were trypsinized and plated in 24-well plates (Limbro, McLean, Virginia) at initial concentrations of 10,000 cells per well in 5% FBS in DME. The cells were allowed to attach for 24 hr; then the seeding medium was replaced with 10% CDHS supplemented phenol red-free DME. Different concentrations of the test compound were added. We stopped the assay after 144 hr by removing medium from wells, fixing the cells and staining them with sulforodamine-B (SRB). The staining technique was modified from that described by Skehan (*9*). Briefly, cells were treated with cold 10% trichloracetic acid (TCA) and incubated at 4°C for 30 min, then washed five times with tap water and left to dry. TCA-fixed cells were stained for 10 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Wells were rinsed with 1% acetic acid and air dried. Bound dye was solubilized with 10 mM Tris base (pH 10.5) in a shaker for 20 min. Finally, aliquots were transferred to a 96-well plate and read in a Titertek Multiscan apparatus (Flow, Irvine, California) at 492 nm. We evaluated linearity of the SRB assay with cell number before cell growth experiments. Alternatively, cells were lysed and the nuclei were counted with a Coulter ZM Counter apparatus (Coulter Electronics, Luton, England) according to a technique previously described in detail (*7*).

Results are expressed as means ±SD. Mean cell numbers from each experiment were normalized to the steroid-free control cultures to correct for differences in the initial seeding density. We assessed differences between the chemical compounds and estradiol-17ß groups using Student's *t*-test.

Extraction and determination of estrogenic compounds from lacquer-coated cans. Twenty different brands of canned foods were purchased in supermarkets in Spain and in the United States. Cans were packed in Brazil, France, Spain, Turkey and the United States.

The liquid from selected cans of whole green beans, artichoke hearts, asparagus

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We gratefully acknowledge A.M. Soto and C. Sonnenschein (Tufts University, Boston) for providing MCF7 cells and for helpful discussions and Karen Shashok for improving the English of the manuscript. This work was supported by grant 94/1551 from the Fondo de Investigaciones Sanitarias (FIS), Spanish Ministry of Health.

Received 31 October 1994; accepted 2 March 1995.

spears, corn, peas, mushrooms, palm hearts, peppers, tomatoes, and mixed vegetables was collected and filtered. A 75-mL aliquot was treated with 20 mL of methanol in a decantation funnel under agitation for 10 min. Samples were extracted with 40 mL chloroform and centrifuged at 1200*g* for 15 min; this process was repeated three times. Solvent was removed by concentration (1 mL) under reduced pressure at 60°C; 0.5 mL sulfuric acid was then added and the samples centrifuged. Finally, the organic phase was dried under a nitrogen stream.

Cans containing fatty foods such as sweetened condensed milk, condensed soup, pork and beans, concentrated milk-based infant formula, cheese dip, and tortillas were processed in a different way. Empty cans were filled up with bidistilled water (pH 7.5) and autoclaved for 30 min at 125°C with a total cycle time of 3 hr. The autoclaved water was processed like the liquid from vegetable and fruit cans.

Dried residues were suspended in 0.5 mL ethanol and chromatographed (Waters 501 HPLC System, Millipore, Milford, Massachusetts) using a Lichrocart Merck silica column (20 × 0.4 cm) (Merck, Darmstadt, Germany) at a flow rate of 1 mL/min with a 500 µL loop injector (Waters U6K). After 2 min of isocratic elution with *n*-hexane (Phase A), a gradient was applied from 0 to 40% phase B [*n*-hexane: methanol: isopropanol (40:45:15)], 10 min to 100% phase B, and 10 min to 100% phase A. The elution profile was monitored at 280 nm (Waters 490, Millipore). Fractions collected between 0 and 11 min (fraction α) and from 15 to 25 min (fraction ß) were pooled out, dried down, resuspended in 100 µL ethanol, and tested by the E-screen assay.

Alternatively, we quantified xenoestrogens in samples using a Perkin-Elmer 250 Binary LC with a Perkin-Elmer diode array detector and a Spherisorb silica S5 W column (25 × 0.4 cm) with a 20 µL loop injector (Rheodyne 7125, Perkin-Elmer). Working conditions were the same as described above.

Spectroscopic studies. Mass spectra of extracted cans and technical-grade bisphenol-A were obtained in a mass spectrometry system operating at an ion source temperature of 200°C in a Hewlett Packard 5890 chromatograph. A 30-m methyl silicon column (OV-P) was used with a 1.2 mL/min flow, and helium as the carrier gas. Temperature of the oven was 80–320°C, with a graded increase of 10°C/min.

Steroids and chemical compounds tested. Estradiol-17ß was obtained from Sigma (St. Louis, Missouri). Bisphenol-A was obtained from Aldrich (Albuch, Germany). Chemicals were dissolved in ethanol to a final concentration of 1 mM and stored at -20°C. They were all diluted in phenol red-free DME immediately before use. The final ethanol concentration in the culture medium did not exceed 0.1%.

Results

The addition of estradiol-17ß to CDHS-supplemented medium increased MCF7 cell numbers. Maximum proliferative effect was obtained with ≥10 pM estradiol-17ß (Fig. 1). The cell yield was sixfold greater than in control cultures (6.67 ± 1.21; n = 15 experiments). In the absence of estradiol-17ß, cells proliferated minimally.

Extracts from food packed in lacquer-coated cans were assayed with the E-screen test after chromatographic elution, as described in Methods. The proliferative effect eluted in the ß fraction significantly increased cell numbers in comparison with controls. Figure 2 shows cell yields of MCF7 cells supplemented with extracts from the liquid of peas packed in a lacquer-coated can. The cell yield for fraction ß was fourfold greater than in control. This proliferative effect was 58% of that obtained with estradiol-17ß. Extracts from

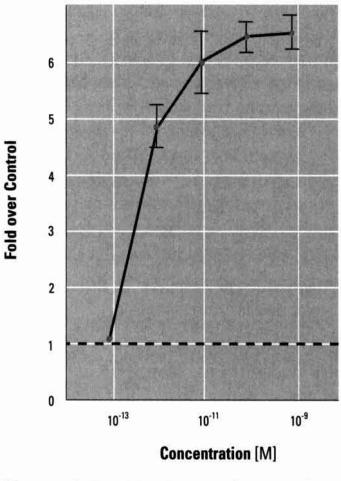

Figure 1. Cell proliferation in MCF7 cells. Cells growing in 10% charcoal dextran-treated human serum-supplemented medium were exposed for 144 hr to different amounts of estradiol-17ß. The points represent quadruplicate cultures; bars indicate standard deviations.

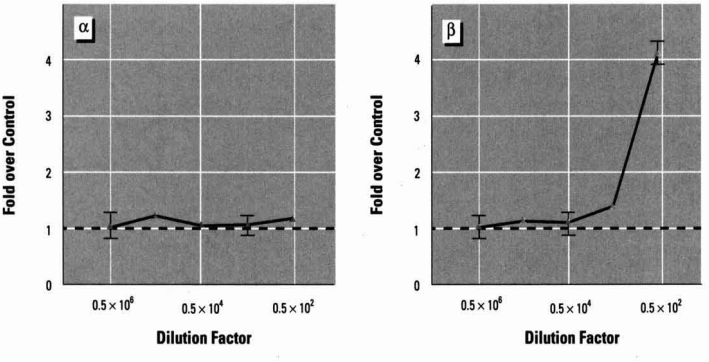

Figure 2. Cell proliferation in MCF7 cells. Cells growing in 10% charcoal dextran-treated human serum-supplemented medium were exposed for 144 hr to different dilutions of extracted liquid from food cans. Fractions α and β were tested. The proliferative effect eluted in the ß fraction significantly increased the cell numbers in comparison with controls. The points represent quadruplicate cultures; bars indicate standard deviations.

Table 1. Bisphenol-A concentration and estrogenic effects (means ± SD) of the liquid phase of vegetables packed in lacquer-coated cans

Vegetable	Can weight (g)	Amount of liquid (mL)	Bisphenol-A (µg/can)	Proliferative effect[a]
Peas	300	50	22.9 ± 8.8	3.9 ± 0.2*
Artichokes	390	150	18.6 ± 6.5	2.2 ± 0.1*
Green beans	400	190	11.9 ± 5.3	2.0 ± 0.2*
Mixed vegetables	450	220	10.1 ± 4.3	1.8 ± 0.2*
Corn	300	15	4.5 ± 2.6	1.5 ± 0.1*
Mushrooms	350	145	4.2 ± 4.1	1.7 ± 0.1*
Asparagus	230	80	ND	—
Palm hearts	500	280	ND	—
Peppers	390	140	ND	—
Tomatoes	390	140	ND	—
Estradiol-17ß (10 pM)	—	—	—	6.7 ± 1.2

ND, not detectable.
[a]Proliferative effect was estimated using 1/200 of the contents of each can.
*Significant difference from control cultures ($p < 0.01$).

