Environmental Health Perspectives

Volume 103
Number 4
Pages 309–408

Journal of the National Institute of Environmental Health Sciences

April 1995

DISCRIMINATION PROHIBITED–Under the provisions of applicable public laws enacted by Congress since 1964, no person in the United States shall, on the grounds of race, color, national origin, handicap, or age, be excluded from participation in, be denied the benefits of, or be subjected to discrimination under any program or activity (or, on the basis of sex, with respect to any educational program or activity) receiving Federal financial assistance. In addition, Executive Order 11141 prohibits discrimination on the basis of age by contractors and subcontractors in the performance of Federal contracts, and Executive Order 11246 states that no federally funded contractor may discriminate against any employee or applicant for employment because of race, color, religion, sex, or national origin. Therefore, the National Institute of Environmental Health Sciences must be operated in compliance with these laws and Executive Orders.

Environmental Health perspectives
Journal of the National Institute of Environmental Health Sciences

Volume 103
Number 4
April 1995

Contents

Environmental Health perspectives

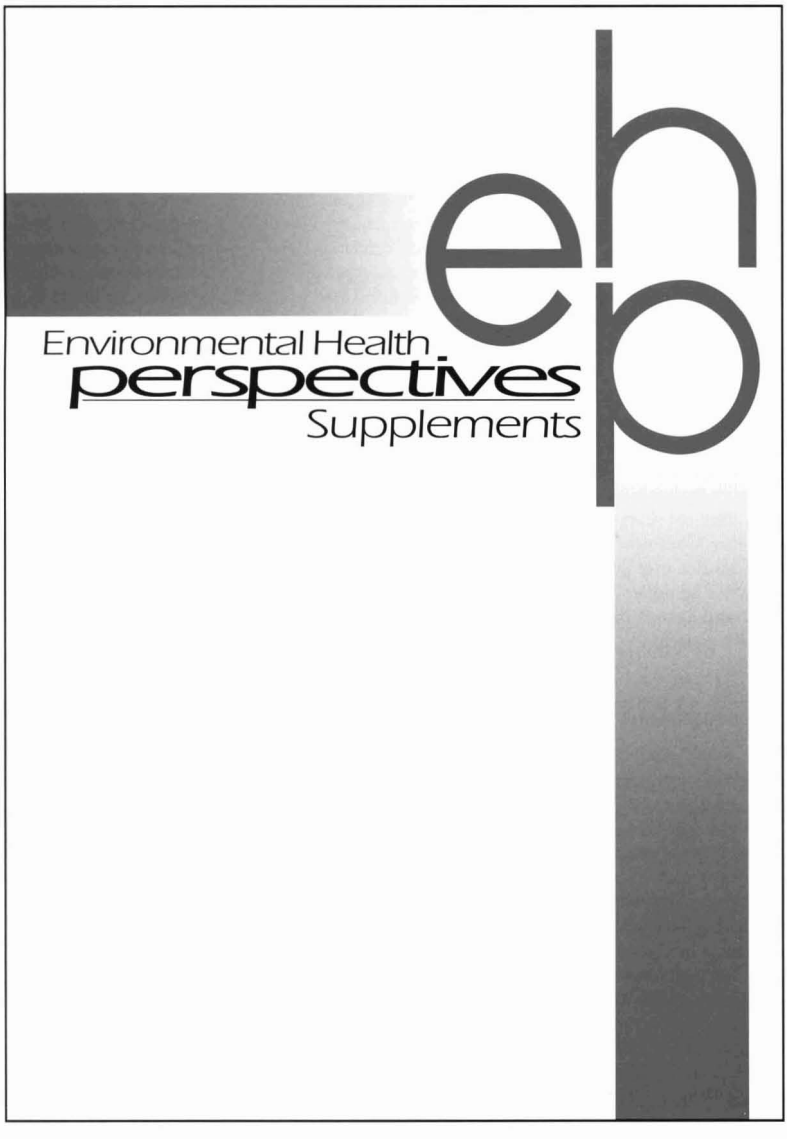
Journal of the National Institute of Environmental Health Sciences

Volume 103
Number 4
April 1995

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 402.

Environmental Health Perspectives has been deemed by the Secretary of Health and Human Services to be necessary in the transaction of the public business required by law of this department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget. All material published in *EHP* is in the public domain; permission to reproduce any portion of its contents is, therefore, not necessary. Questions concerning news information submission and inquiries on other editorial matters should be addressed to the Editors-in-Chief, *Environmental Health Perspectives*, National Institute of Environmental Health Sciences, PO Box 12233, Research Triangle Park, NC USA 27709 (telephone: 919-541-3406).



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. 402

In This Issue

Yellow Light on Green Products

Commercial products touted as environmentally friendly are appearing more frequently on the market shelves (p. 328). Ranging from antifreeze to household cleaners, these products claim to replace potentially harmful ingredients with benign ones. This **Focus** article warns that consumers should take a close look at these "green" products: reformulated products can still be toxic, cost more, and may be ineffective.

A New Hill

Many changes are taking place in Congress, and this means changes for environmental policy. This month's **Spheres of Influence** (p. 332) examines changes in membership of key committees, such as the Transportation and Infrastructure Committee, which has jurisdiction over the Clean Water Act, and the House Commerce Committee, which has jurisdiction over the Safe Drinking Water Act. The possible effects of new priorities and agenda on pending environmental legislation are explored.

Mechanistic Risk Assessment

The **Innovations** article (p. 334) defines mechanism-based toxicology and explores the questions it raises, along with its advantages for assessing risks of potential chemical carcinogens. Chemically induced cancer is thought to occur by a combination of processes including heritable mutations and epigenetic changes resulting in clonal expansion of cells that have a heightened probability of subsequent mutations. Mechanism-based toxicology, which involves measurement and analysis of mutational changes, receptor-mediated effects, pharmacodynamics and pharmacokinetics, biomarkers, and structure–activity analysis, could be used to rapidly screen chemicals and set priorities for studying suspected chemical carcinogens. However, scientists caution that mechanistic data should be used to supplement, not replace, current testing strategies such as chronic animal bioassays and epidemiology. The challenge for scientists and regulators is to determine the best and most cost-efficient methods for collecting risk assessment data.

Learning How Cells Talk

This reprint of a Nobel lecture, "Signal Transduction: Evolution of an Idea," provides a fascinating opportunity to look into the mind of a Nobel laureate and see an original idea borne, ripen, and come to fruition (p. 338). Martin Rodbell, awarded the Noble Prize for Physiology or Medicine in 1994, shares his scientific life between the 1960s and the 1990s. He describes the development of the concept of transducers and their role in cell signaling and concludes with a hypothesis arguing that biological communication requires a complex network of structures, containing, but not necessarily limited to, G-proteins, surface receptors, the extracellular matrix, and a vast cytoskeletal network, all joined together in a community of effort.

The Environmental Estrogens Debate

The concern about estrogenic-acting chemicals in the environment or natural estrogenic chemicals in the diet has received widespread attention in the media. Numerous scientific articles document estrogenizing chemical effects on wildlife species, raising questions about the potential impact of these chemicals on human health. Safe (p. 346) reviews this controversy and concludes that the supposed linkage between dietary and/or environmental estrogens and increases in breast cancer or reductions in sperm count is unproven. Safe suggests that further research is needed before corrective actions are initiated.

Where Are the Frogs?

Boyer and Grue (p. 352) present the argument that declining populations of frogs not only spell trouble for amphibians in general but also for humans, since frogs are considered reliable indicators of environmental quality and, in particular, of water quality. Humans currently consume 65% of the available fresh water on the planet for agricultural use. Our ever-expanding population dictates that sharing and recycling of this precious resource is necessary, and therefore it is vital that the quality of water remains high. The authors suggest that preservation of water quality is necessary for the survival and optimal co-existence of all species.

Nonionic Surfactants

Cserháti (p. 358) describes unique molecules known as surfactants, which are configured so that one end is soluble in water and the other end is not. This property enables nonionic surfactants to interface with innumerable proteins, peptides, amino acids, and phospholipids found in living organisms. The author reviews the effects of surfactants on the structure and activity of biological membranes and emphasizes their function as biological facilitators.

Dioxin Activates HIV-1 Gene

Yao et al. (p. 366) show that induction of oxidative enymes by dioxin stimulates a pathway that generates reactive oxygen intermediates responsible for activation of genes linked to the long-terminal repeat of the human immunodeficiency virus type 1 (HIV-1). The data suggest potential explanations for observations that dioxin increases infectious HIV-1 titers in experimental systems, as well as explanations for epidemiological reports alluding to an association between aromatic hydrocarbon exposure and acceleration of AIDS in humans.

Nitrous Acid and Asthmatics

Nitrous acid is a component of photochemical smog and a common indoor air pollutant released as primary combustion products from gas stoves and portable kerosene heaters. Beckett et al. (p. 372) exposed 11 mildly asthmatic subjects to 650 ppb nitrous acid during three 20-minute periods of moderate exercise. Respiratory impairment was most marked after 25 minutes of exposure and at 85 minutes after onset of exposure. The concentration of nitrous acid and duration of exposure was not outside the range measured in some homes over a 24-hour period or longer.

Apples and Peanut Butter

Finkel (p. 376) reviews the toxicity and exposure data for the food mold aflatoxin, notorious for its role in the great peanut butter debate, and for the pesticide Alar, notorious for the "apple a day keeps the doctor busy" debate, as examples to evaluate the uncertainties of comparative risk assessment. The discussion of relative risks for both aflatoxin and Alar is influenced by their assumed association with children's health, and the compounds have been analyzed by such well-known scientists as Ames and Abelson. Finkel suggests that proper risk assessment must include an estimate of lower and upper bounds of each risk ratio, and when one risk is greater than another, communication of this fact should be reinforced, or if contrariwise, this information should also be stated forthrightly.

International Congress on Hazardous Waste: Impact on Human and Ecological Health

JUNE 5-8, 1995

Marriott Marquis Hotel, Atlanta, Georgia

Sponsored by
U.S. Department of Health and Human Services
Public Health Service
Agency for Toxic Substances and Disease Registry

The Agency for Toxic Substances and Disease Registry and Emory University are hosting an international congress to promote the exchange of findings, ideas and recommendations related to the human and ecological health effects of hazardous waste. The conference will include morning plenary sessions, afternoon platform presentations and evening poster sessions. Deadline for registration is May 15, 1995.

SESSIONS TOPICS:

- EXPOSURE
- HEALTH EFFECTS
- ECOLOGICAL EFFECTS
- EPIDEMIOLOGICAL STUDIES
- RISK ASSESSMENT
- BIOMARKERS
- REGISTRIES
- MODELING
- SOURCE REDUCTION
- SITE REMEDIATION
- META-ANALYSIS
- COMMUNITY INVOLVEMENT
- POLICY

FOR FURTHER INFORMATION CONTACT:

John S. Andrews, Jr., M.D., M.P.H.
Associate for Administrator for Science
Agency for Toxic Substances and Disease Registry
1600 Clifton Road, NE (E-28)
Atlanta, Georgia 30333
Phone 404-639-0708
Fax 404-639-0586
Internet: JSA1@ATSOAA1.EM.CDC.GOV

Attitudes Affect Behavior

Despite the fact that no major piece of environmental legislation was passed in 1994, the environmental movement is not receding. Some deny that environmental problems exist, while others say that they exist but have progressed too far for intervention to be effective. The truth lies somewhere in between. Environmental problems such as acid rain, ocean pollution, destruction of rain forests, depletion of the ozone layer, indiscriminate dumping of hazardous wastes, and misuse of land and water cannot continue indefinitely, and there is no "quick fix." Who can doubt that the major contributors to the degradation of our environment are humans? Environmental problems as instigated by humans are primarily "behavioral problems" as defined by Skinner (*1*) and are, therefore, subject to examination by using applied behavior analysis (*2*). Skinner believed that human behavior is determined to a large extent by its consequences (*3*) and not simply through the transfer of information or advice. That is true of children and nations when the advice involves futures that lie beyond the immediate horizon. Few people would be expected to change behavior based simply on the advice of others but when their quality of life is affected by environmental problems, such as water shortages or air pollution, change can occur very quickly. The mediating factor here is the change in attitude. Attitude change can effectively and quickly lead to changes in behavior. These principles seem to apply not only to individuals; it is becoming increasingly evident that they may apply also at the national and international levels.

There is evidence that attitudes toward the environment are changing. For example, there is little doubt that the earth's climate is undergoing some marked alterations. This knowledge is becoming commonplace as a result of media attention to new information about climate change. Hardly a week goes by that newspapers do not point out some manifestation of change involving the weather, and now much of the public is beginning to believe what atmospheric scientists have been saying for years. Also, extreme weather conditions experienced all over the world have forced us to pay attention to the possibility that something is happening and to the probability that we might be responsible. In June 1992, the Framework Convention on Climate Change was signed by 154 heads of state and governments at the Earth Summit in Rio de Janeiro, Brazil. This was an extraordinary achievement, representing the first time that national governments have acted together to indicate their intention to control widespread and economically important activities in an attempt to reduce risks to the global environment. An attitude change of major proportions has been witnessed, and it seems likely that behavioral changes will follow.

The United States is the home of some of the most influential environmental organizations in the world; however, we are also the source of some of the major global climate changes. For example, we are the largest producer of carbon dioxide and other greenhouse gases (*3*). The path that we have taken to economic well-being is the path that developing nations of the world wish to emulate. Our example is a driving force for many nations as they seek economic improvement and parity. Our attitude has a similar affect on others, and in this regard we must try to be the first to demonstrate that economic development can be achieved without degradation of the environment. Our attitude is of primary importance in how those countries respond to the issues. The bottom line is that the United States must recognize that in order to change the attitude of other nations toward reducing damage to the environment, we have to provide the lead and moderate our own.

This month, on Earth Day, we celebrate the second anniversary of the revised *Environmental Health Perspectives* and commemorate the occasion with the cover showing the earth that appeared on our premiere issue. Once a year we use this picture to remind us of our goals and the interconnectedness of all life and the finite capacities of the planet.

Environmental Health Perspectives is the journal of the National Institute of Environmental Health Sciences, and although our goals are tied to those of the institute and include human health and the environment, they extend beyond these immediate objectives and address the complete spectrum of life and the environment. The goals of the journal include not only understanding the mechanisms through which xenobiotics interfere with the biochemistry of the cell and living systems but extend across the complete range of environmental health sciences from atmospheric physics and engineering to the most basic biochemistry and molecular biology.

In this last year we have attempted to address numerous items of critical concern to environmentalists and other scientists. We have examined environmental issues facing developing nations around the world. We have focused on the controversies surrounding environmental estrogens and celebrated the discovery of the *BRCA1* gene. We were especially proud to feature a cover with our own Martin Rodbell, who received the Nobel Prize in Physiology or Medicine for his discoveries in the field of signal transduction. In the *EHP Supplements* we have published foundation volumes in the fields of oxygen radicals, ecotoxicology, risk assessment, boron, and mechanisms of metal toxicity.

The question for us continues to be what can we do, as a journal, to encourage changes in attitude toward the environment? Our task is to communicate scientific information. While information alone may not be the prime mover in changing attitudes toward environmental issues, it seems reasonable to suppose that once a change is imminent, perhaps because of other more threatening circumstances, information must play a primary role in formulating the final shape of that attitude. In this last year we began a policy of giving the journal free of charge to any scientific institution in any developing country. At present *EHP* is sent to nearly 2000 institutions in developing nations around the world. In this way we are working to ensure that the issues are presented to as wide an audience as possible.

The next stage is to make the journal even more accessible. In this next year we will bring the journal on-line and make it accessible over the Internet. We hope to enhance the exchange of scientific information by making the journal as interactive as possible. We will explain difficult issues using the clarifying power of multimedia expression. We will also run electronic conferences so that people around the world can attend without leaving their laboratories or offices. In addition, such conferences will enable the general public to ask questions directly of scientists actively involved in the examination of some of the difficult issues facing us in the world today. The dissemination of scientific information about environmental health issues will be increased by orders of magnitude, and, hopefully, we will see attending changes in colleagues and the public as their attitudes are shaped by understanding.

Gary E. R. Hook and George W. Lucier
Co-Editors-in-Chief

References

1. Skinner BF. Upon further reflection. Englewood Cliffs, NJ: Prentice-Hall Inc., 1987.
2. Geller ES. The human element in integrated environmental management. In: Implementing integrated environmental management. (Cairns J, Crawford TV, Salwasser H, eds). Blacksburg, VA:Virginia Polytechnic Institute and State University, 1994;5–26.
3. Nitze WA. A failure of presidential leadership. In: Negotiating climate change: the inside story of the Rio Convention (Mintzer IM, Leonard JA, eds). Cambridge, MA:Cambridge University Press, 1994;187–200.

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:

Secretaria del Congreso
Instituto Nacional de Seguridad e Higiene en el Trabajo
Calle de Torrelaguna, 73
E-28027 Madrid-Spain
Tel. 34-1-404 57 36
FAX 43-1-326 78 55

Earth Day will have to be extended to Earth Year, Decade, Generation if the poisoning of water, air, and soil is to be halted or even appreciably slowed down.

Eric Sevareid
CBS Evening News
22 April 1970

Forum

Mission to Planet Earth

Stark images of earth from 135 miles away can help health officials in Mexico pinpoint outbreaks of malaria before they occur, distinguish deadly oil spills from the viscous sheen produced by plankton, and warn people when the crops they eat might contain radiation. These are but a few of the actual and expected pay-offs from the recent shuttle flights run by the National Aeronautics and Space Administration (NASA) under the rubric of Mission to Planet Earth (MTPE).

The mission is a campaign to study the earth as a global environmental system that will continue into the 21st century. Some of the program's signature flights last year are yielding tangible results for scientists interested in the relationship between environmental change and human health, said Robert Harris, director of the science division of MTPE at NASA headquarters in Washington.

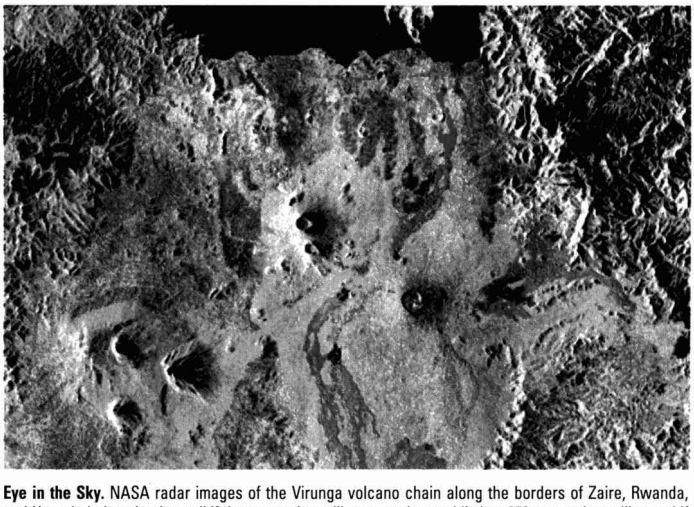
"We need to devote most of 1995 to analyzing mountains of data from highly successful MTPE shuttle flights in 1994," said Harris, who thinks the latest MTPE data can impress Congress and the public into supporting the revamped, streamlined program. Harris points, for example, to data from two 10-day shuttle flights in April and October that revealed the earth as it might be seen without vegetation and scoured its lower atmosphere for evidence of deterioration. The flights, called Space Radar Laboratory I and II, featured two MTPE instruments: the U.S.–German–Italian Spaceborne Imaging Radar-C/X-Band Synthetic Aperture Radar (SIR-C/X-SAR), and a device known as MAPS (Measurement of Air Pollution from Space).