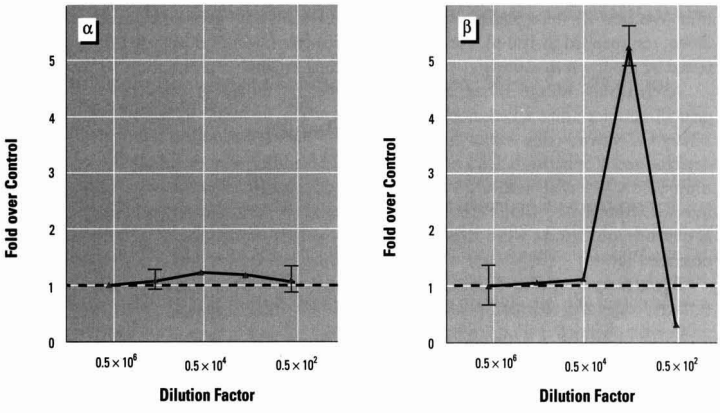

Figure 3. Cell proliferation in MCF7 cells. Cells growing in 10% charcoal dextran-treated human serum-supplemented medium were exposed for 144 hr to different dilutions of water from autoclaved cans. Fractions α and β were tested. The proliferative effect eluted in the ß fraction significantly increased the cell numbers in comparison with controls. The points represent quadruplicate cultures; bars indicate standard deviations.

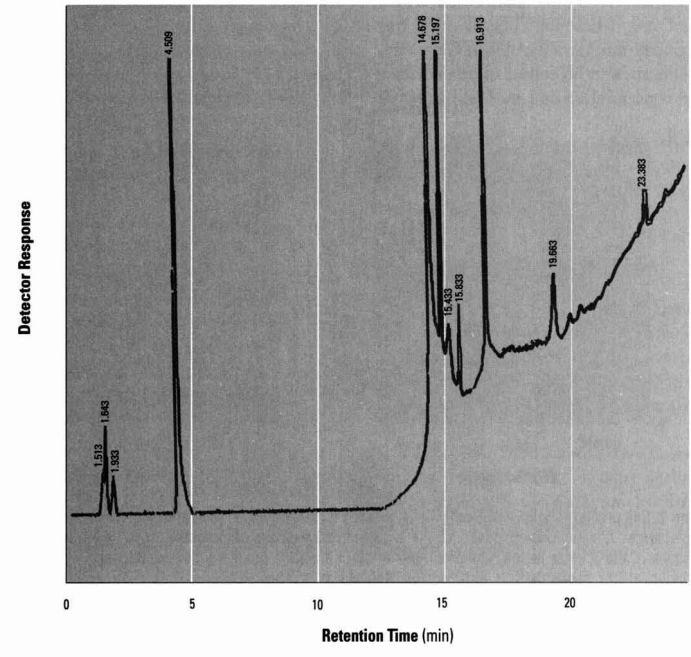

Figure 4. Chromatogram of extracts of lacquer-coated cans showing estrogenic effect in the E-screen bioassay. Fractions collected between 0 and 11 min (fraction α) were negative in the E-screen test, whereas fractions from 15 to 25 min (fraction ß) were estrogenic.

peas, artichoke hearts, corn, mushrooms, whole green beans, and mixed vegetables packed in lacquer-coated cans also showed estrogenic activity (Table 1).

We conducted several experiments to assess whether the estrogenic chemicals were leached from the walls of lacquer-coated cans during the autoclaving process or were released from the vegetables. First, empty cans containing water were autoclaved for 25 min at 125°C; the autoclaved water was processed and then tested with the E-screen assay. Second, fresh fruits and vegetables that were positive in the bioassay when packed in lacquer-coated cans were extracted and tested with the E-screen assay. No estrogenic activity was detected in fresh vegetables (results not shown). In contrast, water autoclaved in cans in which estrogenic activity was found also showed estrogenic activity (Fig. 3).

Some of the lacquer-coated cans that had contained fatty foods also released estrogenic chemicals into water after autoclaving. For example, extracts from cans that had contained sweetened condensed milk increased cell proliferation by as much as 70% of the maximal effect of estradiol-17ß.

Extraction efficiency was assayed with three solvents for bisphenol-A. Different amounts of bisphenol-A, ranging from 3.15 to 10 µg were extracted with hexane, hexane:ethyl ether (1:1 v/v), or chloroform. Hexane extraction failed to recover bisphenol-A; however, with hexane:ethyl ether, percentage recoveries were from 60.2 ± 5.6% to 63.1 ± 4.1%. Chloroform showed the highest extraction efficiency, which ranged from 88.4 ± 6.4% to 89.2 ± 4.5%. We chose chloroform to extract bisphenol-A from the liquid phase of canned foods and simulants.

Recovery rates were also tested before investigating bisphenol-A content in canned foods. The liquid from vegetables packed in glass containers was spiked with known amounts of bisphenol-A ranging from 10 to 70 µg and then extracted according the protocol described in Methods. Recovery rates varied from 82.4 ± 6.1% to 86.2 ± 4.3%.

The chromatographic profile of an extract of the liquid from a lacquer-coated can containing peas is shown in Figure 4. Among the peaks detected, bisphenol-A was identified as having a retention time of 19.69 min. This compound was present in some extracted foods and in water from autoclaved cans; the peak was present in all extracts with estrogenic activity, and all extracts having bisphenol-A were estrogenic. Bisphenol-A was quantified by a calibration curve made after eluting known amounts of the pure substance (y = 30,769x – 67,366; r = 0.998). Quantitative evaluation showed a range of 4–23 µg of bisphenol-A per can (Table 1). Mass spectrometric analysis identified bisphenol-A in these samples (Fig. 5).

When pure bisphenol-A was assayed in the E-screen test, it was found to be estrogenic, although the concentrations required to produce maximum proliferation of MCF7 cells were 1000-fold higher than those of estradiol-17ß (Fig. 6).

Discussion

Substances with diverse chemical structures have estrogenic properties (*10*); this diversity hinders the use of structure as the basis to predict estrogenicity. Chemical diversity thus represents a considerable setback to regulatory efforts aimed at safeguarding the public from the harmful effect of xenobiotics. Simple, reliable biological assays such as the E-screen test (*8*) have been proposed to remedy this situation. This bioassay is based on the ability of chemicals that are estrogenic to induce the proliferation of

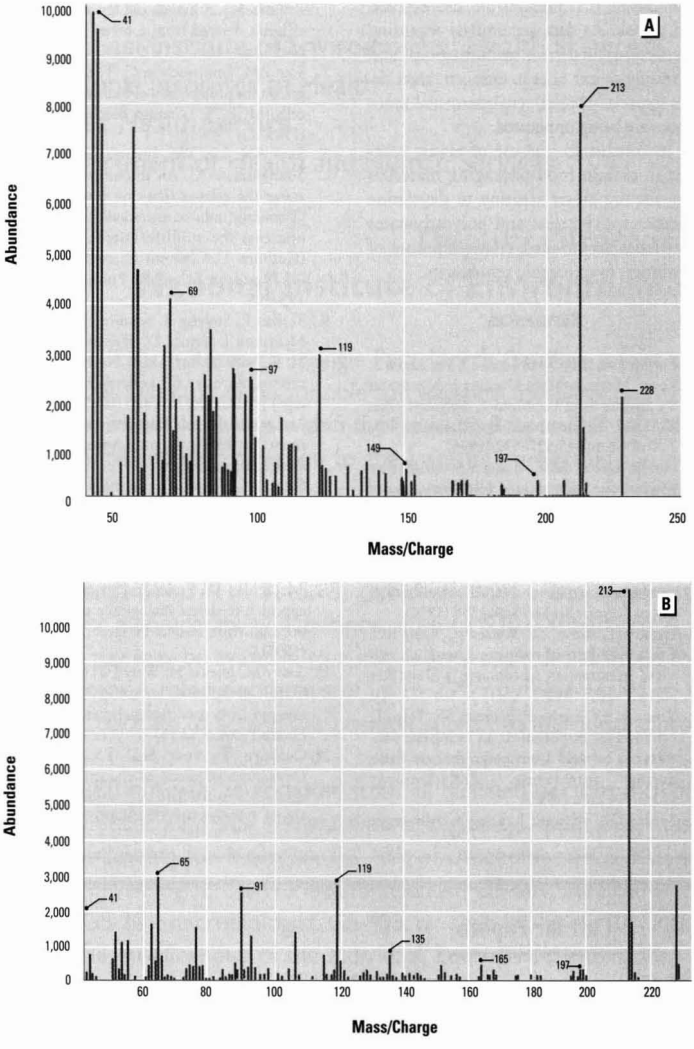

Figure 5. (A) Mass spectrum of extracted lacquer-coated cans positive in the E-screen bioassay. (B) Mass spectrum of bisphenol-A.