The $400 million radar, the most sophisticated of its kind, has the ability to make measurements of the globe over virtually any region at any time, regardless of weather conditions. It can "see" through vegetation cover to map the earth's crust, oceans, and watersheds. "Our main goal is to monitor the earth's health and see how it is doing," said the project's chief scientist, Diane Evans, of the Jet Propulsion Laboratory. The radar can forecast natural disasters by tracking pools of water that form in depressions on a volcano's slope, and can pick out natural pollutants, like algae scum, from man-made hazards like oil spills. It can trace environmental disasters, such as damage to wetlands left by the Chernobyl nuclear power plant explosion.

The data can even predict the outbreak of some diseases, said Evans. University of California at Fresno Professor Jack Paris uses radar data to develop models to predict when stagnant water will produce mosquitoes that carry malaria. So far, he and other researchers have forecast conditions in both Belize and on Mexico's Yucatan Peninsula that produced outbreaks. Such information can also be used to predict outbreaks of yellow fever and snail-borne schistosomiasis, a parasitic infection that is a major health problem in Asia, Africa, and South America. "It's an incredibly useful resource for developing countries," Paris said.

The MAPS instrument measures the global distribution of carbon monoxide in the lower atmosphere. Measuring carbon monoxide indicates how well the atmosphere can cleanse itself of greenhouse gases that can increase the atmosphere's temperature, said project scientist Vicky Connors, of NASA's Langley Research Center. It also assesses loss of ozone protection against ultraviolet radiation, she said. "If ozone is depleted, as we see it can be in the southern hemisphere, UV increases can result in increased skin cancer, but also in DNA damage in crop plants—a very compelling concern." New data released by NASA in December and again in January confirms that the atmosphere is slowly warming and that seas are rising one-tenth of an inch each year. The findings combine Space Radar Laboratory flights data with other information, including information from a third MTPE shuttle flight last year, the Atmospheric Laboratory for Applications and Science (ATLAS).

So far, the MAPS instrument is slated to fly on a satellite in two years, said Harris, but the future of SIR-C/X-SAR is uncertain. It will have to compete with other MTPE instruments vying to fly on a small platform in 1998. Although NASA spent $1.2 billion on the MTPE last year and is expected to spend another $1.3 billion this year, its scope has been considerably scaled down. Now, instead of grand space platforms from which arrays of instruments would fly by the year 2000, smaller, cheaper platforms are being designed to appeal to Congress, which



NASA/JPL/SIR-C/X-SAR Team

Eye in the Sky. NASA radar images of the Virunga volcano chain along the borders of Zaire, Rwanda, and Uganda help scientists tell if the vegetation will support the world's last 650 mountain gorillas and if eruptions may threaten area villages.

slashed the initiative three times this decade—from $17 billion to $7.5 billion—saying it was too risky and unjustifiably expensive. Harris is worried it will happen again: "Any more cuts and we will drastically reduce our objectives and probably lose some international partners who are helping us fund it now."

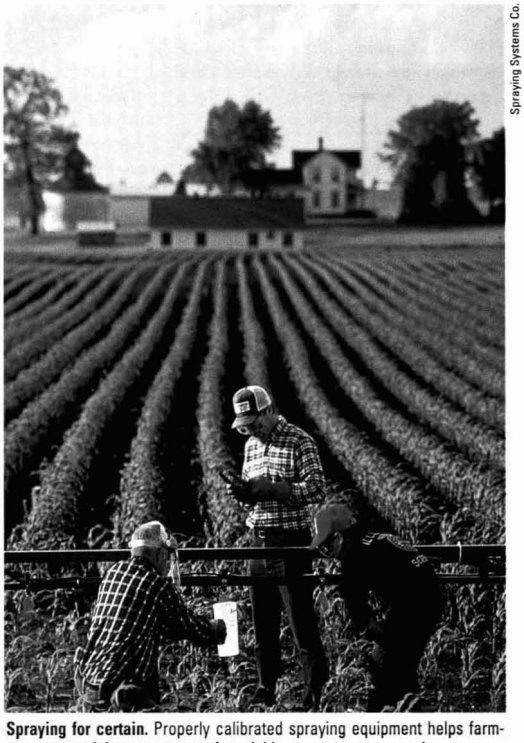
Decline in Pesticide Use by Canadian Farmers

Farmers in Ontario are spraying smaller amounts of pesticides on their crops than in the past. From 1983 to 1993, pesticide use dropped by a dramatic 28.3%, according to the Ontario Ministry of Agriculture, Food, and Rural Affairs (OMAFRA). By comparison, pesticide use in the United States fell 15% between 1982 and 1992. Under the Food Systems 2002 project, OMAFRA has worked with farmers and agricultural and environmental groups since 1987 to cut pesticide use in half by the year 2002.

Declining North American pesticide use resulted, in part, from the application of environmentally "safer" chemicals, although greater amounts of such chemicals might be necessary to achieve the same effect. Today, Ontario farmers use one-third as much atrazine on corn crops to control quackgrass as they did 10 years ago. This one million kilogram decrease represents nearly half of Ontario's total reduction in pesticide use. Many Ontario growers, concerned about atrazine's environmental persistence, eliminated fall applications. On many farms, newer, short-lived herbicides, sprayed at rates of grams per acre rather than kilograms per acre, have replaced atrazine, said Ken Hough of the Ontario Corn Producers Association. There is evidence of a similar trend in the United States, where reduced herbicide use accounts for over 60% of the total decline in pesticide use.

In the early 1980s, the farming community's rising concern about escalating pesticide applications prompted the Ontario government to support lower pesticide use, said Jeff Wilson, chairman of AGCare (Agricultural Groups Concerned About Resources and the Environment), which represents 45,000 growers in Ontario. "It was an evolutionary process, beginning with initiatives from growers," said Wilson, "without a Big Brother or heavy-hand syndrome." Bruce Archibald, manager of OMAFRA's Environmental Health Program, concurs. "It was a win–win situation, with a shift in thinking on the part of the growers and the government providing resources."

Archibald credits much of Ontario's reduced pesticide use to the certification program for purchasing pesticides. Initiated as a voluntary program in 1988, certification became mandatory throughout Ontario in 1991 at the growers' request. To become certified, growers attend a full-day course on proper label reading, mixing, and applying pesticides, and pass an exam every three years. Before this awareness-raising initiative, said Wilson, farmers applied up to 20% more pesticide to their crops than necessary because of poorly calibrated spraying equipment. In the United States, applying a "restricted use" pesticide requires a license. However, many commonly used pesticides are not restricted, and licensing requirements, developed by each state, vary throughout the country.



Spraying Systems Co.

Spraying for certain. Properly calibrated spraying equipment helps farmers ensure minimum amounts of pesticides necessary are used.

Ontario's certification course also introduces the principles of integrated pest management (IPM), such as crop rotation, mechanical pest removal, use of natural pest predators, and targeted use of pesticides, to help Ontario farmers manage pests more efficiently. During the growing season, farmers using IPM monitor their crops for the appearance of pests or weeds. When pest infestation exceeds a threshold level, the farmer applies a pesticide specifically targeted for that pest. This approach contrasts with traditional methods of applying a broad-spectrum pesticide several times a year.

Wilson, a potato and apple grower, pays $14 an acre to have scouts monitor his crops for pests. A pesticide specialist provided by OMAFRA sets threshold criteria and advises him on the timing of pesticide applications. Using this service has eliminated two pesticide sprayings per growing season, which, he said, saves money and protects the soil and water from excess toxic chemicals.

In 1993, the Clinton administration set a goal of implementing IPM practices on 75% of U.S. cropland by the year 2000. By the end of the 1993 fiscal year, formal integrated crop management agreements (which integrate IPM with soil conservation and nutrient management) had been implemented between the USDA and 1092 farms, covering 176,000 acres. Although this represents only one-tenth of 1% of the nation's total cropland, a survey by the U.S. Department of Agriculture indicates that at least some IPM practices are being used on a large portion of the farm acreage in America. The results of this survey, released in September of 1994 in an agricultural information bulletin, show that some form of IPM is being used on 60% of planted acreage of fruits and vegetables and 75– 80% of field crop (corn and potato) acreage.

Historically, the USDA taught farmers to use pesticides as an insurance policy for maintaining crop yields. Getting farmers to change their crop protection methods requires re-education, said Betty Marose, an IPM specialist and Maryland Cooperative Extension agent. "A lot can be done if you have the resources for an extension agent to demonstrate these methods," said Marose, "but it requires time and money." Unfortunately, funding for IPM education and implementation has remained level for the past 10 years.

Ontario's pesticide-container recycling program also contributed to reduced pesticide use. When containers are triple-rinsed before recycling, the rinse, containing as much pesticide as 6% of the tank volume, is saved and applied to crops. A similar U.S. program, initiated by pesticide vendors in 1992, now has participation in 45 states.

With seven years to go, Ontario is well on its way to meeting the Food Systems 2002 goal. Future reductions in pesticide use will be fine tuning, Wilson says, which depends on new research and technology.

Is Bottled Water Better?

Bottled water is one of the fastest-growing beverages on the market. In 1992, consumption of bottled water surpassed that of tea, wine, liquor, powdered drinks, and juice. In 1993, 2,257.7 million gallons of bottled water were sold in the United States, according to Lisa Prats, vice president of the International Bottled Water Association, the trade association of the bottled water industry. Consumers of bottled water cite taste as their primary reason for buying bottled water, but other reasons are safety and concerns about chemicals in tap water, says Prats. The question is, is bottled water worth the difference in cost, at an average cost of 700 times more than plain tap water?

A majority of Americans say they are pleased with the quality of the water that comes from their taps, according to a 1993 national survey on how Americans rate their drinking water by the American Water Works Association. As reported in the winter 1994 issue of *On Tap*, the AWWA survey found that 62% of Americans rate the quality of their drinking water as good (41%) or excellent (21%), while 75% believe that the water in their local community meets (57%) or exceeds (18%) the federal standards for quality and safety. Still, the AWWA survey found that 43% of respondents drink bottled water at least some of the time, although tap water is still their main source of drinking water, and 8% use bottled water exclusively.

Strict regulations govern both bottled and tap water industries. Unlike well water, which isn't subject to regulation, public water supplies are regulated by the EPA. Bottled water, on the other hand, is considered a food, and is regulated by the Food and Drug Administration. In 1989, the Environmental Policy Institute concluded that the "regulations for bottled water were not on par with those for tap water," say Linda Allen and Jeannie Darby of the University of California-Davis in an April 1994 article in the *Journal of Environmental Health*. In addition, regulations for tap and bottled water are not standardized: tap water has uniform national regulations, but "bottled water is still subject to federal regulations with limited applicability and inconsistent state regulations," say Allen and Darby.

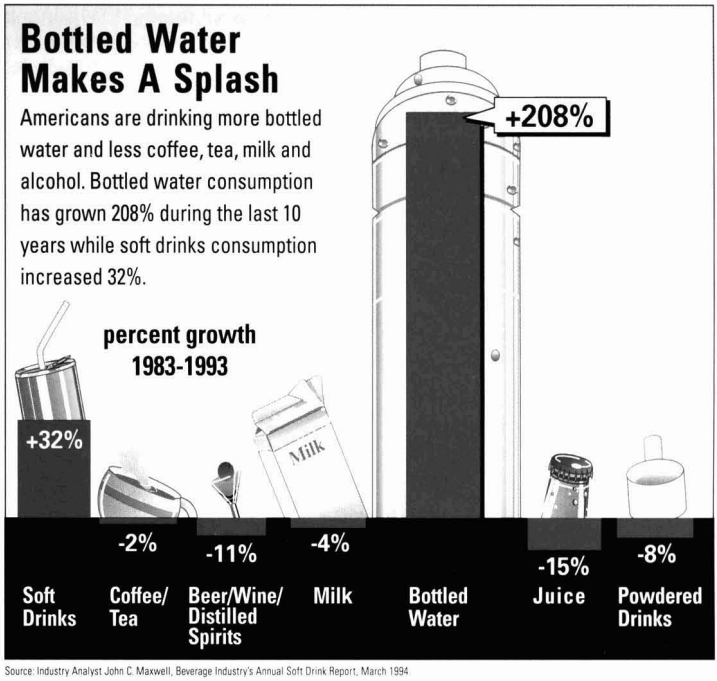
In 1989 the Environmental Policy Institute concluded that bottled water is not necessarily any safer than tap water. In fact, EPI says that storage of bottled water, often for weeks or months at room temperature and higher, promotes bacterial growth in the water. Elevated levels of bacteria in water can cause health problems for infants, the elderly, and immunocompromised people. Still, Stephen Schaub, senior microbiologist in the EPA's Office of Groundwater and Drinking Water, stresses that although studies are inconclusive on the issue, bacteria in bottled water doesn't seem to be a significant problem.

However, an incident in February 1990, in which benzene, a chemical known to cause cancer in humans, was detected in bottles of Perrier at levels that exceeded by four times the EPA standards for tap water, points out that bottled water may have other problems. Perrier recalled more than 170 million bottles as a result of the contamination, and the incident prompted the U.S. General Accounting Office to charge the FDA with failing to set "adequate safety standards for chemical contamination of bottled water."

In 1994, the FDA passed regulations that impose the same standards on bottled water as the EPA imposes on tap water. An exception is lead: lead content may not exceed 5 parts per billion in bottled water, whereas EPA limits lead in tap water to 15 parts per billion. Bottled water may help to bypass other potential problems brought about by the practice of public water suppliers of adding chlorine to drinking water to remove bacteria. Although chlorine kills bacteria effectively, it can react with organic matter in water to form by-products such as trihalomethanes which have been linked to bladder and rectal cancers. Chlorine is not used as a disinfectant in bottled water, according to Prats.

Despite almost half (49%) of the respondents to the AWWA survey saying they believe bottled and tap water to be equal in quality, 37% responded that bottled water is safer and healthier to drink than tap water, as opposed to only 10% who said the opposite, a perception most chalk up to clever advertising by the bottled water industry.

Americans drink bottled water primarily for aesthetic reasons: the taste, smell, and appearance of the water. Tap water supplies are often treated with chlorine, which can leave an aftertaste or odor. Bottled water, on the other hand, is usually treated by ozonation and filtration, processes that leave no aftertaste. Besides taste considerations, the EPA says that drinking bottled water is appropriate when the levels of contaminants in the local water supply exceed health standards, and when household problems, such as lead in the pipes, can cause contamination. Otherwise, researchers argue that bottled water just isn't worth the price, especially considering that it must be purchased, transported, and stored by the consumer. Canadian water researcher Pierre Payment, of the Armand-Frappier Institute, said in an article by the Associated Press that municipalities should advertise the quality



Source: Industry Analyst John C. Maxwell, Beverage Industry's Annual Soft Drink Report, March 1994

of their water the way bottled water companies do, because "North American tap water is the best you can get."

Studying Cell Death

Apoptosis is an ancient Greek word meaning "the falling off" of petals from flowers or leaves from trees. In modern scientific terms, apoptosis refers to the natural or programmed death of cells, as opposed to death caused by injury or necrosis. The failure of programmed cell death to occur has been linked to a variety of illnesses including cancer, dementia, and even AIDS. NIEHS scientist John Cidlowski has been exploring pathways by which apoptosis occurs in hopes of developing screens to reveal how environmental agents affect this process.

"I liken apoptosis to editing," says Cidlowski. "It's what causes us to lose the webbing between our fetal fingers and toes. It's also responsible for male pattern baldness in adults. It even has a suspected role in Alzheimer's disease."

Apoptosis is an energy-demanding process, requiring ATP and the activation of an endonuclease that degrades the chromosomal DNA into small particles. This process culminates in fragmentation of the cell into discrete membrane-bound apoptic bodies that are engulfed by surrounding cells and macrophages.

In contrast, necrosis occurs in response to a variety of harmful conditions and toxic substances. It typically affects groups of contiguous cells, and an inflammatory reaction usually develops in the adjacent viable tissue in response to the release of cellular debris.

There are numerous pathways by which apoptosis can occur, as evidenced by the diversity of signals that stimulate the process. Cidlowski estimates there are 400–500 different ways to stimulate apoptosis, and different stimulants yield varying responses in different cell types. "In some cells, the oncogene c-*myc* is an inducer of apoptosis, yet in other cells it prevents it," Cidlowski says. "Our picture of the signal transduction pathways is not clear. They are going to be cell specific."

Cidlowski has spent the past 16 years researching apoptosis at the University of Vermont and the University of North Carolina, where he focused on three areas: how apoptosis is induced by stress; physiological adaptations to stress, particularly the role of glucocorticoid receptors, in activating apoptosis in lymphocytes and maintaining homeostasis in the rest of the body; and the role of nutrients, especially vitamins, and gene expression in apoptosis.

It is known that certain environmental chemicals, including dioxins, heavy metals, and peroxides, lead to apoptosis in a variety of cells. At the NIEHS, Cidlowski is testing various chemicals to see what components of apoptosis pathways they activate. "We are analyzing the motors of apoptosis," he says. "We are focusing on the enzymes and genes that lead to cell shrinkage, DNA fragmentation, chromatin condensation, protein and RNA turnover, and inhibitors of apoptosis."

The role of apoptosis in cancer, AIDS, Alzheimer's, and other diseases has spawned an explosion of research on the subject. Cidlowski estimates there were as many as 1,000 articles on apoptosis published last year, as opposed to perhaps 50 papers a decade ago. But he cautions against the implication that apoptosis is the answer to everything. "There may be other mechanisms of cell death," Cidlowski says. We are testing whether apoptosis is the physiological counterbalance to mitosis."

Although Cidlowski emphasizes that he and his colleagues are not cancer researchers, some of their research is of particular interest to cancer researchers, such as analysis of the effects of various chemicals, including steroids, metabolic poisons, and immunosuppressants, on apoptosis in the immune system. It was originally thought that such chemicals simply inhibited cell replication. Cidlowski's team has been able to separate inhibition of replication from cell death and has proven that these chemicals act on cancers by inducing apoptosis.