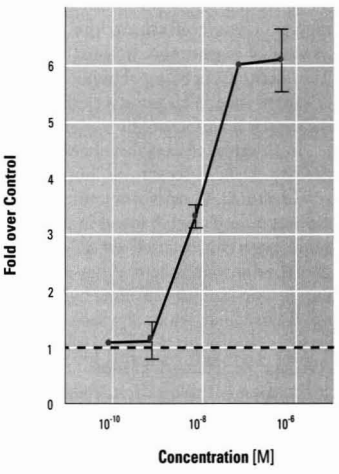

Figure 6. Cell proliferation in MCF7 cells. Cells growing in 10% charcoal dextran-treated human serum-supplemented medium were exposed for 144 hr to variable dilutions of bisphenol-A. The points represent quadruplicate cultures; bars indicate standard deviations.

cells of the MCF7 breast tumor cell line (*6*) under well-defined, reproducible conditions (*7*). The E-screen test has been credited with the identification of pesticides whose estrogenic properties were unsuspected (*8,11*), and it represents a valuable tool in environmental toxicology (*12*).

The data reported in this paper strongly suggest that some foods preserved in lacquer-coated cans acquire estrogenic activity. This biological activity may be related to estrogenic substances such as phytoestrogens or estrogenic pesticides contained in vegetables before they are canned, or plastic monomers or additives used in the manufacture of food containers. Phytoestrogens and organochlorine pesticides were not found in the foods packed in the cans we studied, nor were they detected in fresh vegetables. To test the second possibility, cans in which the vegetables were packed were filled with distilled water and autoclaved to determine whether this treatment released estrogenic components from the inner plastic coating. The plastic monomer bisphenol-A was found as a contaminant not only in the liquid of the preserved vegetables, but also in water autoclaved in these cans.

Monomers and oligomers may be released during the setting period, when conversion of the oligomer into a polymer is incomplete, and when polymerized resin is degraded by high temperatures, autoclaving, enzyme hydrolysis, etc. Bisphenol-A was reported to leach from polycarbonate tubes during autoclaving. Krishnan et al. (*5*) found concentrations of up to 15 mM of this compound (equivalent to 2.3–3.5 µg bisphenol-A/L water) in distilled water after autoclaving. Because acidic water (pH 5.5) was used, whereas hydrolysis of polycarbonate resins is favored by alkaline pH, the amount of bisphenol-A extracted in the study by Krishnan and colleagues may underestimate the amount of this chemical that is potentially extractable.

Foods packed in lacquer-coated cans are sterilized by autoclaving; thus the experiments described by Krishnan et al. (*5*) are to a substantial degree reproduced in the processing of canned food. The amount of bisphenol-A we extracted from the liquid of canned foods ranged from 0 to 33 µg per can. The higher concentrations are above the values found by Krishnan and colleagues. Food in the lacquer-coated cans we studied was in contact with the polymer for a longer time than was the water in flasks used the experiments by Krishnan et al. After cans are autoclaved by the manufacturer, they are usually stored for months before they are sold. This prolonged period may favor the accumulation of bisphenol-A. Leaching of bisphenol-A may also be related with the type of polymer, the sterilization procedure, and the type of food contained. Krishnan and colleagues do not state how many times they attempted to detect bisphenol-A from reautoclaved flasks; however, we found that this compound continued to be released from lacquer-coated cans after a second autoclaving.

The directive of the European Union establishes a specific limit of migration of

the monomer bisphenol-A into food of 3 mg/kg. Cans containing the highest amount of bisphenol-A weighed 0.30 kg. This means about 80 µg of bisphenol-A/kg of canned food. This value is clearly below the highest limit for specific migration.

In all extracted cans that showed estrogenicity in the E-screen test, bisphenol-A was identified by mass spectrometry. The amount of bisphenol-A found in the estrogenic extracts accounted for all the hormonal activity measured. After autoclaving, in one kind of can dimethyl bisphenol-A was found, which also showed estrogenic activity in the E-screen assay. Although the presence of other xenoestrogens cannot be ruled out, it seems reasonable to attribute all the estrogenic activity found in these cans to bisphenol-A leached from the lacquer coating. Studies are in progress to identify other resin components with estrogenic activity. Interestingly, we have found dimethacrylate of bisphenol-A, a component of composite resins used as restorative materials in dentistry, to be estrogenic in the E-screen assay at 10- to 100-fold lower concentrations than bisphenol-A (Pérez et al., manuscript in preparation). Alternative sources of these xenoestrogens such as laboratory contamination (*13*) were ruled out.

The impact of certain estrogenic xenobiotics on the reproductive system, development and health of animals has been clearly documented (*14*). Findings such as ours demonstrate that humans are also exposed and at risk. As data accumulate regarding infertility, genital tract malformations, and increasing cancer rates in estrogen target tissues (especially breast), environmental xenobiotics are being implicated.

In conclusion, the use of plastic coatings in certain food-packaging materials may require closer scrutiny to determine whether epoxy resins and polycarbonates contribute to the inadvertent exposure of consumers to estrogenic xenobiotics.

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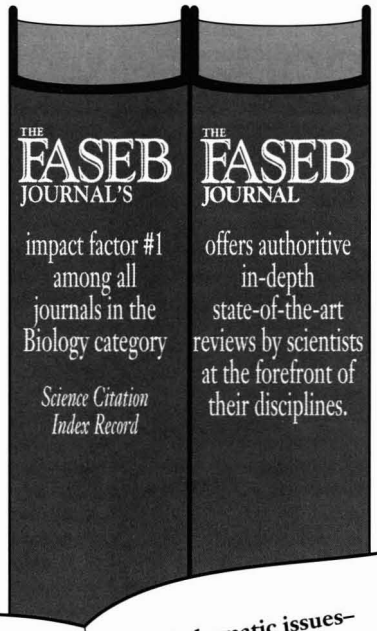

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July 15–20, Sat–Thu
Minneapolis, Minnesota
Information: American Solar Energy Society, 2400 Central Avenue G-1 Boulder, CO 80301
(303) 443-3130
FAX (303) 443-3212

The Ninth International Congress of Immunology
July 23–29, Sun–Sat
San Francisco, California
Information: FASEB Office of Scientific Meetings and Conferences, 9650 Rockville Pike, Bethesda, MD 20814-3998

Engineering Solutions to Indoor Air Quality Problems; An International Symposium
July 24–26, Mon–Wed
Sheraton Imperial Hotel, Raleigh, North Carolina
Information: Kelly W. Leovic
U.S. Environmental Protection Agency,
(919) 541-7717
FAX (919) 541-2147 or
Air and Waste Management Association
(412) 232-3444
FAX (412) 232-3450

August

American Chemical Society Meeting
August 20–25, Sun–Fri
McCormick Place Convention Center, Chicago, Illinois
Information: ACS Meetings, American Chemical Society,
1155 16th Street, NW
Washington, DC 20036
(202) 872-6059
FAX (202) 872-6128

Second International Conference on Environmental Mutagens in Human Populations
August 20–25, Sun–Fri
Prague, Czech Republic
Information: Radim J. Sram, Laboratory of Genetic Ecotoxicology, Prague Institute of Advanced Studies, University of Michelskeho lesa 366 140 00
Prague, Czech Republic
(422) 472-4756
FAX (422) 472-4757

Fourth International ISSX Meeting: Xenobiotic Interactions
August 27–31, Sun–Thu
The Westin Hotel,
Seattle, Washington
Information: ISSX Meeting/Convention Services Northwest, 1809-7th Avenue,
Suite 1414
Seattle, WA 98101
(206) 292-9198
FAX (206) 292-0559

33rd International Congress on Forensic (TIAFT) and 1st Congress on Environmental Toxicology: Gretox 1995
August 27–31, Sun–Thu
Thessaloniki-Macedonia-Greece
Information: Anastasios Kovatsis, Laboratory of Biochemistry, Aristotelian University of Thessaloniki,
540 06 Thessaloniki, Greece
(30) 31-999851
FAX (30)-31-999851, -200392, or -206138

September

International Symposium on Tumor Markers
September 1–3, Fri–Sun
Guest Hotel, Bei-Di-He,
He Bei Provence, China
Information: Mei Yuan, General Secretary BRTIMB 1995, Cancer Research Laboratory, General Hospital of PLA,
Beijing 100853, China
FAX 861-821-7073

Fifth International Congress on Hormones and Cancer
September 17–20, Sun–Wed
Quebec City, Quebec, Canada
Information: Fifth International Congress on Hormones and Cancer, Laval University Medical Center, 2705 Laurier Boulevard, Sainte-Foy, Quebec, GIV 4G2
Canada
1 (418) 654-2244
FAX (418) 654-2714

Reclaim 95: Landfill Mining & Reclamation
September 28–30, Thu–Sat
Holiday Inn Turf,
Albany, New York
Information: Richard Will, The Coordinate Group, Inc.,
Box 3356,
Warrenton, VA 22186-1956
(800) 627-8913
FAX (703) 349-4540