Many chemotherapeutics act by stimulating apoptosis. A major problem with this treatment, however, is that over time cells tend to develop resistance to some chemicals. Cidlowski theorizes that this resistance may result from a loss in the capacity of the tumors to undergo apoptosis. If this theory is true, it places in doubt the prospect that chemotherapy can be refined to be the ultimate treatment for cancer. "We don't know what causes this resistance," Cidlowski says. "If cells lose one of the fundamental motors of apoptosis, it may be difficult to bypass resistance to chemotherapy. Then we'll have to look to new alternatives."

Cidlowski's goal is to define the key components in apoptosis and develop screens for these components in tumors.

EHPnet

April 22 marks the 25th anniversary of Earth Day. A World Wide Web site allows Internet surfers to explore and retrieve information related to a variety of environmental issues and interests.The site, located at http://akebono.stanford.edu/yahoo/Environment_and_Nature/, has something for everyone.

For the environmentally health conscious, there are links to organizations concerned with health effects research related to the release of hazardous substances. Most of the links are directed to the Agency for Toxic Substances and Disease Registry (ATSDR). ATSDR provides information on its biennial reports to Congress, conferences, congressional testimony, and services such as ToxFAQs, a series of summaries about hazardous substances excerpted from the ATSDR Toxicology Profiles and Public Health Statements, and the Science Corner, a simple support tool for scientists searching the Internet for environmental health information.

For those who religiously recycle glass, paper goods, and other materials and have always wondered how these materials are reincarnated, there is a link to the Recycle Cycle, an electronic exhibit based on a display of the same name by the Northwestern University Recycling Program. The exhibit explores the stages materials go through during the recycling process from disposal to their reemergence as new products.

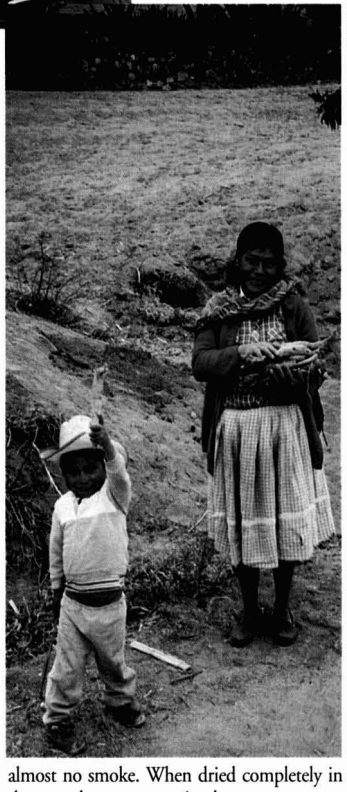
For those in search of inspiration during Earth Day, there is the John Muir exhibit, (http://ice.ucdavis.edu/John_Muir/Sierra_Club_fact_sheet_on_John_Muir.html). The exhibit documents the history of conservationist John Muir and his profound effect on heightening the public perception of nature. The exhibit includes a biography, images, and tributes.

For those who want to sound off on environmental issues, there is an on-line soapbox onto which they may climb called Envirochat. Envirochat allows all users logged into the site to talk about any issue pertaining to the environment.

In remembrance of Earth Days past, users might ponder the "40 Tips to Go Green" pamphlet distributed by the Jalan Hijau ("Go Green" in Malay) Environmental Action Group during Earth Day 1992 in Singapore. Although the pamphlet is three years old, the information is still relevant and serves as a pleasant reminder to keep up "green" practices.

Finally, for those really thinking globally, there is a link to the Enviroweb—a project of the Envirolink Network, the largest on-line environmental information service on the planet, which reaches over 400,000 people in 93 countries.

Eugene B. Shultz



Eugene B. Shultz

Digging for answers. Shultz (above left) examines roots with farm foreman. Women and children near Mexico City test ease of gathering roots (right).

Then, he says, "we can . . . see if key components remain functional and, if so, we can proceed with alternative inducers of apoptosis."

The Root of the Solution

For many, the combination of Native Americans and fire conjures images of pow-wows around a communal blaze. For the 60% of Navajo households that rely on wood or coal-burning stoves as their source of heating and cooking, one reality of that combination is a variety of respiratory illnesses caused by smoke from leaking stoves. However, researchers have discovered a solution in the form of an alternative fuel that burns more slowly than wood and produces almost no smoke: the root of the buffalo gourd.

Eugene B. Shultz Jr., a professor of technology and human affairs at Washington University in St. Louis, Missouri, has spent a large part of his career looking for alternatives to wood as fuel in dry, deforested lands, primarily Third World countries. It was while distilling the roots of the buffalo gourd (*Cucurbita foetidissima*) to make ethanol, not burning them, that Shultz happened upon them as a source of fuel. Shultz explains that the person operating the still had been given too many roots and so threw them out to rot, expecting to take them home for compost. The roots did not rot, the operator reported to Shultz, but dried in the sun "as hard as wood." The revelation prompted Shultz to study the roots as alternatives to wood fuel.

With funding from Western Regional Biomass Energy Program, a program of the U.S. Department of Energy, two projects were begun last May: one at Washington University to test how the buffalo gourd root performed as a fuel, and one at New Mexico State University to test whether it could be grown as a solid fuel crop.

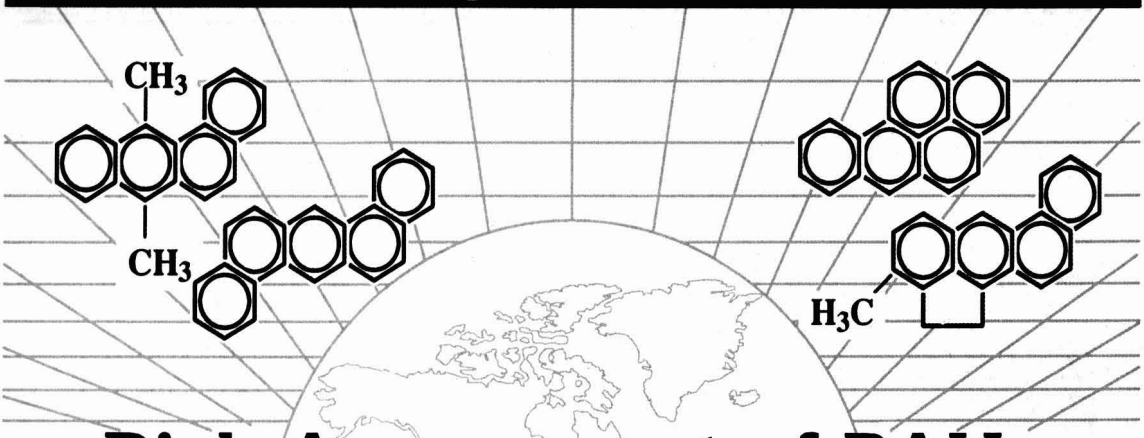
Results of Shultz's studies showed that the roots burn one-third as fast as wood and produce almost no smoke. When dried completely in the sun, the roots contain almost no water, a necessary component of smoke, unlike wood that retains almost 20% moisture even after drying. In addition, unlike wood, the roots contain almost no lignin, the source of by-products of incomplete combustion including carcinogens and respiratory irritants. These two factors make the roots, native to New Mexico, a potential solution to a persistent and devastating health threat to the state's Native American population; respiratory illnesses such as asthma and interstitial lung disease make up the third greatest cause of death among the Navajo. A 1990 study at the Tuba City (Arizona) Indian Hospital found that Navajo children under the age of two living in homes with wood/coal stoves were five times more likely to develop acute lower respiratory tract infections than children in homes without such heating systems. Because the Navajo live in a relatively pristine environment, free from pollution, and because only 10% of Navajos smoke, medical personnel were baffled by the magnitude of the problem of respiratory illnesses among the population. Said Louise Able, a physician with the Indian Health Service, in an article by the Associated Press, "When I began looking at it, I realized that the one common factor was chronic smoke inhalation leading to chronic respiratory illness . . ."

Although the buffalo gourd grows in relative abundance in the low-lying areas of New Mexico, the mile-high plateau of most of the Navajo Nation is too arid to support the plant. The challenge for researchers at the New Mexico State University Research Station was to see if the plant could be coaxed with irrigation to grow at that elevation. Researchers found that, with irrigation, the plant actually thrives in the climate, and crop yields exceeded those previously published in the literature. Although it is difficult to estimate needs because family sizes vary, it might take as little as one-half to one acre of growing area to supply a family with all of their fuel needs. And because the roots are lightweight, even children can help to harvest and handle them.

The potential problem with using the roots as fuels is cost. Although much healthier than wood or coal, the roots would have to compete with the free supplies of coal often provided to the Navajo by companies that mine on Indian land. "The roots would have to be subsidized," says Shultz, "although 'subsidy' may be considered a bad word in today's political climate, so we may have to find another word." According to Shultz, the problem comes down to the fact that because 57% of Navajos live below the poverty line, buying new stoves that don't leak smoke is out of the question for the majority of them, so an alternative fuel only makes sense. "You can solve the problem by buying them decent stoves or by providing them with cleaner fuel." Either way, continues Shultz, the health of the Navajo is the compelling factor. "These people don't deserve these illnesses."

Rootfuel studies are also being conducted in Mexico, Brazil, Zimbabwe, and India. Due to increasing deforestation, it is estimated that by the year 2000, 2.7 billion people worldwide will have inadequate access to wood fuel.

Meeting Announcement



Risk Assessment of PAHs in the Environment

June 26 - 28, 1995

Hyatt Regency Airport
San Francisco, California

Sponsored by:
U.S. Environmental Protection Agency

Co-Sponsors:
U.S. Department of Energy • National Institute of Environmental Health Sciences
Agency for Toxic Substances and Disease Registry • American Petroleum Institute
National Center for Toxicological Research • Electric Power Research Institute
Society for Occupational and Environmental Health • Gas Research Institute

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
215-643-5466 FAX 215-643-2772

Communicating Science in the New Millennium

A two-day conference in January, "NIH: Communicating Science and Health in the New Millennium," underscored NIH's renewed emphasis on ensuring that information from basic and applied biomedical research is made immediately available to citizens, health providers, health educators, and decision-makers. The conference, hosted by NIH Director Harold Varmus, brought together 300 members of the NIH communications community, along with outside researchers, journalists, and administrators. The conference was the first one held in the Natcher Building, a new, state-of-the-art meeting and conference facility on the NIH campus.

In his opening remarks at the meeting, Varmus charged attendees to help NIH address three challenges in communicating research: recognizing what needs to be said, delivering the message, and identifying and responding to the audience NIH must reach, which includes a diverse public with varying levels of literacy. Anne Thomas, director of the NIH Office of Communications and chair of the conference, presented a video during the opening session that highlighted NIH communications and featured scenes of the press conference conducted by Varmus, NIEHS Director Kenneth Olden, and NIEHS scientists following announcement of the discovery of the breast cancer susceptibility gene, as well as a segment on Martin Rodbell, NIH Scientist Emeritus at NIEHS and co-recipient of the 1994 Nobel Prize in Medicine.

Kathleen Hall Jamieson, dean of the Annenberg School of Communication at University of Pennsylvania, delivered the conference's keynote presentation. Jamieson pointed out that NIH must leave behind the old transmission model of communications, where messages are simply imparted to the audience. Current technology and culture demand an interactive model of communications, she said, providing new structures of access to information and allowing a dialogue with the public rather than a lecture platform from which to speak to passive listeners. Jamieson noted that the news media relies upon drama and conflict to define news, and these elements sometimes upstage or distort information about health and science in the news. She also noted that NIH trust and credibility are invaluable, and that in partnerships with private industry to deliver health messages, NIH should take care to avoid the appearance of compromise which may lead to a loss of public trust.

The conference featured a series of panel discussions followed by work group sessions on topics such as health communications, news/mass media, and reaching audiences of ethnic populations, youth, patient cohorts, and low literacy groups. In the work group sessions, NIEHS reported pioneering efforts in outreach to ethnic, low-income, and underserved rural and urban communities. The NIEHS outreach program serves as a model for other NIH efforts.

Samuel Silverstein, president of the Federation of Applied Science and Experimental Biology, asserted that NIH grantees have a responsibility to acknowledge the source of their research funding and to explain not just the results of their experiments but the implications of their findings to the diagnosis, prevention, and cure of disease.

Burroughs Wellcome

Gertrude Elion

Women at the Bench

Women scientists, their accomplishments, and their career paths are the focus of the NIEHS Distinguished Women Scientists Seminar Series that will feature five seminars, including one by Gertrude Elion, 1988 Nobel Laureate and Scientist Emeritus at Burroughs Wellcome Company. Suzanne M. Snedeker, a scientist at NIEHS and chair of the organizing committee, said, "In addition to presenting a formal research seminar, the speakers also will lead an informal discussion about the paths their careers have taken. We hope this dialogue presents a rare opportunity for senior women scientists to talk to junior scientists about their research, and how they successfully broke through the 'glass ceiling' that has affected the advancement of women scientists."

Sponsored by the NIEHS Division of Intramural Research Women Scientists and the NIEHS Office of the Scientific Director, the series will continue from March through September and is open to the public. The research seminars will be held at 10 a.m. in building 101, conference room B, at NIEHS's South Campus in Research Triangle Park, North Carolina. The schedule follows:

- March 2: Patricia K. Donahoe, chief, Pediatric Surgical Services, Massachusetts General Hospital. Donahoe was the NIEHS Hans L. Falk Memorial Lecturer in 1992 and will speak on "TGF-beta Receptor Downstream Interactors in Growth and Differentiation."
- March 31: Alice Huang, dean for science, New York University, will speak on "Neuronal Pathways Utilized by Vesicular Stomatitis Virus after Intranasal Inoculation: Determination of Site(s) for Interference."
- May 8: Elaine Faustman, professor and associate chair, Department of Environmental Health, University of Washington at Seattle, will speak on "*In Vitro* Developmental Toxicity Assessments: Application for Mechanistic Evaluations."
- June 28: Thea Tlsty, associate professor, University of North Carolina at Chapel Hill, will speak on "Disruption of Genomic Integrity in Tumor Progression."
- September 7: Gertrude Elion, 1988 Nobel Laureate and Scientist Emeritus, Burroughs Wellcome Company, "Challenges of Pharmaceutical Research."

For further information about the seminars, contact Claudia Thompson, (919) 541-4638.

International Groups Consult on Prioritizing Chemicals

In January, 60 representatives from 14 countries, including international and intergovernmental organizations and industrial associations, met at the NIEHS to give advice on prioritizing chemicals for international risk assessment and related issues. The meeting was co-sponsored by the International Programme on Chemical Safety (IPCS) and the Organisation for Economic Co-operation and Development (OECD) and supported by the NIEHS, the EPA, and the governments of Japan and Canada.

Since 1973, 284 chemicals have been recommended to the IPCS to be internationally evaluated for health and environmental effects, and over 80% of these chemicals have been evaluated in some manner. Criteria adopted previously for selecting chemicals for evaluation include:

- Adverse effects: data support the conclusion that the substance presents a potential hazard for human health and/or the environment;

- Exposure: the use, persistence, accumulation, or degradation of the substance shows that there may be signficant exposure of humans or the environment;
- Targets: the size and nature of the population at risk (human and other species) and the risks for the environment should be taken into account; and
- International concern: the substance should be of major interest to several countries.

Target chemicals for evaluation were set by U.N. member countries at the first meeting of the International Forum on Chemical Safety in 1994. OECD has a program to test and assess high production volume (HPV) chemicals which began in 1990 and currently includes 220 HPV chemicals; evaluations have been finalized for 45. Results of these evaluations will be published in cooperation with the IPCS. For additional information on this joint effort, contact George C. Becking, World Health Organization, at (919) 541-7537.

Tribal Environmental Council Meets

Native American tribal leaders met in December to confront growing concerns about the environment on tribal lands at the second annual meeting of the National Tribal Environmental Council (NTEC) in Reno, Nevada.

Jerry Pardilla, interim executive director of the council, noted that NTEC was founded by 7 tribes in 1991 and has now grown to 53 member tribes from 16 states. "While there is great diversity in our tribal cultures, geography, and governance," Pardilla said, "we are striving to develop a tribal environmental strategy which respects our differences and builds upon our common experiences."

The meeting was co-sponsored by the NIEHS. Kenneth Olden, director of NIEHS, was the keynote speaker. Olden's address, entitled, "Protecting the Environment, Protecting the Children," highlighted the research initiatives of the NIEHS focused on the issue of environmental justice. Olden said, "Institute efforts recognize that residents in a local area are the starting point for effective research as well as prevention and intervention programs in environmental health sciences . . . When scientists work collaboratively with grassroots groups such as the National Tribal Environmental Council, we do science that more immediately improves people's health."

The NIEHS involvement in the conference is just one aspect of the institute's efforts to address concerns of Native Americans. Last spring, the NTEC hosted a two-day visit by Olden and members of his staff to Pueblo homes near Albuquerque, New Mexico. Leadership of the NTEC also participated in the NIEHS Environmental Justice Symposium in February 1994 that brought together grassroots environmental groups from around the country with key environmental officials from the federal government. During that meeting, President Clinton signed an Executive Order establishing a government-wide environmental justice initiative.

NIEHS

On the front line. K-12 teachers and NIEHS staff met at a recent workshop on science literacy.

Earth Day Environmental Careers Symposium

For the third year in a row, NIEHS will co-host approximately 200 high school students and their teachers for a series of presentations, lunch, and a high-tech, interactive "arcade" of environmental science-related exhibits. The April 26 event is designed to help participants learn more about the many careers relating to the environment including scientific research, public policy, communications, and other fields. Speakers will include Bill Leslie, environmental reporter for WRAL television in Raleigh, a CBS affiliate.

The students from each high school will attend a series of morning and afternoon presentations. The winners of a special essay contest on an environmental subject, sponsored by the NIEHS and North Carolina State University's College of Forest Resources, will receive prizes of U.S. Savings bonds at the opening session.

Michael Hogan, chair of the NIEHS Science Education Committee, which plans the event, noted that plans this year will expand services to enable hearing-impaired students to attend. Hogan noted, "The responses we receive from students and their teachers indicate that the symposiums have generated strong interest in research and other environmental careers among students."

NIEHS Hosts Teacher Workshop

K-12 classroom teachers are on the front line for science education. NIEHS joined with other major research organizations in Research Triangle Park, North Carolina, in hosting a series of teacher workshops titled, "Rx for Science Literacy: The What, Where, How and Why of Biomedical Research." The series, sponsored by the North Carolina Association for Biomedical Research, runs from January through April.

Explaining the purpose behind the conferences, Karen Hoffman, president of NCABR, said, "Teachers are in a unique position to introduce their students to the role biomedical research has played in prevention and treatment of human disease and dysfunction. These workshops give teachers a foothold in enhancing science literacy among their students."