October

European Conference on Combination Toxicology
October 11–13, Wed–Sun
Congress Centre Koningship Veldhoven, The Netherlands
Information: Secretariat Flora de Vrijer TNO Toxicology,
PO Box 360, 370 AJ Zeist The Netherlands
31 3404 44218
FAX: 31 3404 52224
E-mail: vrijer@voeding.tno.nl

Arkansas Toxicology Symposium: New Horizons in Chemical-Induced Liver Injury
October 19–20, Thu–Fri
The Doubletree Hotel,
Little Rock, Arkansas
Information: Jack A. Hinson, Director, Division of Toxicology
University of Arkansas for Medical Sciences,
Little Rock, AR 72205
(501) 686-5766
FAX (501) 686-8970

Eighth International Conference of the Society for Human Ecology: "Livelihood and Liveability"
October 19–22, Thu–Sun
Lake Tahoe,
Tahoe City, California
Information: Nancy L. Markee, University of Nevada Reno,
MS 199,
Reno, NV 89557
(702) 784-1674
FAX (702) 784-1142

Mechanisms and Prevention of Environmentally Caused Cancers
October 21–25, Sat–Wed,
Santa Fe, New Mexico
Information: Alice M. Hannon, The Lovelace Institutes, 2425 Ridgecrest Drive S.E.,
Albuquerque, NM 87108-5127
(505) 262-7255
FAX (505) 262-7043

Fifth International Conference on the Chemistry and Biology of Mineralized Tissues
October 22–27, Sun–Fri
Kohler, Wisconsin
Information: L. Keller,
The University of Texas, Health Sciences Center at San Antonio,
7703 Floyd Curl Drive,
San Antonio, TX 78284-7823

Thirteenth International Neurotoxicology Conference
October 29–November 1, Sun–Wed
Hot Springs, Arkansas
Information: Joan Spyker
Cranmer Professor and Conference Chairman, Department of Pediatrics, UAMS #512, Arkansas Children's Hospital,
1120 Marshall Street, Room 207,
Little Rock, AR 72202-3591
(501) 320-2986
FAX (501) 320-3947

The XVIII Symposium of the International Association for Comparative Research on Leukemia and Related Diseases
October 29–November 3, Sun–Fri
Kyoto International Conference Hall, Kyoto, Japan
Information: Secretariat, The XVII Symposium of IACRLRD,

Laboratory of Molecular Oncology,
The Institute of Physical and
Chemical Research (RIKEN),
2-1 Hirosawa, Wako,
Saitama 351-01, Japan
81-48-462-1111 ext. 3161
FAX 81-48-462-4686

November

Living in a Chemical World–The Second Decennial Symposium
November 3–5, Fri–Sun
Hotel Omni-Shoreham, Washington, DC
Information: David Rall,
5302 Reno Road,
Washington, DC 20015
(202) 244-5380
FAX (202) 966-3093

International Symposium: 66 Years of Surfactant Research
November 5–10, Sun–Fri
Vienna, Austria and Budapest Hungary, with poster sessions on board ship from Passau, Germany
Information: B. Lachmann
Department of Anesthesiology,
Erasmus University
Post Bos 1738,
3000 DR Rotterdam, The Netherlands
31 10 4087312
FAX 31 10 4367870

Susceptibility and Risk: The Third Annual Symposium of the Health Effects Research Laboratory
November 6–9, Mon–Thu
Raleigh, North Carolina
Information: 1995 HERL
Symposium Susceptibility and Risk,
c/o RSD Conference Cordinator,
Health Effects Research
Laboratory, U.S. Environmental,
Protection Agency Mail Drop 70,
Research Triangle Park, NC 27711

Ash VIII on Ash Management and Utilization
November 14–15, Tue–Wed
Stouffer Renaissance Hotel, Crystal City, Arlington, Virginia
Information: Richard Will, The
Coordinate Group, Inc.,
Box 3356,
Warrenton, VA 22186-1956
(800) 627-8913 or
(703) 347-4500
FAX (703) 349-4540

The American College of Veterinary Pathologists
November 14–16, Tue–Thu
Marriott Marquis, Atlanta, Georgia
Information: Sue Parker or Nick A. Montana, ACVP Executive Office
875 Kings Highway, Suite 200,
Woodbury, NJ 08096-3172
(609) 384-6287
FAX (609) 853-0411

American Society of Tropical Medicine and Hygiene 44th Annual Meeting
November 17–21, Fri–Tue
San Antonio, Texas
Information: Paulette Anderson,
ASTMH Headquarters,
60 Revere Drive, Suite 500,
Northbrook, IL 60062
(708) 480-9592
FAX (708) 480-9282

Third Congress of Toxicology in Developing Countries
November 19–23, Sun–Thu
Cairo, Egypt
Information: Sameeh A. Mansour
(V-P & SG/3rd CTOX-DC),
National Research Centre,
Dokki, Cairo, Egypt
(202)701211/701362/701433/701499
FAX (202)-700931

December

International Conference on Food Factors: Chemistry and Cancer Prevention
December 10–15, Sun–Thu
Act City Hamamatsu, Hamamatsu, Japan
Information: ICoFF Secretariat,
Japan Institute for the Control of
Aging, Nikken Foods Co. Ltd.,
723-1, Haruoka, Fukuroi,
Shizuoka 437-01, Japan
81 538 49 0125
FAX 81 538 49 1267

International Symposium on Environmental Biomonitoring and Specimen Banking
December 17–22, Sat–Fri
Honolulu, Hawaii
Information: K.S. Subramanian,
Environmental Health Directorate,
Health Canada, Tunney's Pasture,
Ottawa, Ontario CK1A OL2 Canada
(613) 957-1874
FAX (613) 941-4545

1996

January

Integrins and Signaling Events in Cell Biology and Disease
January 5–11, Fri–Thu
Keystone, Colorado
Information: Keystone Symposia,
Drawer 1630,
Silverthorne, CO 80498
(303) 262-1230
FAX (303) 262-1525

Molecular and Developmental Biology of the Extracellular Matrix
January 5–11, Fri–Thu
Keystone, Colorado
Information: Keystone Symposia,
Drawer 1630,
Silverthorne, CO 80498
(303) 262-1230
FAX (303) 262-1525

Small GTP-binding Proteins and Growth Factor Signaling Pathways
January 5–11, Fri–Thu,
Tamarron, Colorado
Information: Keystone Symposia,
Drawer 1630,
Silverthorne, CO 80498
(303) 262-1230
FAX (303) 262-1525

Exploring and Exploiting Antibody and Ig Superfamily Combining Sites
January 5–11, Fri–Thu
Taos, New Mexico
Information: Keystone Symposia,
Drawer 1630,
Silverthorne, CO 80498
(303) 262-1230
FAX (303) 262-1525

Oxidant Stress: From Molecules to Man
January 8–14, Mon–Sun
Santa Fe, New Mexico
Information: Keystone Symposia,
Drawer 1630,
Silverthorne, CO 80498
(303) 262-1230
FAX (303) 262-1525

The Cell Cycle
January 11–17, Thu–Wed
Taos, New Mexico
Information: Keystone Symposia,
Drawer 1630,
Silverthorne, CO 80498
(303) 262-1230
FAX (303) 262-1525

Blood Stem Cell and Bone Marrow Transplants
January 15–21, Mon–Sun
Keystone, Colorado
Information: Keystone Symposia,
Drawer 1630,
Silverthorne, CO 80498
(303) 262-1230
FAX (303) 262-1525

Molecular Biology of HIV
January 17–23, Wed–Tue
Taos, New Mexico
Information: Keystone Symposia
Drawer 1630,
Silverthorne, CO 80498
(303) 262-1230
FAX (303) 262-1525

Hepatitis C and Beyond
January 23–29, Tue–Mon
Burlington, Vermont
Information: Keystone Symposia,
Drawer 1630,
Silverthorne, CO 80498
(303) 262-1230
FAX (303) 262-1525

Tissue Engineering
January 23–29, Tue–Mon
Taos, New Mexico
Information: Keystone Symposia,
Drawer 1630,
Silverthorne, CO 80498
(303) 262-1230
FAX (303) 262-1525

April

American Society of Mechanical Engineers Solid Waste Processing Division Seventeenth Biennial Conference
April 28–May 1, Sun –Wed
Trump Regency Hotel,
Atlantic City, New Jersey
Information: Richard Will,
The Coordinate Group, Inc., Box
3356, Warrenton, VA 22186-1956
(800) 627-8913
FAX (703) 349-4540

May

Fourth International Symposium on Metal Ions in Biology and Medicine
May 19–22, Fri–Mon
Tarragona/Barcelona
Catalonia, Spain
Information:Mercedes Gómez,
Laboratory of Toxicology and
Biochemistry, School of Medicine,
c/San Lorenzo 21,
43201 REUS, Spain
34 77 759 376
FAX 34 77 759 322

September

Biological Monitoring in Occupational Environmental Health
September 11–13, Wed–Fri,
Espoo, Finland
Information: Biological Monitoring,
c/o Finnish Institute of
Occupational Health Symposium
Secretariat, Topeliuksenkatu 41 a A
FIN-00250 Helsinki, Finland
358-0-47-471
FAX 35804747548

Fellowships, Grants & Awards

Postdoctoral Fellowships in Toxicology/Epidemiology

Postdoctoral fellowships are available in a unique NIH-sponsored training program in toxicology/epidemiology of respiratory tract disease caused by environmental agents. Conducted jointly by the Inhalation Toxicology Research Institute (ITRI) and the Department of Medicine, University of New Mexico (UNM), the program provides training focus in either laboratory or epidemiology-based research with cross-training in the other discipline. The program develops research skills for investigative careers, incorporating interdisciplinary laboratory-human extrapolation. ITRI-based participants will undertake postdoctoral laboratory research and receive lecture and field cross-training in epidemiology and toxicology jointly with UNM-based fellows in epidemiology. Programs are tailored to individuals. Laboratory research or pathogenesis of disease can focus on one of several disciplinary areas, including cell biology, molecular biology, biochemistry, immunology, pathology, physiology, toxicology, radiobiology, aerosol science, or mathematics modeling, depending on interests and qualifications. Annual stipend of $30,800 plus health insurance, tuition and travel costs.

Contact:
Dr. David E. Bice
Education Coordinator
Inhalation Toxicology Research Institute
PO Box 5890
Albuquerque, NM 87185
or call (505) 845-1257 for application materials.
We are an Equal Opportunity Employer.

European Cancer Centre Two-Year Fellowships for Oncologists

The European Cancer Centre was founded in Amsterdam in 1991. Its major goal is to improve oncologic care by developing an international research network through collaborative research. The ECC focuses on organizing early clinical research, placing emphasis on translating basic laboratory research into clinical phase I and phase II studies.

The ECC invites young clinical specialists with a proven interest in research to apply for the ECC Fellowship Programme, which is funded by trade and industry. A substantial part of this two-year fellowship will be spent in the laboratory, performing basic research. The fellows work in the Amsterdam oncologic centres participating in the European Cancer Centre under the supervision of the principal investigator of the study.

Eligibility Criteria: Candidates must meet the following conditions:
- Maximum age 35 years
- Medical degree with specialization in oncology
- Proven research skills
- At least two publications with first authorship in the international peer reviewed literature
- Guaranteed position in home institute after completion of the fellowship.

It is recommended to support an application with letters of reference from present and former supervisors and/or mentors.

Application Procedures: The Research Groups of the European Cancer Centre submit their research proposals and request for a fellow. The ECC Scientific Board, chaired by Professor H.M. Pinedo, MD, PhD, evaluates the proposal on scientific value and innovative importance. After approval of the project, fellowship candidates can be recommended by members of an ECC Research Group. Those interested can also request information about available projects and send in their application.

To apply, candidates must submit: 1) a letter of application with the completed ECC Fellowship Programme Application Form, 2) a short curriculum vitae listing at least three specialists/scientists willing to supply a reference, 3) no more than five relevant full publications,
4) a letter stating a guaranteed permanent position at the home institute upon return.

Selection Procedure: Twice a year, on March 1 and September 1, the applications are reviewed by a selection committee, considering the aforementioned criteria. Selected fellows are then informed of the available research projects best suiting their curriculum and are introduced to the principal investigators.

They will also be invited for interviews with the selection committee and to give a presentation of their work. After the second deliberation round, the selected fellows will be invited to start their two-year fellowship in Amsterdam within a foreseeable time.

Salary and Stipend: A salary and stipend are provided which include all costs of housing and living. The Board encourages the home institute to provide additional funding.

For more information contact:
European Cancer Centre
PO Box 7057
NL-1007 MB Amsterdam
The Netherlands
31 20 644 4500/4550
FAX 31 20 644 4551

Solar Processes and Hazardous Chemicals

The National Renewable Energy Laboratory (NREL) seeks the participation of U.S. educational institutions in a program of research and development on solar processes for the destruction or removal of hazardous chemicals from air or water. This program seeks to identify new processes or improve already known processes (photochemical, photothermal, photocatalytic).

Areas of interest include but are not limited to: 1) photochemical reactor engineering; 2) process chemistry; 3) catalyst improvements; and 4) physical and chemical mechanisms. Up to six awards are anticipated. For solicitation copy, write to:
NREL, Subcontracts Section
M/S 6320-17/2
1617 Cole Boulevard
Golden, CO 80401-3393,
Attn: Kendra K. Ecton
Or FAX request to (303) 231-1444. Telephone for more information only: Paulette Fontaine-Westhart at (303) 231-7807. Reference: Synopsis No. 4-298.

Earthwatch Field Grants

The Center for Field Research invites field biologists to apply for an Earthwatch field grant. The Center for Field Research encourages and evaluates proposals for support by its international affiliate Earthwatch. Earthwatch is a private, nonprofit organization established in 1971 to fund field research, pro-

mote communication between scholars and the public, improve science education, and enhance public understanding of pressing environmental and social problems.

Through its system of participant funding, Earthwatch supports both basic and applied research. Proposals are welcome for field studies on almost any life science topic, in any country, by advanced scholars of any nationality. The research must have scientific merit and feasibly and constructively involve nonspecialist Earthwatch volunteers in the research tasks.

Earthwatch field grants average $20,000. These funds are derived from the contributions of Earthwatch members who enlist for the opportunity to join scientists in the field and assist with data collection and other tasks. On average, each volunteer contributes $600–900 towards the field grant and spends 12–16 days in the field. A typical Earthwatch project employs 4–8 volunteers each on 3–5 sequential teams. To be economically feasible for Earthwatch, the total number of Earthwatch volunteers participating on a project in one year is usually at least 20.

Earthwatch field grants cover the costs of maintaining volunteers and principal researchers in the field. They also help with other project expenses, except principal investigator salaries, capital equipment, overhead, and preparation of results for publications. Applying for grants is a two-stage process. Preliminary proposals are submitted to The Center for Field Research at least 13 months in advance of anticipated field dates. Full proposals are invited upon review of preliminary materials. Proposals are accepted and reviewed year round. For more information, contact:

Dee Robbins,
Life Sciences Program Director,
The Center for Field Research,
680 Mt. Auburn Street,
Watertown, MA 02172
(617) 926-8200
FAX (617) 926-8532

ILSI

International Life Sciences Institute

The Second International Conference on Nutrition and Aging

September 20–22, 1995
Showa Women's University
Tokyo, Japan

A growing number of industrialized countries are examining the challenges associated with aging societies. Japan, faced with a rapidly aging population, has been a leader in research on this important topic and is a particularly appropriate setting for the Second International conference on Nutrition and Aging.

Conference Objectives

The conference will focus on the eating habits and societal and psychological eating attitudes of the elderly, as well as their nutritional status and the effects of nutrition on physiological changes associated with aging. The conference will evaluate the data and provide opportunities for discussion on:

- The current status of research on aging
- Nutritional requirements of the elderly
- Body changes and nutritional effects associated with aging
- Food product development appropriate for the elderly

Sponsored by International Life Sciences Institute (ILSI)
ILSI Research Foundation—Human Nutrition Institute
ILSI Europe
ILSI Japan
ILSI North America

For more information contact:

In Japan

ILSI Japan—Conference Secretariat
Koike Building
9-11-403, 2 Chome Umezato
Suginami-ku, Tokyo 166, Japan
Telephone: 81-33-318-9663, Telefax: 81-33-318-9554

Outside of Asia

Ms. Lili C. Merritt
International Life Science Institute
1126 Sixteenth Street, NW
Washington, DC 20036 USA
Telephone: 202-659-0074, Telefax: 202-659-3859
email: meetings@dc.ilsi.org

Position Announcements

Public Health Scientist
The Natural Resources Defense Council, a national nonprofit public interest organization, seeks a Senior Scientist to bring scientific analysis and knowledge to advocacy in various forums for the prevention of adverse health and ecological effects of toxic chemical pollution. A PhD or MD/MDH is required, with several years of experience in environmental or public health, or a related field. Candidates should be knowledgeable about cutting-edge toxics issues such as disproportionately impacted subpopulations, endocrine disruption, and other non cancer endpoints, and emerging issues regarding carcinogenesis. The position requires the established ability to keep abreast of scientific advances and work with the public health and academic communities. The ability to conduct outreach activities to build bridges with persons affected by toxics problems is also very important. The salary is $60,000 to $70,000, commensurate with experience. Send resume to: Public Health Program, NRDC, 1350 New York Avenue, NW, Suite 300, Washington, DC 20005. Equal Opportunity Employer.

Open Rank Faculty Position Announcement—Occupational and Environmental Exposure Assessment
University of Michigan invites applications for an open rank, tenure-track faculty position in Occupational and Environmental Exposure Assessment. The primary appointment will be in the School of Public Health, Department of Environmental and Industrial Health and will be at a rank and salary commensurate with experience.