The sessions were designed to update teachers' knowledge about biomedical research and testing and to allow scientists from host institutions to present information about their own career paths and about opportunities for young people in science. Examples of sessions include talks on "The Fidelity of Genetic Reproduction," by William Copeland of the NIEHS Laboratory of Molecular Genetics; "Biotechnology: New Tools of Molecular Toxicology," by Kenneth Tindall of the NIEHS Laboratory of Environmental Carcinogenesis/Mutagenesis; and "NIEHS Animal Care and Use Program" by Mary Goelz of the Comparative Medicine Branch.

During the meetings, teachers toured NIEHS laboratories and received a 300-page teacher reference manual and lesson plans as well supplemental materials and videotapes. Teachers who attended received a unit of renewal credit toward teacher certification.

Green Gimmicks for Greenbacks

Its manufacturer calls it safety freeze. That's because Sierra Antifreeze substitutes propylene glycol for ethylene glycol, the major ingredient in conventional antifreeze. Sierra Antifreeze is just one example of the burgeoning trend to use "environmentally friendly" ingredients in a wide range of products ranging from antifreeze to solvents to paint strippers to pesticides.

The "green marketing" push began in the late 1980s and has continued, tapping in on consumers' concerns about not only protecting the environment but also about using products reputed to be safer and less toxic than traditional chemical alternatives. But just how much of this marketing is valid and how much is simply an attempt by manufacturers to cash in on consumer concerns? Answering that question is not easy.

Poison control centers around the country handled more than 3,500 cases of ethylene glycol exposure in 1993, the most recent year for which statistics are available. If swallowed, ethylene glycol can cause severe kidney damage. Propylene glycol, on the other hand, is on the FDA's GRAS (Generally Regarded as Safe) list and is used in small quantities in a variety of foods and confections. However, propylene glycol can cause temporary grogginess and nausea. Although the American Association of Poison Control Centers doesn't list cases of exposure to propylene glycol, it does report that in 1993 poison control centers handled 1,446 cases of exposure to glycols other than ethylene glycol.

So just what is meant by "environmentally friendly" or "environmentally safe"? "It's really hard to make a sweeping generalization, it's such a wide category of products and product changes," said Ned Groth, director of technical policy and public service at the Consumer's Union.

"There is a valid concept buried in here. The concept is designing products that have minimum health hazards associated with their use and minimum adverse environmental impacts," says Philip Dickey, director of the Household Toxics Project of the Seattle-based Washington Toxics Coalition.

But Dickey warns that specific criteria are needed for the concept to have any real meaning. Looking at household chemical products, such as cleansers, he ticks off a number of factors which have to be considered. "You could look at things like the toxicity of the product through various routes of exposure, such as accidental ingestion or inhalation; potential for skin irritation or damage; long-term health implications; whether the product is chemically reactive; that is, is there a potential for accidents by mixing the product with other household products?" he says.

Citing the example of Sierra Antifreeze, made by Safe Brands of Omaha, Dickey notes that while the product itself is less toxic than conventional antifreeze, there are other factors to consider.


Green Seal

Arthur Weissman—An environmentally friendly label can give products a competitive edge.

"My concern with that product is that people will want to buy a less toxic product, but then they may be under the illusion that the spent material that comes out of the car is so harmless they can dispose of it any way they want to," he says. After any antifreeze is flushed from a car's radiator it can be hazardous, since it becomes contaminated simply by being used.

"There are heavy metals [in used antifreeze] which become contaminants. Heavy metals have been implicated in nerve damage, kidney and liver damage," says Jon White, chief of the Environment and Product Safety Section of the Wisconsin Department of Agriculture, Trade and Consumer Protection.

Government Regulations

Concern about protecting the environment and human health has spawned manufacturers to develop more benign products (as well as marketing claims), which in turn has spurred government efforts to investigate such claims to make sure that consumers aren't misled.

In the late 1980s the Federal Trade Commission began examining so-called green claims and in 1992 issued guidelines to regulate them. Since 1992 the FTC has brought 28 orders against companies that have used misleading or deceptive advertising claims to tout their products' "environmentally friendly" characteristics.

For instance, in 1994 the FTC forced Orkin Exterminating Company, Inc. of Atlanta to stop advertising that "its lawn care pesticides are as safe as some common household products such as suntan lotion or shaving cream" and that the "pesticides when used as directed are practically nontoxic and do not pose a significant risk to human health or the environment" unless Orkin

had scientific evidence to back up the claim. The FTC charged Orkin had no reliable scientific evidence to substantiate those claims.

Though the FTC guidelines governing the use of green claims do not have the force of law, they are designed to help companies conform to legal requirements on advertising, labeling, and other forms of consumer marketing.

"The commission looks at advertising in terms of what is conveyed to consumers. The commission looks at all reasonable interpretations that consumers may take from the claim and in each case the advertiser has an opportunity to substantiate all such reasonable interpretations. The intent of the manufacturer is not an issue. The only issue is what message the consumer takes and whether or not it can be substantiated," says FTC attorney Michael Dershowitz.

The 1992 guidelines governed several environmental marketing terms including "recyclable," "degradable," and "environmentally friendly." Essentially, the commission required manufacturers to be able to substantiate these claims with "competent and reliable scientific evidence."

While the FTC monitors advertising claims, another government agency, the Consumer Products Safety Commission, monitors warning labels on products. Such labels describe the hazards products might pose, whether consumers should avoid inhaling or swallowing the products, and what should be done in such cases.

"We're concerned with the hazards that are associated with the product. The cleaners are usually, but not always, eye irritants or skin irritants," says Mary Toro, a CPSC compliance officer.

States are also actively checking into green claims made by manufacturers. An ad hoc task force of nine state's attorneys general began examining green claims in 1989. It was this effort which led to the FTC's 1992 guidelines. The task force, which now includes 11 states, remains active in bringing actions against manufacturers making unsubstantiated green claims. For example, in 1991, task force actions led to several companies each paying $50,000 to 10 states for making unsubstantiated and confusing claims that their products were "ozone friendly" or "ozone safe."

According to the settlement with one company, while the hairspray it made did not contain chlorofluorocarbons, which deplete the stratospheric ozone layer, it did contain volatile organic chemicals, which contribute to the formation of ground-level ozone, which can impair breathing.

"There is definite overlap between the FTC and the task force," says Wisconsin Assistant Attorney General Barbara Tuerkheimer, that state's representative to the task force. However, she said efforts between the two have been cooperative, with states deferring to the FTC or vice versa on occasion.

Private Vigilance

Government regulators are not the only ones evaluating green claims. Private groups are also active in the area. The Council of Better Business Bureaus works to keep manufacturer's advertising truthful. Last year the CBBB's National Advertising Division objected to Sierra Antifreeze's slogan, "Don't give me another toxic antifreeze, give me something different," because it implied that Sierra was completely nontoxic. As a result of the objection the slogan was dropped, according to division spokesperson Lynne Collins.

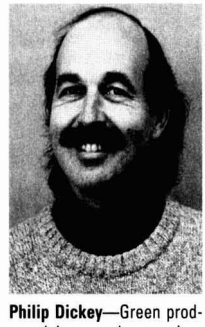
Some private groups are also involved in product evaluation. Green Seal, a five-year-old Washington, D.C.-based nonprofit organization, tests products to see if they are legitimately "environmentally friendly."

"That's not the mission of the FTC or the Consumer Products Safety Commission," says Arthur Weissman, Green Seal's vice president of standards and planning. "The FTC guidelines are to ensure that the claims made by manufacturers are truthful, accurate, and nondeceptive. That doesn't tell a consumer whether the product with the claim is overall better than other products they find in the market. "

Working with a variety of authorities, including industry experts and independent scientists, Green Seal develops its own protocols to assess a class of product's environmental impacts and contracts with Underwriters Laboratory in Chicago to determine if products meet those standards.

The process of earning Green Seal's approval is both voluntary and confidential, Weissman says. "If a company's product doesn't meet the standard it can learn how to improve the product; if it does get the seal, it should attract a large percentage of consumers who want to do things right environmentally in the marketplace. And the theory is the manufacturer gains a competitive edge in the market," says Weissman.

The Washington Toxic Coalition's Household Toxics Project has evaluated items ranging from adhesives to toilet bowl cleaners by comparing information from product labels, federally required material safety data sheets, toxicology texts, and manufacturer brochures to arrive at ratings ranging from "lowest toxicity and environmental impacts" to "highest hazard." And Scientific Certification Services of Oakland, California, has been independently verifying manufacturers' environmental claims since 1990. For products that meet the manufacturer's claims, SCS will provide a statement on the package that the product meets the maker's claim.



Washington Toxics Coalition

Philip Dickey—Green product claims may be meaningless in terms of safety.

But the effort to check such claims, as they affect health, is a rather small one. "Many of them will slip through the net and not be subject to verification in any way to let the consumer know that what they say is true," says Michael McCloskey, chair of the Sierra Club. "I think probably in the majority of cases environmental groups and consumers have no way of knowing whether the claims are true."

The terms that are bandied about by manufacturers provide little help. For example, the term "nontoxic," which is frequently found on labels and in product advertising, sounds simple but may hide multiple meanings. Dickey notes that the term doesn't tell consumers whether acute or long-term toxicity is being described. Also, "nontoxic" is not legally defined in the federal Hazardous Substance Act, though the act does set standards for defining toxicity.

"'Nontoxic' is an interesting claim," says the FTC's Dershowitz. He notes that the commission did not deal with the term in its 1992 guidelines, but may when it reviews them later this year, "since it's become more prevalent. The question is what level of safety is conveyed to consumers and perhaps does it undermine any precautionary statements that are used in conjunction with it that are on the product," he says.

One product that is advertised as nontoxic is a stain remover called "Goo Gone." According the material safety data sheet, the product, a mixture of petroleum naphthas and citrus oils, does not meet the definition of toxicity in the federal Hazardous Substance Act. The label on Goo Gone does, though, still have this warning for consumers: "Harmful or fatal if swallowed. Keep out of reach of children."

"We're still obliged as a manufacturer of . . . a spot and stain remover to warn consumers to still take caution with the product . . . Our product differs from others because it doesn't contain chlorinated solvents. If a child would drink several ounces, they wouldn't have permanent damage," says Scott Zeilinger, vice president of the Magic America Corporation, which makes Goo Gone.

And one toxicologist, when told the contents of the product, praised it. "It's a vast improvement on old-style cleaning agents," said Patricia Field, emeritus toxicology section chief of the State Laboratory of Hygiene of Wisconsin.

Goo Gone is also described as 100% organic, a description that raises questions in the mind of some authorities. "'Organic' is probably misused most of the time. I don't know what manufacturers mean when they say a product contains organic ingredients. At best they don't know what they're talking about, at worst they're out to deceive people," says Dickey. Referring to household cleaners, Dickey says organic may be meaningless in terms of safety. Organic, he notes, can simply mean containing carbon. "Certainly household cleaners contain organic chemicals, but that is by no means an indication of their safety. Organic chemicals include all kinds of toxic things, from PCB to benzene," Dickey says.

The FTC has not examined the term, though Dershowitz has questions about its meaning and use. "It's something we know is out there. I'm not sure I know what it conveys to consumers. I don't think I'd be remiss in saying that it probably conveys something positive about the product," he says.

Sierra Club

Michael McCloskey—Many green claims may not be verified.

Green Products

Among the environmentally friendly products on store shelves are those that use citrus oils as a base. In a concentrated form, these oils, derived from the peels of citrus fruits, are effective as solvents and degreasers, and they are less hazardous than other components of solvents such as toluene and xylene, which can be toxic when inhaled.

But they are not totally benign. Citrus oils can irritate the skin; if swallowed they can irritate the gastrointestinal tract. Warnings of these effects appear on the label of the citrus oil-based solvent Citra Solv. The label also urges "immediate medical attention" if the product is swallowed, gets in the eyes or on the skin. It's also flammable.

As to its efficacy, an examination by *Consumer Reports* magazine in 1993 described the performance of Citra Solv as "worthy, if at an exorbitant price." On an ounce-for-ounce basis it's about three times the cost of conventional cleaners, illustrating that manufacturers believe consumers will pay a premium for green products.

In 1993 Green Seal established standards for household cleaners. Besides meeting a battery of criteria to determine their effectiveness, the cleaners cannot be classified as toxic or highly toxic as defined by the Consumer Product Safety Commission. Green Seal also requires strict limits on the amount of heavy metals in the cleaners. And while the products may have chlorinated organic compounds, they can only be in concentrations that are below 10 times the applicable maximum contaminant levels in the national drinking water standards. Only one cleaner, BCD Ultra Concentrated Cleanser, has been certified by Green Seal.

Green Seal's criteria for paint calls for it to be made without a whole host of toxic ingredients, including benzene, xylene, cadmium, and mercury. Removing such ingredients can reduce exposure to potential carcinogens and lower the risk of liver and kidney damage and eye and lung irritation.

"It's very good to minimize exposure to such compounds in products. It's prudent public health policy," says Ronald White, director of environmental health at the American Lung Association.

Green Seal certification also means the product must be effective. "We do have performance requirements. We don't want a Green Seal certified product to be ineffective. That would frustrate consumers. In the case of paints that don't have good hiding power it will cause consumers to put multiple coatings and totally neutralize the benefits by adding to the VOC [volatile organic compound] levels," says Weissman.

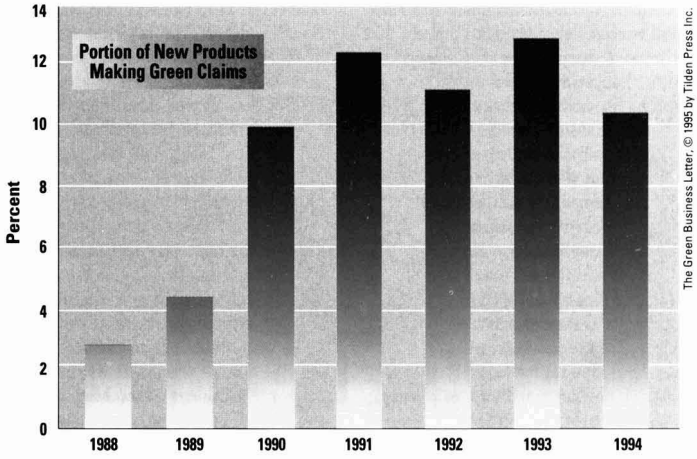
But there may be other trade-offs when some hazardous ingredients are removed. For example, one paint stripper on the market, Safest Strip made by 3M, has no methylene chloride. Methylene chloride is an animal carcinogen and when inhaled can lead to the formation of carbon monoxide in the blood, says Field. But using this water-based product means that paint stripping can take much longer. Safest Strip may take an hour or more to do the job that a conventional stripper could do in 15 minutes, according to 3M. And users should make sure it doesn't get in their eyes, warns the label.

Another manufacturer of household products, SC Johnson Wax, is reformulating a number of its household products to reduce the amount of VOCs. According to the company, its goal is a 25% reduction by the end of the year 2000. Overall the VOC total formula ratio of its products worldwide was down 17% as of 1993, the year for which the most recent data are available. Most of the reduction is in products in Europe and the Americas. However, the percent of VOCs used has actually risen in Africa and the Asian-Pacific region.

Pesticides are also going the environmentally friendly route. According to a 1993 EPA report, toxicity-related claims (such as "no synthetic chemicals") appeared on 22 pesticides and insecticides in 1991.

But the value of this claim is dubious, according to Jean Frane, a specialist in the EPA's Office of Pesticide Programs. "If you take it literally, no synthetic chemicals is not necessarily anything that makes it healthier. However, people will read that as meaning no nasty chemicals. No synthetic chemicals means that it is made with natural ingredients that are derived from plants, or things like that. It does not necessarily reflect that those things are not toxic," she says.

Frane goes so far as saying that the EPA erred in allowing such a statement to appear on the labels. "Probably we should have not permitted it. Right now there is an absolute prohibition on making toxicity-related claims," she says. Frane does say, however, that because these products are likely to have a water-based formulation rather than a solvent-based one, they are "more biologi-



cal in nature and therefore are less likely to be a human health hazard."

Some scientists don't see much of a distinction between the safety of natural and synthetic chemicals. "There doesn't seem to be any toxicological reason it should be the case that naturally occurring chemicals are in some way safer than synthetic chemicals. When we look at the chemicals that are tested for carcinogenicity, the proportion that are carcinogenic in rodent studies are similar for natural and synthetic chemicals," says Lois Gold, a biochemist at the University of California, Berkeley.

EPA

Jean Frane—Lack of synthetic chemicals may not mean a product is nontoxic.

Some "natural" products may be quite harmful. Writing in the October 1990 issue of the *Proceedings of the National Academy of Sciences*, Gold and colleague Bruce Ames described how a type of potato bred to be rich in anti-insect toxins had to be withdrawn from the market because these natural toxins were harmful to people.

Gold and Ames argue that many natural pesticides have not been tested for mutagenicity or carcinogenicity and say "their safety compared to synthetically derived pesticides should not be prematurely assumed."

But there are biological methods of pest control that do offer alternatives. According to Janet Andersen, acting director of EPA's new Biopesticides and Pollution Prevention Division, which was established in November 1994, there are slightly more than 400 "active ingredients," or biological pesticides including viruses, bacteria, fungi, and pheromones. Pheromones, for example, are natural chemicals which are sex attractants. Sold as ingredients in products such as Biolure, Pherocon, and Pherotech, pheromones can be used to disrupt insect mating patterns. Andersen, who said the division actively promotes the use of such biological agents because of their safety, acknowledged that they may be slower acting than chemical alternatives and may require more sophisticated management because they are effective against only one or two insects, compared to chemical pesticides that are often targeted more broadly.

Insecticidal soaps are another nonchemical approach to pest control. These soaps, which are harmless to people, says insect specialist Susan Mahr of the University of Wisconsin-Madison, are not effective against all insect stages and must be applied when the insect is actually on the plant to work.

Conclusion

Although it is clear that "green" products are usually less toxic than conventional ones and can perform effectively, it is also clear that products that may pose less of a risk to health are not necessarily benign. Terms such as "nontoxic," "natural," and "organic" are not necessarily synonmous with safety and have yet to be satisfactorily defined. Government agencies are relatively limited in their ability to regulate green marketing claims. And few private groups offer some sort of evaluation of claims which largely depend on manufacturers' willingness to seek product evaluation. For the consumer, the best advice would seem to be to read product labels thoroughly and be skeptical of sweeping claims and undefined terms. As Consumers Union's Groth warns, "*Caveat emptor*": let the buyer beware.

Harvey Black

Harvey Black is a freelance journalist in Madison, Wisconsin.

Erratum

The information following the sentence which begins "Mixtures research . . ."of the Focus article entitled "Strange Brew: Assessing Chemical Mixtures" on page 142 of the February 1995 issue (vol. 103, no. 2) was incorrectly attributed to Victor J. Feron. The correct source of the information is Harold Zenick.

Republicans Take the Helm:

What's Ahead for the Environment

Joseph Tart

As the 104th Congress settles in, environmental advocacy groups are remobilizing their efforts to prepare for a possible war against environmental legislation. It is now unclear the impact that Republicans will have, but sources generally agree that some environmental legislation will be wounded.

The top priority of the new Congress is the Republicans' "Contract with America," the ten-point legislative agenda that includes a promise to protect the nation from environmental legislation "run amok." There are no specifics dealing with environmental issues. Title III of HR 9, the Job Creation and Wage Enhancement Act, seeks to ensure that risk assessments and risk communication are open, objective, and sufficiently informative to serve the needs of decision-makers, the regulated community, and the public. The Republicans are proposing to require risk assessment and cost–benefit analysis for almost all federal regulations, eliminate unfunded mandates, and boost private property protection.

Environmentalists oppose risk legislation because they claim it induces "paralysis by analysis" at federal agencies and prevents new legislation from being implemented by regulatory agencies. EPA Administrator Carol Browner testified before the House Committee on Commerce, saying, "HR 9 would make it more difficult to remove unsafe chemicals from the market, more difficult to introduce safer alternatives, and would stifle industrial innovation. The bill calls for much more extensive risk assessments, cost–benefit analyses, and regulatory impact analyses before the EPA can take action."

Environmentalists also fear that unfunded mandates and private property protection legislation may be the first steps in rolling back environmental legislation, because the federal government would have to finance the legislation. For example, the government would be required to reimburse land owners whose properties are affected by the laws.

In their efforts to downsize the federal government as part of the contract, Republicans made changes in the committee structure which oversees science and environmental issues. They have cut two of the standing committees in the House and reduced the number of subcommittees. The Energy and Commerce Committee was dismantled, passing the entire Department of Energy to the House Science Committee (formerly the Science, Space and Technology Committee). The Merchant Marines and Fisheries Committee, which shared jurisdiction over wetlands and held primary jurisdiction over the Coastal Management Zone Act and the Endangered Species Act, was also abolished and consolidated among other committees.

The dissolution of these environmental committees symbolizes the Republican agenda for environmental legislation—cutting, trimming, and reducing the role of the federal government. As the new committee chairs take their places, environmentalists anticipate weakening of major pieces of legislation that were not reauthorized by the 103rd Congress and remain open for reform.

Clean Water Act

Primary jurisdiction over the Clean Water Act in the House belongs to the Transportation and Infrastructure Committee, which is chaired by Bud Shuster (R-Pennsylvania). In the Senate, jurisdiction belongs to the Environment and Public Works Committee, chaired by John H. Chafee (R-Rhode Island).

Shuster has a fairly poor environmental voting record: he received a score of 20% for 1993–94 and a lifetime score of 20% by the League of Conservation Voters Education Fund, a nonpartisan environmental research and education organization. His clean water bill in 1993 would have decreased requirements of farmers, made watershed protections voluntary, and given communities more time to comply with existing laws. Shuster's priorities for reforming the Clean Water Act this year include redefining wetlands, developing private properties protection, and reducing funding. He hopes to declassify a large number of existing wetlands and let state and local governments define their wetlands. As for property protection, Shuster hopes to require the federal government to pay landowners under the constitutional "takings" requirement. For example, if privately owned land is classified as wetlands and cannot be used by its owner, the government would be required to pay for the loss.

In contrast to Shuster, the Senate leader on the issue has a strong environmental voting record. LCV gave Chafee, who is currently serving his fourth term, a 1993–94 score of 79% and a lifetime score of 74%. He was endorsed by LCV in 1982, 1988, and 1994, and now remains one of the few allies environmentalists have in the Senate majority. Many conservative Republicans opposed his appointment to chair of the environment committee because of his long-time support of environmental protection, but the Senate leadership gave him the chair due to his seniority as the committee's ranking minority member. To check Chafee's power, the leaders packed the committee with conservatives. Of the committee's 16 members, all 8 other Republicans voted with LCV less than 25% of the time last year, and voted 80% of the time to protect private property rights, according to the League of Private Property Voters.

Because Chafee's committee is largely anti-environmental, his efforts to pass some of the legislation he proposed last year may be blocked. Environmentalists are hoping that he will be able to put a check on some of the Republican proposals. "We are hoping Chafee will limit some of the reform to just funding," said Paul Schwartz, national campaigns co-director of Clean Water Action. But the future of the act looks bleak for environmentalists. "We anticipate that the Clean Water Act will be severely weakened," Schwartz said. "The question is to what extent."

Safe Drinking Water Act

Primary jurisdiction of the Safe Drinking Water Act lies in the hands of the Senate Environment and Public Works Committee

and the House Commerce Committee, chaired by Thomas Bliley (R-Virginia). Bliley does not plan to address drinking water until the Contract with America is completed, according to his press secretary Charles Boesel. Boesel said Bliley has not decided which issues he will address in the SDWA reform. Last year, he sponsored HR 3392, Safe Drinking Water Act Amendments of 1994, which was considered anti-environmental by the LCV. Bliley was given a 1993–94 LCV score of 7% and a lifetime score of 15%.

In the Senate, Chafee has made drinking water a top priority and hopes to move quickly on a bill similar to S 2019, the reform bill that unanimously passed the committee and gained Senate-wide support last year. However, other committee members may not be as supportive of such a bill this year.

Schwartz says that last year's Senate bill, S 2019, actually weakens the SDWA, and that it is considered too environmentally friendly for this Congress. Therefore, he predicts a weakening of the act, saying it is unclear whether the Democrats will put up a strong fight or not.

To combat possible blows to the SDWA, a coalition including public health officials, consumer organizations, religious leaders, environmental justice advocates, and environmental advocates is launching a nationwide campaign to educate the public about safe and affordable drinking water. The group will discuss how state and local officials treat the issue, and stress the communities' right to know what is in their drinking water. "We hope this will be a campaign that will change the debate in Congress," Schwartz said.

Clean Air Act

The Clean Air Act also faces change because of current problems with the implementation of its provisions. Primary jurisdiction belongs to the House Commerce Committee, chaired by Bliley, and the Senate Environment and Public Works Committee.

In early January, the act came under fire when House Majority Whip Thomas DeLay (R-Texas) introduced several bills that would repeal all or portions of the acts' 1990 amendments, as well as the acid rain and air toxics programs. DeLay, who is serving his sixth term, has a 1993–94 LCV score of 9% and a lifetime score of 8%. His bills have raised skepticism from several congressional staffers and criticism from the EPA. Hearings were scheduled to be held in late January.

As with the SDWA, Bliley will not address clean air issues until the Contract with America is completed. He has not stated whether he supports any of DeLay's proposals.

Superfund

The Republicans are hoping to reform Superfund as well. In the House, primary jurisdiction belongs to the Commerce, Trade and Hazardous Materials subcommittee of the Commerce Committee, which is chaired by Michael Oxley (R-Ohio). In the Senate, the jurisdiction belongs to the Superfund subcommittee of the Environment and Public Works, chaired by Bob Smith (R-New Hampshire).

Oxley's priorities for Superfund reform include reducing cleanup costs and increasing the number of cleanup sites, according to his press secretary, Peggy Peterson. A major issue for Congress this year will be whether to repeal the retroactive liability provisions of Superfund. Oxley supported a Superfund reform bill last year that did not repeal retroactive liability, but says this is a different Congress and the repeal may garner more support. He has not yet made a decision on the issue, Peterson said. Informal meetings began in late January, but Peterson said it would take some time before hearings were scheduled because Superfund is such a massive project. Peterson said Oxley will work on Superfund informally while he pursues the Contract with America and interstate waste and flow control.

Meanwhile, in the Senate, Chafee and Smith are holding a series of hearings and are working to draft a bill together. Smith is examining several changes that should be made, but is adamant about repealing retroactive liability. Chafee will wait to focus on Superfund after he addresses drinking water and interstate waste issues.

Endangered Species Act

The Endangered Species Act appears to be endangered under the new Republican leadership, who propose cuts to the act. Primary jurisdiction in the House belongs to the Resources Committee, chaired by Don Young (R-Alaska).

Young supports major changes to the Endangered Species Act. He has commissioned a task force chaired by Richard Pombo (R-California) that will hold several hearings throughout the country for citizens who have been affected adversely by the Endangered Species Act, local environmental leaders, or anyone who has something to say about the bill. He will consider the input and work to have a bill out of the committee and on the floor by June 1, according to his communications director Steve Hansen.

Hansen said that Young has developed specific reforms, including a regulation that in order for an animal to be listed as threatened, there must be scientific and biological evidence. Then, once the species is listed, the government must take measures to improve the health and population through the development of a refuge. Once it is biologically proven that the species is healthy again, it should be immediately removed from the list, rather than after a waiting period, as it is now. Young also wants to mandate that if the government deems a habitat on a private landowner's property necessary for the recovery of a species, then the government must reimburse the owner for the loss of the land.

Young is known for his anti-environmental stance. He has an LCV score of 2% for 1993–94 and a lifetime score of 13%. Aside from revising the Endangered Species Act, Young hopes to open Alaska's Arctic National Wildlife Refuge to oil drilling and gas development, to expand logging in the Tongass National Forest, and to build tourist developments around Alaska's national parks.

Budget

In pursuing the Contract with America, the Republicans are looking to make budget cuts wherever possible. Undoubtedly, this will include cuts to areas that deal with environment and health.

The Labor, HHS, and Education subcommittee of the House Appropriations Committee chaired by John Edward Porter (R-Illinois) is examining the National Institutes of Health for possible recisions from the FY 1995 HHS budget. Cuts may be made to duplicative NIH and HHS programs, but Porter will assure that dramatic cuts are not made.

"If you look at the . . . Congress members, you'd be hard-pressed to find a bigger fan of NIH and biomedical research," said Dave Kohn, Porter's press secretary. "He thinks we need to trim spending, but NIH is an important priority."

Porter hopes to have recommendations to the subcommittee by late February.

President Clinton's 1996 budget includes budget increases for certain divisions of NIH and level funding for the EPA, but Congress has the ability to raise or lower his figures.

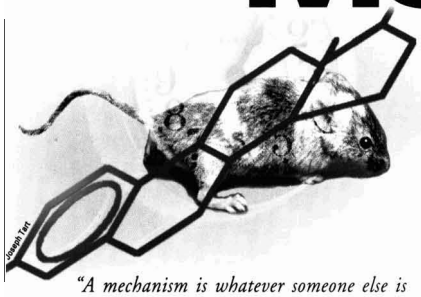
The future of environmental legislation will depend on how quickly and effectively the Republicans attempt to implement their ideas, as well as how strongly the remaining green Democrats oppose them. Although the 103rd Congress had a number of environmentalists, gridlock prevented environmental legislation from being passed. This Congress has the ability to redefine environmental law as it is scheduled to reexamine every major environmental statute enacted in the past 25 years.

Brandy Fisher

Brandy Fisher is a freelance journalist in Chapel Hill, North Carolina.

Mouse or Molecule?

Mechanism-based Toxicology in Cancer Risk Assessment



"A mechanism is whatever someone else is working on at a level lower than I am."

—Anonymous toxicologist

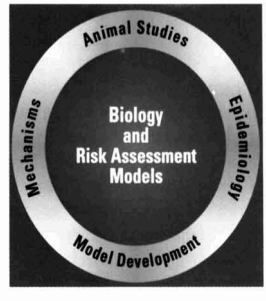
Cancer is a set of diseases characterized by uncontrolled cell growth. It is thought to be a complex process involving multiple steps, any of which may be initiated, altered, and otherwise affected by exposure to chemical carcinogens in the environment. Although an understanding of the process remains incomplete, recent gains in the knowledge of the mechanisms of action of carcinogens both in experimental animals and humans may help refine current risk assessment methodology for identifying and quantifying cancer risks associated with chemical exposure.

For regulatory agencies such as the EPA, the FDA, and the Occupational Safety and Health Administration (OSHA), a certain urgency exists to improve cancer risk assessment. Along with continued concern from the public about the safety of environmental chemicals and tremendous pressure from industry to provide more solid scientific rationales for specific regulatory decisions, there are the numbers: of the 70,000 substances in commerce, adequate toxicological data are available for only 10–20%. And of the 50 top-production chemicals in the United States (which total nearly 700 billion pounds per year), more than two-thirds have yet to be evaluated for carcinogenicity in animals.

These pressures, of course, also have an impact on that portion of the scientific community charged with providing public policy makers with the most complete information on the environmental components of human disease and on the biological mechanisms by which these diseases occur. In terms of cancer risk assessment, the problem is compounded by time and money. The conventional 2-year rodent bioassay, which typically forms the basis of cancer risk assessment, costs between $2 and $4 million and requires 4–6 years to complete. "At present, with current resources, we can test only 10 to 15 chemicals by this approach each year," says George Lucier, director of the Environmental Toxicology Program at the NIEHS.

Another important issue is uncertainty. Cancer risk assessment of chemical exposure relies heavily on tumor data from animal carcinogenicity bioassays conducted at high doses. Positive bioassay findings are frequently followed by mathematical extrapolation to the much lower exposure levels anticipated for environmental exposures of humans.

Along the way to a carcinogenicity assessment, and in the absence of knowlege that demonstrates otherwise, certain conservative inferences or "defaults" are assumed such as the conservation of biological processes among species, the inference of similar susceptibility between animals and humans; the assumption that susceptibilities within a population do not differ by age, gender, or genetics; and the assumption of low-dose linearity—that chemicals act like radiation at low doses to induce cancer, and that a single mutational change can result in adverse effects down the road. And so, when actual or more accurate data are either unavailable, incomplete, or inadequate, use of defaults add their own varying degrees of uncertainty regarding the carcinogenicity of a particular chemical. Thus, says Lucier, regulatory agencies are often forced to make decisions on chemicals and safe exposure levels without an adequate science base.



Modes of Action

"We are charged with decreased use of animals, charged with developing alternatives for screening chemicals more rapidly and to set priorities for further testing," says J. Carl Barrett, head of NIEHS's Environmental Carcinogenesis Program. "Mechanism-based toxicology presents one type of alternative." According to Barrett, mechanism-based toxicology (MBT) involves "the use of knowledge of mechanisms both of disease process and of chemical effects to better predict the toxicological potential of chemicals, to estimate risk at low doses, to extrapolate between species, and to quantitate interindividual differences in response."

Support for the use of MBT data can be found in EPA's proposed revisions to its *Guidelines for Carcinogen Risk Assessment* (August 1994). In its draft document, the agency leaves room for inclusion of more biologically based data as a means of reducing the uncertainty associated with extrapolation of risk. While acknowledging that the mechanisms by which any chemical causes cancer may never be completely detailed, scientists involved in formulating the proposed EPA revisions generally agreed that this should not preclude the use of scientifically supportable "mode of action data" in the risk assessment process.

Barrett points to three fundamental modes or mechanisms of action by which chemically induced cancer can arise: heritable mutations in critical target genes, heritable epigenetic changes in cellular phenotype, and clonal evolution or expansion of cells influencing the probability of subsequent mutations occurring either spontaneously or through exogenous exposure. An example, explains Barrett, is diethylstilbestrol (DES) exposure *in utero.* Developmentally timed exposure of this synthetic

estrogenic compound can change the tissue differentiation pattern throughout the lifetime. "If you give DES during the first five days of development, adult animals have a totally different pattern, a different differentiation, than nonexposed animals. So there are critical periods in development where exposures can change the progression of growth and control of cells that may lead to the cancer process," says Barrett. "These are not mutually exclusive. A chemical can exert multiple mechanisms or modes of action."

Advantages

As Barrett points out, advantages of MBT are that it allows many more substances or chemicals to be tested for biological activities, provides an alternative to the use of animals, and provides more efficient design of toxicology studies. "For example, if you know a chemical exerts an impact on cell–cell communication, then you might look at its impact on cancer or noncancer endpoints," Barrett says.

MBT may also be applied to testing the validity of certain default assumptions; for example, the conservation of biological processes, including mechanisms. "If you look at how the steroid hormone receptor functions, it doesn't matter if it's a yeast or a man; it functions the same way," says toxicologist Linda Birnbaum of EPA's Health Effects Research Laboratory. "And I think if you understand how an effect or part of an effect is brought about, you can look at whether those mechanisms are working in man as well as in experimental animals." Birnbaum adds: "Sometimes there are much shorter assays that demonstrate carcinogenic potential. Maybe by applying mechanistic approaches one can understand under what circumstances that potential is realized."

In the initial stages of risk assessment, MBT can be used to more rapidly screen chemicals and to set priorities for further studies. In terms of mutational activity, for example, current knowledge of critical suppressor genes, such as p53, might be exploited to understand the carcinogenic mechanisms of specific chemicals. Another example is receptor-mediated pathobiology, the knowledge that some chemicals, such as dioxin and estrogens, seem to cause diseases by interacting or binding with specific receptors. Molecular proteins such as the Ah receptor may help predict the toxicological impact of other chemicals. And in terms of structure–activity relationships (SAR), the knowledge that certain types of chemicals do or do not share structural or biological properties associated with mechanisms critical to carcinogenesis can help strengthen or weaken concern about an agent's carcinogenicity. Mutational and SAR analysis as well as knowledge of receptor-mediated activity may also offer a rationale for reasonably assuming hazard and may preclude costly and time-consuming bioassays.

Another use of MBT in cancer risk assessment is in determining dose–response relationships for chemical effects at low doses. Dose–response assessment refers to the process of estimating the relationship of dose of a substance to degree of effect. MBT can be used to explore and identify mutational activity, receptor-mediated effects, pharmacodynamics, and pharmacokinetics.

Robert Wood Johnson Med.

Michael Gallo—We have to take a hard look at receptor-mediated mechanisms.

Michael Gallo, director of the NIEHS Center of Excellence at New Jersey's Robert Wood Johnson Medical School, says he sees a greater emphasis on the role played by receptor-mediated mechanisms involved in carcinogenesis, particularly the hormonal component. "We now know that the receptor-mediated mechanisms may be the classic of all cancer promoters. The receptors we're studying are involved in growth regulation—the Ah receptor, the estrogen receptor, the epidermal growth factor receptor, the thyroid hormone receptor. We have to take a hard look at them." Thus, it follows that dose–response assessment for an effect other than mutagenesis or tumor incidence may be useful for assessing potential environmental carcinogens. If carcinogenic effects are secondary to precursor molecular effects, such as disruption of hormonal activity, such precursor events may be more relevant than tumor incidence for risk assessment. Gallo says a major area of concern today is the role played by hormonal or endocrine disruptors in the environment. "If we can show that disruption or modification of a hormone is in fact an effector for cancer, then there are secondary or tertiary mechanisms involved."