Desired candidates will hold wither a PhD in industrial hygiene, epidemiology, environmental health, molecular genetics or other relevant disciplines or an MD with experience in such disciplines. Candidates should have an active interest in innovative and interdisciplinary solutions to theoretical and applied problems in exposure assessment in environmental and occupational settings. Examples of areas of interest include the application of environmental and occupational exposure assessment to exposure-response modeling and risk estimation, and the integration of measures of target organ dose in exposure modeling. Successful candidates will have a demonstrated ability to attract competitive external funding, to publish original research in the peer reviewed literature, and to teach at the graduate level including doctoral level students or medical students.

The University of Michigan actively encourages interest from women and minorities and is an Equal Opportunity/Affirmative Action Employer.

Letters of application, accompanied by a curriculum vitae, statement of research and teaching interest, and the names and addresses of three references should be sent to: Thomas Robins, MD, MPH, Associate Professor,
The University of Michigan School of Public Health,
Department of Environmental and Industrial Health,
1420 Washington Heights,
Ann Arbor, Michigan 48109-2029
e-mail: trobins@umich.edu
FAX (313) 763-8095.

Postdoctoral Research Opportunities at the National Institute of Environmental Health Sciences
Listed below are outstanding opportunities to conduct research with leading scientists in Research Triangle Park, North Carolina.

To apply, please send a cover letter, curriculum vitae, bibliography, and names of three references to the hiring scientist at the maildrop and laboratory listed using the following address: NIEHS, PO Box 12233, Research Triangle Park, North Carolina 27709. In your cover letter, list the position title and the HNV number.

Minorities, women and handicapped individuals are encouraged to apply. All applicants receive consideration without regard to race, religion, color, national origin, sex, physical or mental handicap, political affiliation, age (with statutory exceptions) or any other nonmerit factor. Positions are open until filled.

Molecular Mechanisms of DNA Repair (HNV88)
Miriam Sander
(919) 541-2799
Laboratory of Molecular Genetics,
Maildrop D3-04
Mechanisms of DNA repair in Drosophila are being investigated with focus on the *in vivo* and *in vitro* functions of Rrp1 (recombination repair protein 1). This protein is potentially important in DNA repair and homologous recombination. Future studies will include enzymatic, physical, and genetic characterization of Rrp1.

Mammalian Molecular/ Developmental Genetics (HNV89)
Steven S. L. Li
(919) 541-4253
Laboratory of Genetics,
Maildrop D3-05
The organization and developmental regulation of mammalian genes, including neurogenic genes, are being investigated. Applicants should have a strong background in genetics, biochemistry or molecular biology.

Molecular Neurobiology (HNV94)
J.S. Hong
(919) 541-2358
Laboratory of Environmental Neurosciences,
Maildrop E1-01
The signal transduction pathways regulating the expression of neuropeptide and cytokine genes in neural and glial systems are being investigated. Studies on the effects of neuropeptides on the biosynthesis and release of cytokines in microglial cells and potential roles of cytokines in neurodegeneration will be conducted. Applicants should have experience in neuropharmacology, neurochemistry or molecular biology.

Ion Homeostasis and Cell Injury (HNV95)
Elizabeth Murphy
(919) 541-3873
Laboratory of Molecular Biophysics,
Maildrop 17-05
Changes in ion transport and homeostasis appear to be involved in apoptotic cell death. Studies focus on measuring changes in intracellular calcium, pH, sodium and magnesium in isolated cells using fluorescent indicators in cells stimulated to undergo apoptosis. Alterations in

signal transduction pathways which are responsible for the ionic alternations are also under study.
Applicants must have experience in ion measurements using fluorescent indicators or experience with cell culture or molecular biology.

Molecular Dosimetry and Epidemiology (HNV96)
George W. Lucier
(919) 541-3802
Laboratory of Biochemical Risk Analysis,
Maildrop A3-02
Knowledge and techniques in molecular biology are applied to investigations designed to determine effects of low-dose exposures to environmental agents. Animal models, cell systems and human samples are used.
Studies encompass mutation analysis and signal transduction elements.

Molecular and Cellular Biology (HNV97)
Anton Jetten
(919) 541-2768
Laboratory of Pulmonary Pathobiology,
Maildrop D2-01
The action and function of several nuclear (orphan) receptors in the regulation of gene expression and differentiation are being investigated. Studies involve characterization of response elements, interaction with other transcriptional factors and gene knockouts. Applicants must have training in molecular biology techniques.

Mechanisms by Which Organisms Produce Mutations (HNV99)
Roel M. Schaaper
(919) 541-4250
Laboratory of Molecular Genetics,
Maildrop E3-01
Studies are aimed at understanding the mechanisms by which organisms produce mutations. Specific projects involve the isolation and molecular characterization of *antimutator* mutants in the bacterium *E. coli;* the genetic and biochemical analysis of DNA replication fidelity in this organism; and a structure-function analysis of the *dnaE* and *dnaQ* genes (encoding, respectively, the DNA polymerase and exonucleolytic proofreading activity).

Mechanisms of DNA Replication (HNV100)
William Copeland
(919) 541-4792
Laboratory of Molecular Genetics,
Maildrop E3-01
The regulation and mechanism of human DNA polymerases involved in the replication of nuclear and mitochondrial DNA is being investigated. Attention is on the mutation rate of the mitochondrial and nuclear genome by understanding the enzymology of the mitochondrial and nuclear DNA polymerases. Future studies will include the regulation of these essential enzymes in the cell.

Reproductive Biology and Toxicology (HNV104)
Masahiko Negishi
(919) 541-2404
Laboratory of Reproductive and Developmental Toxicology,
Maildrop E4-07
The molecular events underlying the abnormal development of the reproductive system associated with exposure to xenobiotic estrogens such as diethylstilbestrol (DES) are being investigated. Particular interest is the biochemical and molecular analysis of transient and permanent alterations in the estrogen-responsive (e.g., lactoferrin) and metabolizing (e.g., sulfotransferase) genes and the implications for human health disease.

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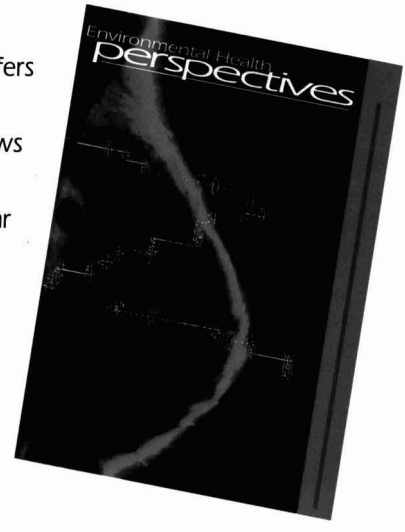

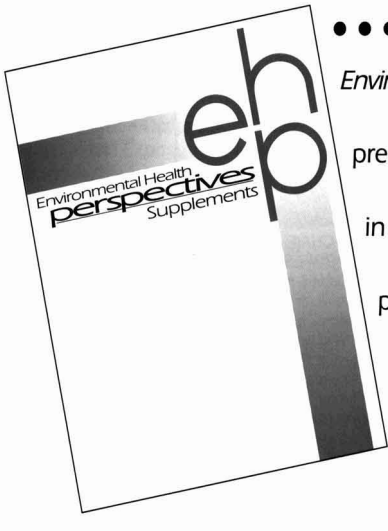

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Editorial Policy

Environmental Health Perspectives is intended to be a forum for the discussion of issues in environmental health, and several formats have been devised for that purpose. In addition, several formats are available for the publication of scientific articles and scientific discussion. All scientific articles are subject to peer review. The primary criteria for publication are environmental significance and scientific quality.

Environmental science is made up of many fields, and therefore we are prepared to consider scientific progress in all of them. Cross-fertilization and serendipity have proven to be extremely important processes in the advance of science in general, and this must hold true for the science of environmental health. We will consider for publication articles ranging from the most basic molecular biology to environmental engineering. We particularly encourage those researchers concerned with mechanisms of toxic action and new approaches for detecting and/or remedying environmental damage.

Opinions and ideas based on scientific observation and argument are welcome. While the expression of opinions may lead to debate and disagreement, such reactions are healthy and can lead to new research and discoveries. Presentations of ideas and opinions will be promoted, but our policy will be to strive for objectivity and balance.

In addition to scientific articles and discussion, we publish news of the environment. We will consider factual articles about issues that affect the environment and human health. We summarize legislative and regulatory developments, grant information from NIEHS and other granting agencies, new research areas, environmental problems, technological advances, and information about the National Toxicology Program and other important programs. Presentations of news strives for objectivity and balance and is based on the strength of scientific evidence.

Our policy is to give the corresponding author of each published article 200 free reprints.

Scientific Research

Scientific articles are subject to rigorous peer review. Two formats are available for the publication of scientific articles:

Research Articles are original manuscripts reporting scientific research and discovery in the broad field of environmental health. Research articles may come from any field of scientific research, from the most basic molecular biology and biochemistry to atmospheric physics, ecology, and engineering. The criteria for publication are weighted toward scientific quality and environmental significance. The work will be assessed according to its originality, scientific merit, and experimental design; the manuscript will be evaluated based on its conciseness, clarity, and presentation. We also attempt to address certain ethical problems during the review process. We require assurances that all human and animal subjects have been treated humanely and with due regard for the alleviation of suffering. Manuscript review also considers scientific integrity as part of the process.

Research Advances are concise articles intended to address only the most recent developments in a scientific field. Lengthy historical perspectives are not appropriate in this category. Clarity of presentation is of primary importance because these articles are intended to be educational though targeted to the expert audience.

Opinions, Ideas, Perspectives

The journal is a forum for the expression of ideas and opinions. Opinions and ideas should be carefully considered and based on scientific principles. Several formats are offered:

Editorial statements are published by our editors, members of our editorial boards, and occasional guest editors. These statements are intended to focus attention on important or neglected areas of environmental health, offer opinions and ideas, and stimulate discussion.

Reviews & Commentaries are up-to-date, narrowly focused review articles that may present commentaries offering perspective and insight on a particular topic. Only recent developments in a field should be addressed.

Correspondence is encouraged. Opinions, perspectives, and insight are welcome. Comments on articles published in *Environmental Health Perspectives* are also welcome, but criticism will always be balanced by the opportunity for defense and clarification. Letters to the Editor cannot exceed 1200 words.

Meeting Reports are short summaries of conferences, symposia, or workshops in which the scientific objectives and achievements of a meeting are described.

Environews

The news section provides up-to-date information on important issues in environmental health covering a variety of areas including policy, legislative, and regulatory actions; innovative technological and conceptual research advances; conference and meeting summaries; and emerging environmental problems. The news section consists of several components:

Forum articles are brief reports on matters of potential environmental health significance such as chemical spills and contamination episodes. Brief reviews of recent scientific advances are also included.

NIEHS News summarizes significant activities or accomplishments at NIEHS and the National Toxicology Program.

Focus articles are substantive news items about important issues in environmental health. Examples include reports on risk assessment, risk management dilemmas, women's health initiatives, environmental equity, relevance of animal models to toxicity testing, and structure–activity approaches to toxicity evaluation.

Spheres of Influence is a legal/regulatory column that presents reports on significant events and decisions involving the executive branch, Congress, and regulatory agencies. Examples include new directions of White House policies, impact of Clean Air Act legislation, and coverage of congressional hearings on environmental health issues.

Innovations presents emerging opportunities in environmental health based on new discoveries or approaches in biology, chemistry, engineering, or information sciences. Examples include the use of transgenic animals in toxicity testing, new advances in molecular biology, development of more rapid and efficient methods for clean-up of hazardous wastes, and methods for early detection of environmental damage and environmentally mediated diseases.

Announcements includes a calendar of upcoming events such as conferences, workshops, and public hearings. Appropriate listings are made for industrial, academic, regulatory, and legal activities. This section also includes listings of fellowship and grant announcements and positions available.

Environmental Health Perspectives Supplements

During the last 20 years, we have focused on the development of a series of monographs that have generally arisen from symposium or conference proceedings. We continue to publish monographs, but they now appear as supplements to the main journal. Six to eight supplements are published per year. Four to six of these consist of conference, workshop, or symposium proceedings, and two issues are dedicated to the publication of solicited and unsolicited comprehensive reviews on environmental health. All articles published in the supplements, regardless of their source, are peer reviewed.

Each supplement resulting from a conference, symposium, or workshop should address a specific problem, an area of concern, a research problem, or a particular scientific issue. Supplements will, in general, be dedicated to scientific issues and not programmatic themes. It is intended that each collection of manuscripts form a landmark statement for a particular subject. Each supplement must be an up-to-date, balanced source of reference material for researchers, teachers, legislators, and the informed public. Publication of conference proceedings in *Environmental Health Perspectives Supplements* requires the submission of a proposal as described in Instructions to Authors.

Supplement Articles from conferences are generally the result of research investigations, reviews, or a combination of both; however, brief reports and commentaries are also appropriate.

Perspective Reviews are targeted to the one or two specific issues of *Environmental Health Perspectives Supplements* set aside for the publication of reviews in environmental health sciences. Perspective reviews are in-depth, comprehensive review articles that address developments in specific scientific areas. Perspective reviews must not be simply a compilation of the literature. Perspective reviews should be scholarly, landmark statements offering a complete and balanced perspective as well as insight into the environmental significance of the research.

Instructions to Authors

To ensure fairness, objectivity, and timeliness in the review process, we routinely request three reviews. Therefore, authors must submit four copies of each manuscript. All manuscripts must conform to the instructions to authors; those that do not will be returned without review.

All manuscripts must be typed, double-spaced, in English. Type the article on white paper, 216 ×279 mm (8.5 × 11 in) or ISO A4 (212 × 297 mm), with margins of at least 25 mm (1 in). Type only on one side of the paper. Number pages consecutively, beginning with the title page. If the manuscript is accepted for publication, a computer disk copy must be submitted along with two hard copies of the revised manuscript. Organizers of conference, symposium, or workshop proceedings will receive 25 free copies of the published supplement. Corresponding authors will receive 100 free reprints after publication.

Organization of Manuscripts

Research Articles are manuscripts reporting scientific research and discovery in the broad field of environmental health and may come from any field of scientific research. Criteria for publication are weighted toward quality and environmental significance.

Title Page: List title, authors (first or second names spelled out in full), full address of the institution where the work was done, and affiliation of each author. Indicate author to whom galley proofs and reprints should be sent (include complete address for express mail service, telephone and FAX numbers).

Second Page: Provide a short title (not to exceed 50 characters and spaces) that can be used as a running head. List 5–10 key words for indexing purposes. List and define all abbreviations. Nomenclature and symbols should conform to the recommendations of the American Chemical Society or the International Union of Pure and Applied Chemistry (IUPAC). Include acknowledgments and grant information.

Abstract: Place a double-spaced abstract on the third page. The abstract should not exceed 250 words. The abstract should state the purpose of the study, basic procedures, main findings, and the principal conclusions. Emphasize new and important aspects of the study or observations. The abstract should not include details of materials and methods or references.

Introduction: Begin the introduction on a new page. State the purpose of the research and give a brief overview of background information. Do not include data or conclusions from the work being reported.

Methods: Begin on a new page. Describe the materials used and their sources. Include enough detail to allow the work to be repeated by other researchers in the field or cite references that contain this information.

Results: Begin on a new page. Present your results in logical sequence in the text. Do not repeat materials and methods, and do not repeat data in tables or figures. Summarize only important observations. Results and Discussion may be combined if desired.

Discussion: Begin this section on a new page. Emphasize new and important aspects of the study and the conclusions that follow. Relate results to other relevant studies. Do not simply recapitulate data from the Results section.

References: Begin this section on new page. References are to be numbered in order of citation in the text and should be cited in the text by number in parentheses. The style for references is as follows:

Journal Article:

1. Canfield RE, O'Connor JF, Birken S, Kirchevsky A, Wilcox AJ. Development of an assay for a biomarker of pregnancy in early fetal loss. Environ Health Perspect 74:57–66 (1987).

Book Chapter:

2. Lohman AHM, Lammers AC. On the structure and fiber connections to olfactory centers in mammals. In: Progress in brain research: sensory mechanisms, vol 23 (Zotterman Y, ed). New York:Elsevier, 1967;65–82.

Book:

3. Harper R, Smith ECB, Jones DB. Odour description and classification. New York: Elsevier, 1968.

Editor as Author:

4. Doty RL, ed. Mammalian olfaction, reproductive processes, and behaviour. New York: Academic Press, 1976.

Conference Proceedings:

5. Ames B, Shigenaga MK, Gold LS. DNA lesions, inducible DNA repair, and cell division: three key factors in mutagenesis and carcinogenesis. In: Proceedings of the conference on cell proliferation, 14–16 May 1992, Research Triangle Park, NC. New York:Xavier, 1993; 35–44.

Government Report:

6. Melvin DM, Brooke MM. Laboratory procedures for the diagnosis of intestinal parasites. Report no. 75-8282. Atlanta, GA:Centers for Disease Control, 1974.
7. U.S. EPA. Status of pesticides in reregistration and special review. EPA 738-R-94-008. Washington, DC:Environmental Protection Agency, 1994.

Other Publications:

8. IARC. Arsenic and arsenic compounds. In: IARC monographs on the evaluation of carcinogenic risk of chemicals to man, vol 23. Some metals and metallic compounds. Lyon: International Agency for Research on Cancer, 1980;39–141.
9. Spiegelhalder B, Preussmann R. Nitrosamines and rubber. In: N-nitroso compounds: occurrence and biological effects (Bartsch H, O'Neill IK, Castegnaro M, Okada M, eds), IARC scientific publications no. 41. Lyon:International Agency for Research on Cancer, 1982;231–243.