Measurement of biochemical or molecular events following chemical exposure has also sparked considerable interest in the use of biomarkers in risk assessment. MBT may be useful in evaluating the quantitative and qualitative relationships of these markers to toxic effects. At the recent NTP Workshop on Mechanism-based Toxicology in Cancer Risk Assessment, a dose–response work group considered the value of biomarkers for risk assessment. They said some biomarkers may help determine exposure to a carcinogen and its effect. For example, biomarkers reflect gene mutations directly related to carcinogenesis or to alterations in gene expression. Comparisons of animal model and human molecular biomarkers were viewed as potentially useful for establishing interspecies dosimetry and susceptibility.

Another NTP workshop subgroup concluded that it would be useful and appropriate to apply mechanistic information to assessing the relevance of the rodent bioassay for humans. Participants pointed out that the collection of relevant mechanistic data can either support the interspecies extrapolation default or could otherwise provide a

NIEHS

Meeting on mechanisms. A recent meeting on mechanism-based toxicology brought together leaders in environmental health. (left to right) George Lucier, Lynn Goldman, EPA assistant administrator for prevention, pesticides and toxic substances, NIEHS Director Kenneth Olden, and J. Carl Barrett.

rationale for revising it. They also concluded that mechanistic information could be used prospectively in the design of chronic rodent bioassays as an aid in selecting route of administration, dosing, species selection, and endpoints. If, for example, the data indicated compound-induced cell proliferation or cytotoxicity, this would provide a basis for monitoring those endpoints in the bioassay. The workshop subgroup saw biomarkers of susceptibility, such as those indicative of polymorphisms of metabolism, detoxication, and DNA repair, as presenting "a promising opportunity for interindividual and interspecies extrapolation," as well as having possible utility for evaluating age and/or sex-related differences in human susceptibility.

NIOSH

Brian Hardin—Scientific data can be used to delay action.

What is Enough?

Mechanism-based toxicology appears to offer tremendous potential for cancer risk assessment, but how much evidence is enough? As Barrett points out, in spite of MBT's potential, what is needed is a comfort level with some sort of minimum data for risk assessment decisions. Toxicologist Ellen Silbergeld of the University of Maryland, agrees. "It is important to come up with some understanding as to what is the minimum amount of data that would allow both scientists and regulators to proceed together with some degree of confidence, with some ability to communicate to the public, the nature and extent of risk." She cautions that unless some limits are set, "We are in danger of being attracted into the very gray area between risk assessment-related research and basic biology, which is the endless quest for knowledge but may not be exactly consonant with the needs as well as the resources to make informed and practical decisions. I think of an analogy to clinical medicine, and I am aware now that as I teach medical students that they don't have to understand everything about molecular biology and disease process in order to make a clinical decision. It seems to me we've been remiss in establishing that same type of strategic outlook in how we bring science into the regulatory process."

For Brian Hardin, senior scientist in the Office of the Director at NIOSH, the issue can be viewed from another perspective. "Pursuit of more and better scientific data can be used very effectively by forces whose interests are served by avoiding action, by delaying action . . . 'paralysis by analysis'." He says these forces can make skillful and plausible appeals for more and better science before some government intervention is allowed that would disturb the established order, perhaps at the expense of public health. "Whose risk is being minimized?" he asks.

Hardin, along with other scientists, also expresses concern that resources gained for mechanistic research will come at the expense of whole-animal bioassays and epidemiologic studies. "Those sorts of studies provide the most convincing and most powerful tools we have today for protecting human health. Despite the faith we all have in mechanistic work, I predict it will be many, many years before it is possible to regulate any chemical in commerce as a carcinogen in the absence of epidemiologic or animal evidence of carcinogenicity," Hardin says.

Birnbaum points out that conventional long-term bioassays may still be controversial after their completion. She says the use of short-term MBT studies that are highly predictive of a compound's likelihood of carcinogenicity could be used to support two-year bioassay findings. "That in fact could result in agreement that the compound is a bad actor and should not be used. In that sense we would have done a good job of protecting the public health."

U. of Maryland

Ellen Silbergeld—There needs to be an understanding about the minimum amount of data needed for risk assessment.

Recommended by Birnbaum and others is a more iterative and integrative approach to data collection for risk assessment purposes. At the NTP workshop, Silbergeld reminded participants that the bioassay is an extremely rich source of information and offers the only lifetime surveillance opportunity of an animal model. She recommends expanding the bioassay to provide more mechanistic information as it is proceeding. "That may allow you to stop at certain times, evaluate where you are, but at the same time preserve an established structure and possibly be more economical overall because you'll be able to eliminate as you go along," Silbergeld adds. "I don't think the problem here is including mechanistic information. The problem here is making justifiable selection among an enormous array of potential mechanistic approaches that could be applied."

Uses of MBT

- To more rapidly screen chemicals and set priorites for further studies
- As a basis for reasonably assuming hazard (rebuttable presumption)
- To determine quantitative dose–response relationships
- To understand species, strain, and individual differences in susceptibility
- For species extrapolations
- For more efficient experimental design

Few would argue that increased use of mechanism-based toxicology should mean severely diminished use of the conventional bioassay. Rather, MBT should be used in addition to current testing strategies, not as a substitute for them, and study designs should be developed to provide the information needed for the best-informed risk assessments.

Today, several key impediments exist to the use of mechanistic data in risk assessment for cancer: comfort among scientists and regulators with the status quo; constraint by regulatory legislation; concern with the implications for making costly decisions. It has been said that science needs to evolve to increase its own confidence in mechanistically based predictions. For this work to continue, it is imperative that confidence also be increased among legislators, regulators, and the public.

Leslie Lang

Leslie Lang is a freelance journalist in Chapel Hill, North Carolina.

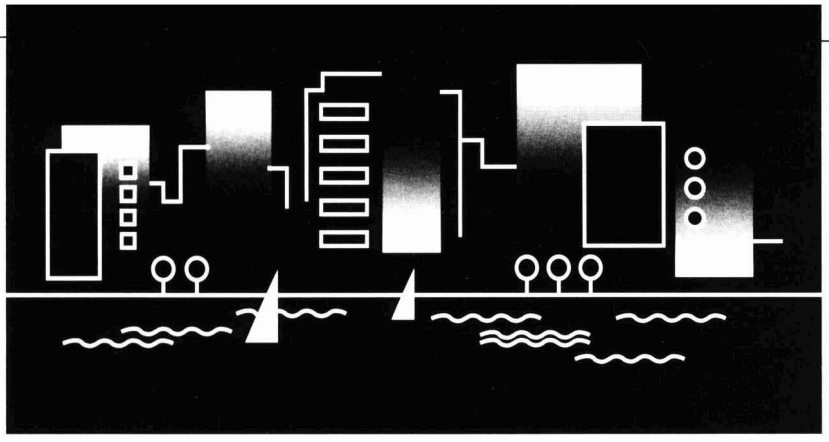
Suggested Reading

Ashby J, Tennant RW. Prediction of rodent carcinogenicity for 44 chemicals: results. Mutagenesis 9:7–15 (1994).

Barrett JC. Mechanisms of multistep carcinogenesis and carcinogen risk assessment. Environ Health Perspect 100:9–20 (1993).

National Research Council. NRC science and judgment in risk assessment. Washington, DC: National Academy Press, 1994.

U.S. EPA. Report on the workshop on cancer risk assessment guidelines issues. Washington, DC: Environmental Protection Agency, 1994.



New England Epidemiology Summer Program
1 and 2 week courses
June 5-30, 1995

15th annual summer program at Tufts University in Medford/Boston offers 17 courses reviewing established concepts and new developments in epidemiology. Registrants may also enroll for CME, CEU, Nursing CEU, Industrial Hygiene CM or graduate credit. The following courses will be offered:

June 5-9:

• Introduction to Epidemiology	Philip Cole, University of Alabama, Birmingham
• Health Care Utilization Research	Malcolm Maclure, Harvard School of Public Health
• Cancer Epidemiology	Dimitrios Trichopoulos, Harvard School of Public Health
• Pharmacoepidemiology	Wayne Ray, Vanderbilt University
• Conducting Epidemiologic Research	Harris Pastides, University of Massachusetts, Amherst
• Epidemiology in Developing Countries	Nicolaus Lorenz, Swiss Tropical Institute

June 12-23

• Theory & Practice of Epidemiology, Level I	Anders Ahlbom, National Institute for Environmental Medicine, Karolinska Institute
• Theory & Practice of Epidemiology, Level II	Kenneth Rothman, Boston University
• Biostatistics for Epidemiologists	Harland Austin, Emory University
• Regression & Categorical Data Methods	Stanley Lemeshow, University of Massachusetts, Amherst
• Clinical Research	Albert Hofman, Erasmus University Medical School

June 25-30

• Occupational & Environmental Epidemiology	Kyle Steenland, National Institute for Occupational Safety and Health
• Exposure Assessment for Occup/Env Epidemiology	Noah Seixas, University of Washington
• Causal Inference	Stephan Lanes, Epidemiology Resources Inc.
• Survival Analysis	William Louv, Glaxo Research Institute
• Ethics & Epidemiology	Kenneth Goodman, University of Miami
• Perinatal Epidemiology	Lowell Sever, Battelle Seattle Research Center

New England Epidemiology Institute

One Newton Executive Park, Dept. PA-EHP
Newton, MA 02162-1450
Telephone (617) 244-1200 FAX (617) 244-9669

Signal Transduction: Evolution of an Idea

Martin Rodbell

National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709 USA

In general there is no set of observations conceivable which can give enough information about the past of a system to give complete information as to its future.

Norbert Wiener

Think simplicity; then discard it.

Alfred North Whitehead

I was born in 1925, a time when there were no talking movies, radio was just emerging as a popular listening device, newspapers printed important information, and libraries were sources of both pleasure and learning. My father's grocery store (above which we lived) was a community center where people from blocks away would come for their groceries and to gossip. We knew or knew about everyone in our neighborhood. In that atmosphere I grew up as a young man feeling the warmth of this community. Retrospectively, I have come to realize how important this long-gone community and the intense human relationships have been to my development as a scientist. My scientific neighborhood encompasses a place where cultural and language differences have been melded seamlessly and with synergy to promote communication, to expand knowledge with a kinship of purpose, and to create new thought. Nature, which we often equate with our genetic make-up, and Nurture, which symbolizes our environment, interact mutually and synergistically in this community. These are the forces that have given meaning to life; i.e., the parable of which comes first, the chicken or the egg, is not of biological importance.

My lecture symbolizes my interest in societal/cellular relationships and concerns the broad issues of biological communication. The first half deals with the development of the concept of transducers and their role in cell signaling. Since this concept is still at an evolutionary phase, I conclude with a hypothesis which in its simplest message argues that biological communication consists of a complex meshwork of structures in which G-proteins, surface receptors, the extracellular matrix, and the vast cytoskeletal network within cells are joined in a community of effort, for which my life and those of my colleagues is a metaphor.

Receptors, Allostery, and the Second Messenger Theory

The concept of receptors as sensory elements in biology has a long history. Early in this century Paul Ehrlich realized the importance of surface receptors and postulated a "lock-and-key" theory to explain their interactions with antigenic materials and drugs. Today, it is understood that receptors are proteins with the patterns of design and malleability of structure required for discriminating between an extraordinary variety of chemical signals. My interest in receptors began in the early 1960s, when I uncorked the means of freeing adipocytes from their tissue matrix by collagenase treatment and found that insulin at physiological concentrations stimulated glucose uptake (*1*). Searching for the possible site of action of the hormone, I tested the effects of treating adipocytes with phospholipases and proteases on the assumption that, if the surface or plasma membrane contains insulin receptors, these digestive enzymes might prevent insulin action. Surprisingly, phospholipases mimicked the known actions of insulin on glucose utilization and protein synthesis (*2,3*). Based on such observations I postulated that insulin might act by stimulating phospholipases (*4*), which was not a bad hypothesis in view of the accumulated evidence of the importance of phospholipases in mediating the actions of a variety of hormones (*5*).

During the 1960s, two major theories influenced the course of my research on hormone receptors. One was the "second messenger" theory (*6,7*). This theory suggested that extracellular or primary messengers in the form of hormones or neurotransmitters act through receptors that regulate the production of 3′5′-adenosine monophosphate (cyclic AMP), considered to be the intracellular messenger that mediates the actions of hormones on all aspects of cellular metabolism, growth, and differentiation. The perceptions of Monod and colleagues that led to their incisive theory of allosteric regulation (*8*) blended beautifully with Sutherland's theory that receptors are structurally and functionally linked to the regulation of cyclic AMP production. Overwhelmingly persuasive was the notion that adenyl (now adenylate or adenylyl) cyclase is an allosterically regulated enzyme system consisting of two distinct sites, receptors and catalytic. Located at the surface or plasma membrane of cells, the assymetric positioning of these sites—the allosteric hormone-sensing sites on the exterior and ATP-utilizing catalytic sites at the interior surfaces of the membrane—provided a logical framework for investigating the molecular basis for hormone action. My attention shifted from insulin to those hormones known to stimulate the production of cyclic AMP in fat cells.

Multireceptor Adenylate Cyclase System in Adipocytes

At the time, the only specific assay for cyclic AMP production relied on a complicated, time-consuming bioassay. Krishna et al. (*9*) and later Salomon et al. (*10*) developed relatively simple chromatographic assays which for the first time allowed rapid, multiple assays of adenylate cyclase. When Lutz Birnbaumer arrived in my laboratory in 1967, that assay literally danced under his extraordinary prowess, yielding information that laid the foundation for the concept of transducers. Before he joined my laboratory, I had developed a rapid method for obtaining fat cell membranes (called "ghosts") responsive not only to insulin but also to various hormones that stimulate cyclic AMP production and resultant lipolysis in fat cells (*11*). These hormones included epinephrine, adrenocorticotropin hormone (ACTH), thyroid-stimulating hormone, leutinizing hormone, secretin, and glucagon, and fluoride ion. The latter, shown previously to stimulate adenylate cyclase in a variety of cell membranes (*6*), activated the fat-cell system by a magnesium-dependent process displaying a Hill coefficient of 2.0, suggesting that the system may contain at least two sites of magnesium action, one certainly an Mg–ATP complex at the catalytic site. That a regulatory site for magnesium exists was suggested by the finding that both ACTH and fluoride markedly reduced the concentration of magnesium ions necessary for stimulation of activity (*12*). The kinetics of ATP action proved too complicated for interpretation at the

This article is dedicated to all my colleagues, former and present, who contributed heavily to the concept of signal transduction. Without their efforts, the field of G-proteins would not have existed.

This article was delivered as a Nobel lecture on 8 December 1994 by M. Rodbell upon receipt of the Nobel Prize in Physiology or Medicine.

time. Not realizing that ATP was contaminated with GTP, we couldn't interpret what later proved to be the stimulatory and inhibitory actions of GTP on adenylate cyclase systems. The lesson is clear to me today: never attempt to interpret a hyperbolic curve; it describes the behavior of the entire universe!

Demonstration of Distinct Hormone Receptors

Much of our energy and time was devoted to delineating the receptors for the hormones that stimulated the cyclase system. The pharmacology of the peptide hormone receptors was essentially unknown and necessitated a variety of indirect tests, including the effects of proteases, inhibitory analogs, and differential ion dependencies, which combined suggested that each of the hormones stimulated cyclase through distinct receptor types. Since the enzyme system and the receptors were contained in the same cell, these findings allowed us to test a fundamental question: do all of the hormones operate on the same enzyme or, as depicted in the Sutherland model, is each hormone receptor coupled to separate cyclase models? The various hormones were tested at maximal and submaximal concentrations alone or combined with the other hormones. Synergy was seen with some combinations, but, most importantly, additivity of response was not obtained with maximal concentrations of the hormones (*13*). Similar findings were reported simultaneously (*14*). Although not proof, we argued that it is likely that the fat-cell cyclase system consists of multiple receptors interacting with a common catalytic unit. Conceptually, the picture that emerged is that each receptor contains specific binding regions and some common structural element that interacts with the catalytic component to stimulate conversion of MgATP to cAMP. At that time we considered that the catalytic component contains the regulatory site for magnesium ions and is the site of action of fluoride ion. Lipids were somehow involved in the structural interactions between receptors and catalytic unit because, unlike fluoride action, hormone action was exquisitely sensitive to agents (phospholipases, detergents) that affect membrane structure (*15*). It was clear that hormone action involved a more complex structural and regulatory enzyme system than originally conceived. It was inconceivable to me that several hormone receptors could be structurally annealed to the same enzyme (I referred to this problem as "too many angels on a pinhead"). A new concept of hormone action had to be considered.

Informational Processing: The Concept of Transduction

At that time my thinking on the subject of how hormonal information is transferred across the cell membrane and translated into action was greatly influenced by the theories of informational processing proposed by Norbert Wiener (*16*), the originator of cybernetic theory. This subject was introduced to me by Oscar Hechter, who had previously proposed several important theoretical considerations concerning hormone action. Hechter was the first to question the proposition that hormones directly acted on the adenylate cyclase enzyme (*17*). Through lengthy discussions at a downtown hotel bar in Washington, DC prior to a meeting that I had organized at NIH to honor Sutherland, we arrived at the concept of transduction as a means of coupling information between signal-activated receptor and regulation of adenylate cyclase. Given the paucity of knowledge at that time, the concept of informational processing was put in abstract cybernetic terms: discriminator for receptor, a transducer, and an amplifier representing adenylate cyclase because of the large increase in cyclic AMP generated when converted to its activated state. The transducer is a coupling device designed to allow communication between discriminator and amplifier. At the meeting I presented this idea, illustrated (but without participation of magnesium and GTP at that time) in Figure 1. We considered the possibility that magnesium ions and lipids participated in the transduction process, but we realized that the transducer concept required fleshing out with more evidence on the structure/functional relationships between receptor and enzyme.

Actions of GTP and Glucagon on Liver Cyclase

Because of the experimental complexity of studying the multireceptor adenylate cyclase system in rat adipocytes, my colleagues (Birnbaumer, Pohl, Krans) and I turned our attention to the glucagon-sensitive adenylate cyclase system in liver. To some extent this change was made because of the historical significance of the hepatic system in hormone action and, coincidentally, because Neville (*18*) at NIH had reported purification of rat liver plasma membranes by a relatively simple procedure. As importantly, we radiolabeled glucagon with ^{125}I, making it possible to investigate both the nature of the glucagon receptor and the relationship between hormone binding and hormonal activation of adenylate cyclase.

Michiel Krans began the glucagon-binding studies with our findings that hormonal activation of adenylate cyclase in liver membranes rises within seconds and falls rapidly when the hormone is displaced by an antagonist such as des-his-glucagon, which proved later to be a weak, partial agonist. Our expectations were that binding of ^{125}I-glucagon would proceed rapidly (within seconds) and would be reversed easily by washing the membranes free of medium containing the hormone. Instead, Krans observed that binding was extremely slow, requiring at least 20 min before reaching a plateau. Extensive washing under a variety of conditions failed to remove the bound material. None of the binding characteristics fit with the kinetics of hormone action. However, the medium used for binding contained nothing but salt and buffer, whereas the cyclase assay medium contained multiple components including the substrate, MgATP. A dramatic change resulted when all of the cyclase-ingredients were added to the hormone-binding medium. The level of bound hormone at steady-state was drastically reduced; maximal binding was attained within seconds. We subsequently found that ATP was the principal culprit. Realizing from painful experience as a graduate student that commercial preparations of ATP contain a variety of contaminating nucleotides, I tested many types of purine and pyrimidine nucleotides. GTP, GDP, and ITP were the only nucleotides that mimicked the effects of ATP. Most importantly, the guanine nucleotides acted at concentrations much lower (two to three

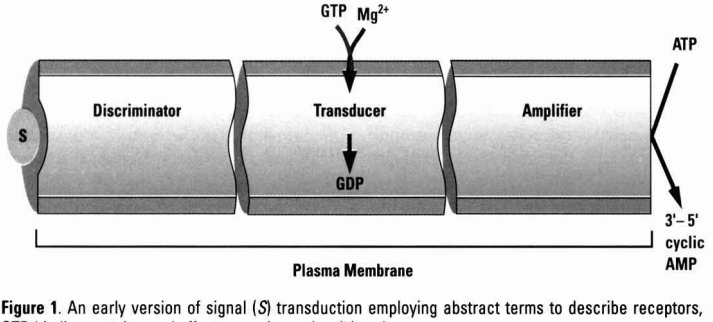


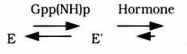
Figure 1. An early version of signal (*S*) transduction employing abstract terms to describe receptors, GTP-binding proteins, and effectors such as adenylyl cyclase.

orders of magnitude) than ATP. GppCp, a poorly hydrolyzable analog, also acted, although its effects required much higher concentrations compared to GTP or GDP. Each of the nucleotides induced rapid release of prebound glucagon from its receptor. We established that guanine nucleotides act by lowering the affinity of receptor for the hormone (*19*).

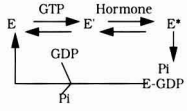
At that point the central question was the possible relationship of this effect of GTP on hormone binding to the actions of glucagon on adenylate cyclase activity. To avoid the problem of contaminating GTP in the assay for the enzyme, we prepared ^{32}P-App(NH)p as substrate using a biosynthetic method. This analog proved stable to degradation by ATPases in the membrane. Under these conditions, glucagon did not stimulate adenylate cyclase unless GTP was present in approximately the same concentrations that affected the affinity of the receptor (*20*). Subsequently, Lin and Salomon (*21*) demonstrated that hormone and GTP concertedly and rapidly induced the active form of the enzyme. Glucagon, moreover, reduced the small lag in activation given by activating nucleotide alone. The die was cast; logically GTP acts at the transduction process along with magnesium ions (Fig. 1). Although the components of the informational processing system remained unknown, there was little doubt in our minds that a transducer exists and that this crucial component mediates the transfer of information between receptor and enzyme.

GTP Hydrolysis

Because GTP was susceptible to hydrolysis by nucleotidases in membranes, our next objective was to substitute GTP with a nonhydrolyzable derivative. Taking a cue from our experience with App(NH)p, Gpp(NH)p was synthesized. A few months later, we found that Gpp(NH)p caused the enzyme's activity to "take off" to an extent not even seen with fluoride ion. To our amazement, the normally unstable cyclase system remained fully active even after 3 days at room temperature. We then tested Gpp(NH)p on a variety of cyclase systems using every cell membrane preparation we could obtain. All showed the same phenomenon (*22*). Gpp(NH)p, unlike hormone plus GTP, stimulated activity following a rather lengthy lag period which was shortened considerably when hormone was added (*21*). Salomon investigated the binding of ^{32}P-Gpp(NH)p to liver membranes and found substantial guanine nucleotide-specific binding, far in excess of the number of glucagon receptors (*23*). These findings were discounted by others because of the seeming disparity in the levels of glucagon receptor and guanine nucleotide binding sites. However, it was not understood at the time that multiple types of receptors interact with several types of GTP-binding proteins; that story evolved nearly 10 years later. The key elements of signal transduction gained from these findings were that Gpp(NH)p binds to the liver membranes in the absence of hormone, whereas glucagon quickened the ability of the nucleotide to activate adenylate cyclase, not vice versa. These findings, plus modeling of the kinetics of Gpp(NH)p/Mg (*24*), gave rise to a three-state model (Eq. 2) in which hormones act by promoting the conversion of the nucleotide-bound E' state to the activated state (E*).



(1)



(2)

However, with 21 parameters using just Mg^{2+} and Gpp(NH)p concentrations as variables, we realized that this model yielded only an approximation of what must be a very complicated system.

At about the same time, Schramm, in a series of beautifully executed experiments, demonstrated that Gpp(NH)p acted in a pseudo-irreversible fashion; i.e., removal of the nucleotide from the medium after incubation resulted in retention of the high level of cyclase activity (*25*). Based on this finding with Gpp(NH)p taken together with the inability of GTP alone to stimulate activity, we proposed that the transducer must have the capacity to hydrolyze GTP. When GTP was substituted for Gpp(NH)p in the modeling of the liver system's kinetics (Eq. 2), the data fit with the activated state (E*) being the state in which GTP was converted to GDP + P_i. In this fashion, it could be understood why activation by GTP and hormone involved essentially no lag period, whereas with Gpp(NH)p + hormone, the lag was shortened but persisted. GTP turnover, in this model, is required for the rapid, reversible actions of the hormone. A few years later, Cassel and Selinger, in a brilliant set of experiments, showed conclusively that hormones stimulated the hydrolysis of GTP to GDP + P_i. From these findings, they elaborated the theory that hydrolysis of GTP to GDP is the "turn-off" reaction and the resultant bound GDP converts the transducer to its inhibitory state (*26*). Hormones promote the displacement of GDP and its exchange with GTP; this exchange reaction is the key to hormonal activation of G-proteins. Nucleotide exchange and GTP-hydrolysis are fundamental to the regulation of all types of G-proteins that have been examined to date. Not considered in this theory, however, is that the overall turnover of GTP is a complex set of reactions including hydrolysis and subsequent release of phosphate from a bound state. In a detailed study of the light-activated rhodopsin system (*27*), it was suggested that hydrolysis of GTP is a very rapid process, whereas the rate-limiting step is the release of inorganic phosphate from its binding sites on transducin, the G-protein responsible for activation of phosphodiesterase in rod outer segments. This proposal fits with the prolonged activation by fluoride (complexed with aluminum or magnesium ions), which most likely acts by binding to the same magnesium–phosphate binding sites on transducin.

Dual Stimulatory and Inhibitory Actions of GTP and Fluoride

The multireceptor fat-cell system proved invaluable not only for investigating the multiple actions of hormones. It provided the first insight that adenylyl cyclase is both inhibited and stimulated by two independent processes involving GTP and fluoride. Löw and Harwood found that fluoride ion and both GTP and Gpp(NH)p induced stimulation and inhibition of the enzyme as the concentrations of these agents were increased (*28,29*). The mechanism was elusive until Hirohei Yamamura (*30*) noted marked differences in the properties of the stimulatory and inhibitory phases. Subsequent characterization of the dual process (*31*) and the discovery (*32*) that the fat cell contained adenosine receptors that induce inhibition of adenylate cyclase via a GTP-dependent process finally placed the inhibitory role of guanine nucleotides on the same level of importance as the stimulatory process. From these studies arose the new concept of dual regulation of adenylate cyclase by hormones, guanine nucleotides, and fluoride ion (*33*). Implicit in the argument was the understanding that transduction involving stimulation and inhibition must be exercised through distinct GTP-binding proteins. We called them "nucleotide regulatory proteins" (abbreviated N) because ITP was also active. Thus arose the nomenclature N_s and N_i, now popularly known as G_s and G_i.

One logical consequence of these findings is that G-proteins are independent of both receptors and adenylate cyclase. Pfeuffer's purification of a 42 kDa protein that he could label by incubating membranes with ^{32}P-NAD and cholera toxin (*34,35*) provided the first tangible evidence for the existence of G_s, the cyclase stimulatory transducer. It had been earlier discov-

ered that cholera toxin greatly increased the production of cAMP in intestinal cells, suggesting that the toxin acts on the adenylate cyclase system [reviewed by Gill (*36*)]. Later, pertussis toxin (*37*) provided the means for detecting and purifying G_i and G_o. Meanwhile, in the laboratory of Tompkins, it was found that treatment of cultured lymphoma cells (rat S49) with cyclic AMP resulted in cell death (*38*). Based on this phenomenon, Tompkins and co-workers isolated surviving mutant forms, one of which was eventually shown to lack the ability of Gpp(NH)p and fluoride ion to stimulate the enzyme; epinephrine action was also abolished (*39*). Using the mutant called AC^- (because it was mistakenly thought to lack adenylate cyclase), Gilman and his colleagues (*40,41*) subsequently demonstrated that supplementation with extracts from wild-type cells restored both hormonal action in a GTP-dependent fashion and the actions of Gpp(NH)p and fluoride. This assay proved useful for the first purification of what was then called G/F factor, now known as $G\alpha_s$, the transduction protein(s) responsible for stimulating adenylate cyclase.

During this period, studies in our lab (*42,43*) showed that hormone receptors linked with G_s displayed very different physical and kinetic properties from those observed when adenylate cyclase was linked (after activation) with G_s, suggesting either different states or different forms of the GTP-regulatory process. Finally, and perhaps most critically, was the discovery by Bitensky and colleagues (*44*) that light-activation of a cyclic GMP phosphodiesterase in rod outer segments was mediated by a guanine nucleotide-dependent process, similar to the actions of guanine nucleotides on adenylate cyclase. By 1980 it was clear that the actions of guanine nucleotides were not confined to the adenylate cyclase system. In a brief overview (*33*), I proposed that there must be several types of GTP-binding proteins which I called N_s, N_i, N_t (now transducin), and N_x, that mediate the actions of hormones on a number of effector systems. N_x was postulated when I learned that GTP affected the binding of agonists to receptors known to alter calcium uptake in liver cells (*45*). By 1990, those predictions were proven correct. However, the number and variety of GTP-binding proteins involved in signal transduction are now greater than I had imagined.

General Characteristics of Guanine Nucleotide Action

Within the decade of the 1970s, some of the fundamental characteristics of receptor systems coupled through GTP-binding proteins had been delineated. What followed in the ensuing 20 years was the elaboration of the types of G-proteins, now about 20. Beginning with transducin (*46*), it emerged that G-proteins are constructed of three types of subunits, an α-subunit uniquely capable of binding and degrading GTP and a tightly knit complex of β and γ subunits. This discovery, eventually established for all G-proteins coupled to receptors (*47*), opened up a new chapter in signal transduction which, in recent years, has helped to explain the pleiotropic actions of hormones.

Topological Disposition of Components

One of the most difficult problems in membrane biology is to understand how the membrane's components are organized or structured within the plane of the membrane. The topological relationship of membrane proteins to the exterior and interior components of the cell presents another major problem. The "mobile receptor" concept introduced the notion that receptor proteins are free to move rapidly within the membrane. In the case of receptors linked to G-proteins, this concept gave rise to the hypothesis that hormones act by stimulating the engagement between receptors and G-proteins. The "collision-coupling" model (*48*) attributes the rate of cyclase activation to the frequency and efficiency of collisions between agonist-bound receptors and G-protein; in this manner any one receptor can activate a number of G-proteins due to the free mobility of each component. The rate of activation of G-proteins (and adenylate cyclase) are directly proportional to the number of agonist-occupied receptors.

Although kinetic analysis can provide important insights into mechanism, in reality the fundamental question is how the different components are constructed and distributed in the plane of the membrane so that they interact with the observed efficiency and rapidity. The logistics of the encounters are obviously better if the membrane is packed with receptors, as in the case of rhodopsin in rods or cones which is in large excess of G-proteins and effectors. However, in most cells hormone receptors are present at relatively low concentrations.

For this reason, I have thought that receptors and G-proteins may be precoupled and that hormones act by altering the nature of the coupling process. This notion now seems justified based on biophysical studies which reveal that receptors are complexed with G-proteins and that such complexes are confined within matrixlike, specialized domains (*49*). In fact, receptor-coupled signaling processes in general now seem more Bhudda-like in their structures, both in their stationary setting and the multicomponent structures which appear to interact in a flickering fashion, more in keeping with the ephemeral relationship between action and inaction, between life and death.

The major concern in my laboratory starting in the late 1970s was the structure of the hormone-sensitive cyclase systems as they exist in their native membrane environment. I had learned of target or irradiation analysis from a report that target analysis might be useful for discerning the nature of the interactions between the components of the glucagon-sensitive system in liver membanes (*50*). The interpretations of the data were based on the mobile receptor theory. Of major concern to us was the fact that irradiation studies were carried out with freeze-dried material. We had learned that freeze-drying of liver membranes, for example, led to drastic reductions in hormonal regulation of adenylate cyclase. We decided to use this technique employing a different protocol not involving freeze-drying.

Fortunately, on the floor above my lab dwelled a scientist with the necessary credentials. Ellis Kempner had conducted his graduate thesis on the usage of irradiation analysis, knew both its promises and its faults, and became interested in our problem. As importantly, Werner Schlegel, a young scientist from Switzerland trained in biophysics, had just arrived in the lab looking for a suitable research problem. Schlegel and Kempner began a project which became the focal point of our research for the past 15 years.

Target Analysis

Schlegel and Kempner ultimately worked out procedures that fully preserved activity and, indeed, provided the first detailed *functional structure* of each component of the glucagon-sensitive system in liver membranes and the hormone-sensitive, stimulatory and inhibitory structures in rat adipocytes (*51,52*). I emphasize the phrase "functional structure" since the analysis measures the exponential decay in activity in relation to the energy input of electrons that bombard the system; this relationship provides the functional mass. As reviewed recently by Kempner (*53*), irradiation of complex, multicomponent enzyme systems does not cause disruption of complexes, but introduces breakages in the protein backbone along each chain of the complex. Thus, although activity is lost, the decay in activity accurately reflects the loss in functional mass.

Most surprising and initially puzzling were the findings that irradiation of both the liver and adipocyte systems prior to exposure to regulatory ligands (hormones, fluoride ion, guanine nucleotides) displayed functional target sizes of about 1500 kDa for the stimulatory processes

involving glucagon + GTP; an even larger functional size was exhibited by the inhibitory phase of the adipocyte adenosine-receptor-mediated process. Such large sizes did not fit with the estimated sizes of receptors, G-proteins, or adenylyl cyclase. When the systems were exposed first to activating ligands and then analyzed for their target sizes, dramatic reductions in functional mass were observed. For example, in the presence of glucagon and GTP, the functional size was reduced to about 350 kDa. In the presence of fluoride ion or Gpp(NH)p, the size was reduced to about 250 kDa. The size of adenylate cyclase as measured with MgATP as substrate was about 120 kDa, now realized from the structure of cloned cyclases.

Disaggregation Theory of Hormone/GTP Action

Out of these findings arose the postulate that the hormone-sensitive cyclase system is composed of an oligomeric complex of receptors and G (or N) proteins which, upon interaction with hormone and GTP, disaggregate into monomers of the receptor-G complex (*33*).

Most importantly, target analysis led me to the conclusion that the primary signal emanating from the actions of hormones must be a protein; this protein had to consist, minimally, of a GTP-binding protein. Not knowing that G-proteins were heterotrimers, the estimated size of the monomer ranged from about 120 kDa [fluoride- or Gpp(NH)p-activation] to about 220 kDa after glucagon-treatment (correcting for the estimated mass of cyclase). The estimated values obtained after fluoride or Gpp(NH)p treatment were much larger than that of $G\alpha_s$ (43–50 kDa). The larger value obtained after glucagon treatment I conjectured as the combination of the receptor complexed with a monomer of G_s. The monomer complex, considered to be the true "messenger" of hormone action, reacts with adenylate cyclase resulting in either stimulation (by G_s) or inhibition (by G_i). This theory I termed the "Disaggregation Theory of Hormone Action" (*33*). Incorporated are the fundamental ideas that the structure of the receptor–G-protein complex is a multimer of these components, that adenylyl cyclase exists separately from the complex, and that a "monomeric" structure derived from the disaggregation is the messenger that communicates information from the hormone-bound receptor–G-protein complex to the effector or enzyme.

In this model, I had assumed that receptors and G-proteins existed in about equal amounts and were coupled stoichiometrically. Much later when accurate methods became available for measuring the concentrations of receptors and G-proteins in cells, it became clear that in most cells, G-proteins are present in excess of receptors, possibly as much as 10:1. Given such information, clearly the model must be altered in that the largest portion of the mass of the glucagon-sensitive adenylyl cyclase (or the adenosine-sensitive, inhibitory system in adipocytes) must be attributed to that of G-proteins i.e., G-proteins are likely multimeric structures.

The disaggregation theory soon fell into disfavor because of the findings that heterotrimeric G-proteins treated with Gpp(NH)p or the later, more popular GTPγS dissociated into free α-subunits and the βγ complexes (*54,55*). From this arose the "dissociation" theory (Gilman, 1988). On my part, the disaggregation theory clearly needed biochemical evidence for the existence of multimeric forms of G-proteins. The odyssey in this direction began with two approaches: cross-linking experiments with synaptoneurosomes from rat brain and extraction of G-proteins with various detergents followed by sucrose-gradient analysis of the hydrodynamic properties of the extracted material.

Cross-linking Studies

Synaptoneurosome membranes were chosen for most of the studies because brain tissue contains the bulk of all known types of G-proteins. We were greatly aided in these studies by generous contributions from several colleagues (principally, Alan Spiegel at NIH) in the field who had prepared polyclonal antibodies against peptide sequences of the α and β subunits of several types of G-proteins (G_s, G_i, G_o, and G_q), including subspecies of these proteins.

We tested a variety of cross-linking agents for both their efficacy and selectivity of action at low concentrations. Phenylenedimaleimide proved the most satisfactory. In addition to all of the G-proteins tested, multimeric tubulin and F-actin were the only two types of membrane-associated proteins that were detectably cross-linked (*56*). After cross-linking in their membrane-environment, the G-proteins were extracted with sodium dodecyl sulfate and chromatographed on sieving columns that allow separation of proteins over a large range of sizes. In this manner it was found that both α- and β-subunits of G_s, G_i, G_o, and G_q were cross-linked to form structures comparable in size to cross-linked tubulin or actin. We concluded from these studies that G-proteins, most likely intact heterotrimers, are multimeric structures in association with the plasma membrane. Such evidence provided substantial credence to our basic arguments for the disaggregation theory. Most importantly, it appeared that multimeric G-proteins are responsible for the large ground-state structures observed with target analysis.