Abbreviate journal names according to *Index Medicus* or *Serial Sources for the BIOSIS Previews Database*. List all authors; do not use et al. in the bibliography. Include the title of the journal article or book chapter and inclusive pagination. References to papers that have been accepted for publication but have not yet been published should be cited in the same manner as other references, with the name of the journal followed by "in press." Personal communications, unpublished observations, manuscripts in preparation, and submitted manuscripts should not be listed in the bibliography. They are to be inserted at appropriate places in the text, in parentheses, without a reference number.

Figures and Legends: Three sets of publication-quality figures are required. Graphs and figures should be submitted as original drawings in black India ink, laser-printed computer drawings, or as glossy photographs. Electronic versions of figures are encouraged, but should be submitted in addition to, not in lieu of, hardcopies of the figures. Dot matrix computer drawings are not acceptable as original art. The style of figures should be uniform throughout the paper. Letters, numbers, and symbols must be drawn to be at least 1.5 mm (6 points) high after reduction. Choose a scale so that each figure may be reduced to one-, two-, or three-column width. Identify all figures on the back with the authors' names and figure number; indicate TOP. Color figures will be considered for publication if the color facilitates data recognition and comprehension.

Figure legends should be typed on a separate page following the references. Legends should be numbered with Arabic numerals.

Tables: Each table must be on a separate page. Tables should be numbered with Arabic numerals. General footnotes to tables should be indicated by lowercase superscript letters beginning with a for each table. Footnotes indicating statistical significance should be identified by *, **, #, ##. Type footnotes directly after the table. Complex tables should be submitted as glossy photographs.

Computer Disks: Electronic copies of initially submitted manuscripts are not required. Revised manuscripts resubmitted after acceptance for publication must be sent in electronic form together with two hard copies.

Submit electronic formats on 3.5" disks suitable for reading on either PC or Macintosh platforms. Macintosh is the preferred platform, although PCs are acceptable. The file should contain all the parts of the manuscript in **ONE** file.

Label the outside of the disk with the title of the manuscript, the authors, and the number it has been assigned. Name the computer used (e.g., IBM, IBM compatible, Macintosh, etc.) and the operating system and version (e.g., DOS 3.3). Identify the word processing program and version. Microsoft Word format is preferred, and its use will greatly facilitate publication; however, we can convert other formats, including: Microsoft Word, WordPerfect, ASCII, Text Only.

Research Advances are concise articles intended to address only the most recent developments in a scientific field. Lengthy historical perspectives are not appropriate. Begin with the title

page and continue as described for research articles. References, abbreviations, figures, and tables should be handled as described for research articles. Clarity of presentation is of primary importance and the use of color figures is encouraged. Include a photograph (black and white or color) of the author together with a brief biography. If multiple authors or groups are involved, up to three biographies with photographs may be included.

INNOVATIONS are short articles that describe novel approaches to the study of environmental issues. Prepare initial pages as described for Research Advances. Maintain text in a clear and precise manner and wherever possible include color photographs to illustrate strategy and clarify conceptual problems. Some degree of speculation regarding the potential usefulness of a new technique or novel process in other areas of environmental health may be included. References should not be included, but a suggested reading list is required.

COMMENTARIES are short articles offering ideas, insight, or perspectives. Begin with a title page and second page as described for research articles. Include a brief abstract.

REVIEWS & COMMENTARIES are brief, up-to-date, narrowly focussed, review articles with commentaries offering perspective and insight. Begin with a title page and second page as described for Research Articles. Include an abstract and handle references, tables, figures, and abbreviations as described for Research Articles.

MEETING REPORTS should not exceed 2400 words in length. Begin with the title of the meeting and authorship of the report and start text on the next page. Detail when and where the meeting was held, how many people participated, who sponsored the meeting, and any special organizational arrangements. Meeting sponsors and principal participants, such as session chairs, may be listed on a separate page. The report should summarize the contributions of the meeting to scientific knowledge, insight, and perspective; this should not take the form of comments of participants or personalized perspectives. Space is limited, so only the highlights should be mentioned. Novel ideas, perspectives, and insights should be emphasized. Do not describe social aspects of the meeting. Send an electronic copy and four hard copies.

ENVIRONMENTAL HEALTH PERSPECTIVES SUPPLEMENTS

SUPPLEMENT MANUSCRIPTS result from conferences, symposia, or workshops and may take several forms. 1) Manuscripts reporting original research should be formatted as described for Research Articles, 2) opinions and discussion about a particular topic should be formatted as described for Commentaries, 3) manuscripts reviewing a topic or reporting a combination of review and original research should be formatted as described below for Perspective Reviews.

PERSPECTIVE REVIEWS are in-depth, comprehensive reviews of a specific area. They should begin with a title and second page as described for research articles. Introduction and presentation of information should be continuous with specific items and discussion indentified by using subheadings. Abstracts, references, abbreviations, figures, and tables should also be handled as described for research articles.

PROPOSALS for the publication of conference, symposium, and workshop proceedings will be considered; however, space is limited. We turn away many excellent proposals simply because we do not have space to publish them.

All proposals are reviewed and examined with a number of specific questions in mind. In developing a proposal, consider the following: Proposals are assessed according to their originality and scientific merit. Is the supplement needed? Is the subject matter timely and potentially useful to workers in the field? What is the environmental significance of the topic being addressed? Is the proposed supplement a complete representation of the field? Are there other aspects that should be included? Does the proposal contain sufficient information for evaluation? Is the presentation clear? Can the organizers integrate the participants into a cohesive unit? Are the contributors appropriate for the topic listed and do they have scientific credibility?

The source of funding is also considered. Scientific objectivity is extremely important, and it must be clear that organizers are not being used to present a bias favored by the funding body. Contributions from an interested party to a conference need not disqualify a proposal, but it is appropriate that the major source of funding be from a disinterested source or that organizational safeguards be set in place to minimize the intrusion of institutional bias.

All proposals must be submitted at least six months in advance of the conference. In the publication of conference proceedings, timeliness is essential. Because it takes at least six months to publication, no proposal will be considered after the conference has been held.

SUBMISSION OF MANUSCRIPTS AND PROPOSALS

Submit all manuscripts and proposals in quadruplicate to:

Editor-in-Chief
Environmental Health Perspectives
National Institute of Environmental Health Sciences
PO Box 12233
111 Alexander Drive
Research Triangle Park, NC 27709 USA

In your covering letter please provide assurances that the manuscript is not being considered for publication elsewhere and that all animals used in the research have been treated humanely according to institutional guidelines, with due consideration to the alleviation of distress and discomfort. If the research involved human subjects then a statement must be made to the effect that participation by those subjects did not occur until after informed consent was obtained.

Permission to reprint figures or tables from other publications must be obtained by the author prior to submission of the manuscript.

Finally, a statement must be made indicating that all authors have read the manuscript and are in agreement that the work is ready for submission to a journal and that they accept the responsibility for the manuscript's contents.

Inquiries may be made by calling (919) 541-3406 or by FAX at (919) 541-0273.

SUBMISSION OF NEWS INFORMATION

Environmental Health Perspectives welcomes items of interest for inclusion in the Environews, Calendar of Events, and Announcements sections of the journal. All items are published subject to the approval of the Editors-in-Chief. All submission for these sections should be sent to the attention of:

Associate News Editor
Environmental Health Perspectives
National Institute of Environmental Health Sciences
PO Box 12233
111 Alexander Drive
Research Triangle Park, NC 27709 USA

Items submitted for inclusion in the Forum section must not exceed 400 words. Items may be edited for style or content, and by-lines are not attached to these articles. If possible, items should be submitted on computer disk using WordPerfect or Microsoft Word, in straight text without formatting.

Items received for the Calendar of Events will be published in as timely a manner as possible, on a space-permitting basis. Submissions should include all relevant information about the subject, date, time, place, information contact, and sponsoring organization of the event.

Position announcements will be limited to scientific and environmental health positions and will be run on a space-permitting basis. Although we seek to publish all appropriate announcements, the timeliness of publication cannot be guaranteed.

Public information advertisements will be run free-of-cost as space becomes available. All ads are run subject to their appropriateness to the editorial format of the journal. Submissions of advertisements should include full-page, half-page, and quarter-page formats if available. Ads should be camera-ready, black and white positives.

Persons interested in free-lance writing opportunities with Environmental Health Perspectives should submit a cover letter, resume, and writing samples to the address above. For inquiries call the associate news editor at (919) 541-5377.

2nd World Congress on Alternatives and Animal Use in the Life Sciences

October 20–24 1996, Utrecht, The Netherlands

Programme topics:

- Alternatives in :
  - Basic research
  - Toxicology
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