Detergent Studies

The next stage necessitated some means of isolating the multimeric G-proteins, a process necessitating the use of detergents. Aware of the fact that detergents such as sodium cholate and Lubrol extracted intact heterotrimeric structures (*57*); i.e., monomers of the putative multimers, we considered the possibility that these detergents may disrupt the multimeric structure. Accordingly, we tested the sizes of G-protein structures extracted with a variety of detergents, using hydrodynamic properties on sucrose gradients as our assay. Of the seven tested, octyl glucoside (OG), Tween 20, and digitonin yielded structures behaving hydrodynamically larger than those given with sodium cholate or Lubrol, after correcting for the possible contributions of micellar forms of the detergents (*58*). OG extracted from liver membranes structures that were heterodisperse, about 10% sedimenting through sucrose gradients, the bulk remaining soluble in the detergent. When membranes were treated with cholera toxin in the presence of ^{32}P-NAD (the means of specifically labeling $G\alpha_s$), the majority of labeled material appeared in the insoluble fraction (*59,60*). When such labeled material in the membranes was subjected to the combined actions of glucagon and low concentrations of GTPγS, a large portion of the insoluble material became soluble and appeared in a fraction similar to that of purified heterotrimeric G_s.

Based on the cross-linking and hydrodynamic studies, we deduced that G_s is likely multimeric in liver and synaptoneurosome membranes, that only multimeric structures are altered by glucagon and low concentrations of GTPγS in liver membranes, and that one of the primary results of their action is the disaggregation of multimers to monomers, as predicted in the disaggregation theory. In synaptoneurosomes, high concentrations of GTPγS caused dissociation into free α and βγ of heterotrimeric G-proteins dissolved in Lubrol or sodium cholate but not in digitonin (*58*). Hence, our suspicions were confirmed that the native structures of G-proteins are not preserved with detergents used for purifying heterotrimeric forms of G-proteins.

Extended Disaggregation Theory of Hormone Action

Target analysis provided the initial impetus for proposing the disaggregaton theory. However, it has become clear that the the-

ory as originally presented has to be modified to account for the fact that G-proteins are the major component representing the large functional mass; i.e., G-proteins form multimeric structures. We had also established that there are marked differences between the regulation of G-proteins by the coupled receptors and the regulation of adenylyl cyclase by G-proteins (*42,43*). When the structures and regulatory properties of adenylyl cyclases became known (*61*), particularly the fact that these are transmembrane proteins that have a two-cassette structure (i.e., two distinct domains on a 12 membrane-spanning structure,) it became possible to construct a more coherent theory to explain the regulation of the cyclase system (*62*). Two regulatory cycles, one for regulation of multimer to monomer G-proteins, the other for regulation of cyclase by a monomeric G-protein (G_s) are illustrated in Figure 2.

The excursion of receptor along the multimeric G-protein chain is governed by the hormone-induced exchange of GTP and GDP; the GTP-occupied monomer at one end is released, allowing it either to interact with adenylyl cyclase or to return (after hydrolysis of GTP to GDP) to the other terminus of the multimer. In cycle B, the GTP-occupied monomer interacts with the enzyme without necessarily inducing significant changes in enzyme activity. Activity is governed by magnesium-dependent hydrolysis of bound GTP to GDP + P_i. In this theory hydrolysis induces dissociation of α from $\beta\gamma$; the resultant separated subunits interact distinctively with the two cassettes or domains of adenylyl cyclase. Depending on the type of adenylyl cyclase associated with the associated G-protein, activity is governed solely by α_s, synergistically by the combination of α_s and $\beta\gamma$, or by inhibition of α_s- stimulation by $\beta\gamma$. Release of P_i from its binding site on α_s results in reassociation of α_s with $\beta\gamma$. The GDP-bound G_s then reassociates with the multimer to become part of the hormone-regulated cycle. It should be emphasized that both cycles occur in association with the surface membrane. The principal element that differs from other theories of hormone-regulated cyclase systems is that the concerted interactions of enzyme, Mg^{2+} and GTPase are responsible for separation of α_s from $\beta\gamma$. The extent and duration of enzyme stimulation are controlled by the independent actions of the separated subunits and the rate at which P_i is released following hydrolysis.

Most people in the field will argue that hydrolysis is not necessary for activation because nonhydrolyzable analogs of GTP are fully capable of stimulating cyclase activity. However, my view is that allosteric regulation by Gpp(NH)p, a slow, hysteretic process, may involve stabilization of a magnesium-induced disassociation of G_s that normally exists transiently and which does not require any participation by adenylyl cyclase in the dissociative process. In this sense, the nonhydrolyzable analogs of GTP may have misguided many in the G-protein field into thinking that energy derived from the splitting of GTP is not involved in signal transduction. It should be noted in this extension of the disaggregation theory that both disaggregation of multimers and dissociation of monomers are separate but interrelated phenomena, both contributing to the overall dynamics of signal transduction.

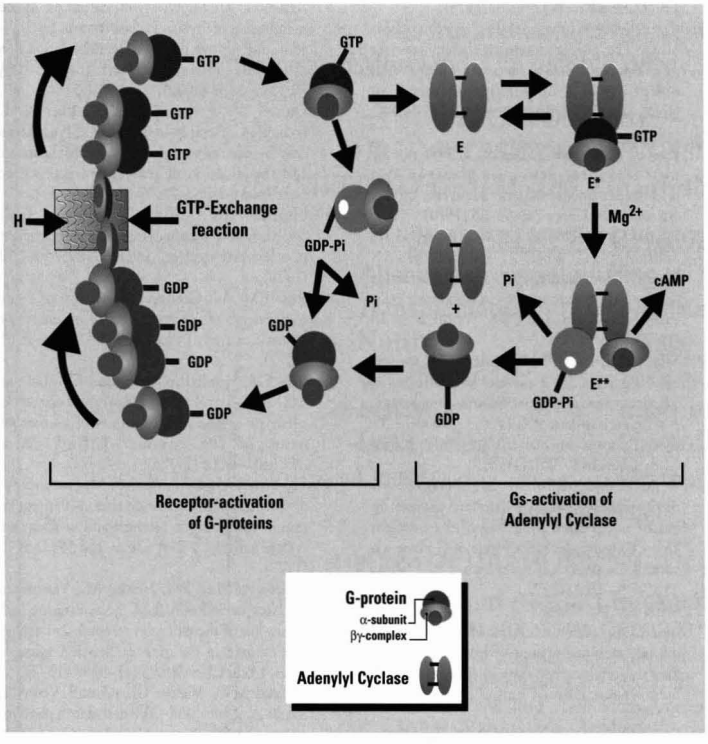


Figure 2. Regulation of the adenylyl cyclase system through receptor activation of G-proteins and the activation of adenylyl cyclase by G_s.

G-Proteins Are Similar to Cytoskeletal Proteins

During these studies, my attention was drawn to the striking similarities in the properties of G-proteins with those of tubulin and actin, the major cytoskeletal elements in cells [which I have reviewed (*63*)]. For example, G-proteins, like actin and tubulin, are associated with the inner aspect of the surface membrane, adhering possibly both through intrinsic membrane proteins, such as receptors, and to membrane lipids. Of particular interest is the fact that all three types of multimeric proteins are subject to regulation by either GTP (G-proteins and tubulin) or ATP (actin) and their hydrolytic products (dinucleotides and P_i). Receptors regulate exchange of bound nucleotides (GDP with GTP) and act catalytically in the process. Similarly, the excursion of a single myosin molecule during muscle contraction along the chain of actin multimers is governed by the exchange of bound ADP with ATP and the hydrolysis of ATP to ADP and P_i; As stated previously, GTP turnover (production of GDP+P_i) is essential for the rapid and sustained actions of hormones; release of bound P_i is the crucial rate-limiting process in the overall dynamics of signaling. The same is true for myosin/actin interactions (*64*).

With these similarities in structure and regulation, G-proteins can be classified as part of the cytoskeletal matrix, with the primary functional difference that G-proteins serve as chemical signaling devices, whereas tubulin and actin serve as mechano-signaling devices. The release of monomers from multimers is the basis for chemical signaling by G-proteins. Dynamic changes in the disaggregation–aggregation cycle of actin and tubulin multimers are fundamental for regulating the interactions or movement between specialized components of cells. Based on evidence accumulated over the

past decade (*63*), all three types of cytoskeletal proteins are connected in some manner to a variety of signaling systems that adhere to the cytoskeletal matrix, including heterotrimeric G-proteins, so-called small molecular weight G-proteins, protein kinases and phosphatases, and other proteins or systems that communicate between the surface membrane and the interior of cells. These components form weblike structures that possibly interact in a flickering manner in response to activation of membrane receptors, including those that are growth promoting.

Given the extraordinary complexity of signaling processes, as viewed at the biochemical level, clearly needed are new investigatory tools. Already promising are the microscopic imaging techniques with immunofluorescent molecules for specifically tagging and viewing structures in their living environment. I suspect that the reductionists with their prowess in molecular biology and X-ray crystallography and those of us attempting to view the living process at the cellular level will merge with our assemblages of ideas and experiences. When this larger, multiplex community of effort finally is consummated, a bright new era in scientific discovery will certainly emerge.

References

1. Rodbell M. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. J Biol Chem 239:375–380 (1964).
2. Rodbell M. Metabolism of isolated fat cells. II. The similar effects of phospholipase C (clostridium perfringens alpha toxin) and of insulin on glucose and amino acid metabolism. J Biol Chem 241:130–139 (1966).
3. Rodbell M, Jones AB. Metabolism of isolated fat cells. III. The similar inhibitory action of phospholipase C (clostridium perfringes alpha toxin) and of insulin on lipolysis stimulated by lipolytic hormones and theophylline. J Biol Chem 241:140–142 (1966) .
4. Rodbell M, Jones AB, Chiappe de Cingolani GE, Birnbaumer L. The actions of insulin and catabolic hormones on the plasma membrane of the fat cells. Recent Prog Horm Res 24:215–254 (1968)
5. Berridge MJ, Irvine RF. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312:315–321 (1984).
6. Sutherland EW, Rall TW, Menon T. Adenyl cyclase. I. Distribution, preparation, and properties. J Biol Chem 237:1220–1227 (1962).
7. Sutherland EW, Robison GA. The role of cyclic-3',5'-AMP in responses to catecholamines and other hormones. Pharmacol Rev 18:145–161 (1966).
8. Monod J, Changeuxc JP, Jacob F. Allosteric proteins and cellular control systems. J Mol Biol 6:306–329 (1963).
9. Krishna G, Weiss B, Brodie BB. A simple, sensitive method for the assay of adenyl cyclase. J Pharmacol Exp Ther 163:379–386 (1968).
10. Salomon Y, Londos C, Rodbell M. A highly sensitive adenylate cyclase assay. Anal Biochem 58:541–548 (1974).
11. Rodbell M. Metabolism of isolated fat cells. VI. The effects of insulin, lipolytic hormones, and theophylline on glucose transport and metabolism in ghosts. J Biol Chem 242:5751–5756 (1967).
12. Birnbaumer L, Pohl SL, Rodbell M. Adenyl cyclase in fat cells. I. Properties and the effects of adrenocorticotropin and fluoride. J Biol Chem 244:3468–3476 (1969).
13. Birnbaumer L, Rodbell M. Adenyl cyclase in fat cells. II. Hormone receptors. J Biol Chem 244:3477–3482 (1969) .
14. Bär HP, Hechter O. Adenyl cyclase and hormonme acton. I. Effects of adrenocortiotropic hormone, glucagon, and epinephrine on the plasma membrane of rat fat cells. Proc Natl Acad Sci USA 63:350–356 (1969).
15. Birnbaumer L, Pohl SL, Michiel H, Krans MJ, Rodbell M. The actions of hormones on the adenyl cyclase system. In: Advances in biochemical psychopharmacology (Greengard P, Costa E, eds). New York:Raven Press, 1970;185–208.
16. Wiener N. Cybernetics. New York:MIT Press, 1961
17. Hechter O, Halkerston IDK. The hormones: physiology, chemistry, and applications. In: The hormones, vol 5 (Pincus G, Thiman KV, Astwood EB, eds). New York:Academic Press, 1964;697–825.
18. Neville D. Isolation of an organ specific protein antigen from cell-surface membrane or rat liver. Biochim Biophys Acta 154:540–552 (1968).
19. Rodbell M, Krans HMJ, Pohl SL, Birnbaumer L. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. IV. Effects of guanylnucleotides on binding of ^{125}I-glucagon. J Biol Chem 246:1872–1876 (1971).
20. Rodbell M, Birnbaumer L, Pohl SL, Krans HMJ. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanylnucleotides in glucagon action. J Biol Chem 246:1877–1882 (1971).
21. Rodbell M, Lin MC, Salomon Y. Evidence for interdependent action of glucagon and nucleotides on the hepatic adenylate cyclase system. J Biol Chem 249:59–65 (1974) .
22. Londos C, Salomon Y, Lin MC, Harwood JP, Schramm M, Wolff J, Rodbell M. 5'-Guanylylimidodiphosphate, a potent activator of adenylate cyclase systems in eukaryotic cells. Proc Natl Acad Sci USA 71:3087–3090 (1974).
23. Salomon Y, Rodbell M. Evidence for specific binding sites for guanine nucleotides in adipocyte and hepatocyte plasma membranes. A difference in fate of GTP and guanosine 5'-(beta, gamma-imino) triphosphate. J Biol Chem 250:7245–7250 (1975).
24. Rendell MS, Rodbell M, Berman M. Activation of hepatic adenylate cyclase by guanyl nucleotides. Modeling of the transient kinetics suggests an excited state of GTPase is a control component of the system. J Biol Chem 252:7909–7912 (1977).
25. Schramm M, Rodbell M. A persistent active state of the adenylate cyclase system produced by the combined actions of isoproterenol and guanylyl imidodiphosphate in frog erythrocyte membranes. J Biol Chem 250:2232–2237 (1975).
26. Cassel D, Levkovitz H, Selinger Z. The regulatory GTPase cycle of turkey erythrocyte adenylate cyclase. J Cyclic Nucleotide Res 3:393–406 (1977).
27. Ting TD, Ho YK. Molecular mechanism of GTP hydrolysis by bovine transducin: presteady-state kinetic analyses. Biochemistry 30:8996–9007 (1991).
28. Harwood JP, Low H, Rodbell M. Stimulatory and inhibitory effects of guanyl nucleotides on fat cell adenylate cyclase. J Biol Chem 254:6239–6245 (1973).
29. Harwood JP, Rodbell M. Inhibition by fluoride ion of hormonal activation of fat cell adenylate cyclase. J Biol Chem 248:4901–4904 (1973).
30. Yamamura H, Lad PM, Rodbell M. GTP stimulates and inhibits adenylate cyclase in fat cell membranes through distinct regulatory processes. J Biol Chem 252:7964–7966 (1977).
31. Cooper DMF, Schlegel W, Lin MC, Rodbell M. The fat cell adenylate cyclase system. Characterization and manipulation of its bimodal regulation by GTP. J Biol Chem 254:8927–8931 (1979).
32. Londos C, Wolff J. Two distinct adenosine-sensitive sites on adenylate cyclase. Proc Natl Acad Sci USA 74:5482–5486 (1977).
33. Rodbell M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature 284:17–22 (1980).
34. Pfeuffer T. GTP-binding proteins in membranes and the control of adenylate cyclase activity. J Biol Chem 252:7224–7234 (1977).
35. Pfeuffer T, Helmreich EJ. Activation of pigeon erythrocyte membrane adenylate cyclase by guanylnucleotide analyoges and separation of a nucleotide binding protein. J Biol Chem 250:867–876 (1975).
36. Gill DM. Mechanism of action of cholera toxin. Adv Cyclic Nucleotide Res 8:85–118 (1977).
37. Katada T, Ui M. Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. Proc Natl Acad Sci USA 79:3129–3133 (1982).
38. Daniel V, Litwack G, Tomkins GM. Induction of cytolysis of cultured lymphoma cells by adenosine 3':5'-cyclic monophosphate and the isolation of resistant variants. Proc Natl Acad Sci USA 70:76–79 (1973)
39. Bourne HR, Coffino P, Tomkins GM. Selection of a variant lymphoma cell deficient in adenylate cyclase. Science 187:750–752 (1975).
40. Ross EM, AG Gilman. Resolution of some components of adenylate cyclase necessary for catalytic activity. J Biol Chem 252:6966–6969 (1977).
41. Ross EM, Howlett AC, Ferguson KM, Gilman AG. Reconstruction of hormone-sensitive adenylate cyclase activity with resolved components of the enzyme. J Biol Chem 253:6401–6412 (1978).
42. Lad PM, Welton AF, Rodbell M. Evidence for distinct guanine nucleotide sites in the regulation of the glucagon receptor and of adenylate cyclase activity. J Biol Chem 252:5942–5946 (1977).
43. Welton AF, Lad PM, Newby AC, Yamamura H, Nicosia S, Rodbell M. Solubilization and separation of the glucagon receptor and adenylate cyclase in guanine nucleotide-senstive states. J Biol Chem 252:5947–5950 (1977).
44. Bitensky MW, Wheeler GL, Aloni B, Vetury S, Matuo Y. Light- and GTP-activitated photoreceptor phosphodiesterase: regulation by a light-activated GTPase and identification of rhodopsin as the phosphodiesterase binding site. Adv Cyclic Nucleotide Res 9:553–572 (1978).

45. Goodhardt M, Ferry N, Geynet P, Hanoune J. Hepatic alpha 1-adrenergic receptors show agonist-specific regulation by guanine nucleotides. Lose of nucleotide effect after adrenalectomy. J Biol Chem 257:1157–1183 (1982)
46. Fung BKK. Characterization of transducin from bovine retinal rod outer segments. I. Separation and reconstitution of the subunits. J Biol Chem 258:10495–10502 (1983).
47. Gilman AG. G proteins: transducers of receptor-generated signals. Annu Rev Biochem 56:615–649 (1987).
48. Tolkovsky AM, Levitzki A. Mode of coupling between the beta-adrenergic receptor and adenylate cyclase in turkey erythrocytes Biochemistry 17:3795–3810 (1978).
49. Neubig RR. Membrane organization in G-protein mechanisms. FASEB J 8:939–946 (1994).
50. Houslay MD, Ellory JC, Smith GA, Hesketh TR, Stein JM, Warren GB, Metcalfe JC. Exchange of partners in glucagon receptor-adenylate cyclase complexes. Physical evidence for the independent, mobile receptor model. Biochim Biophys Acta 467:208–219 (1977).
51. Schlegel W, Kempner E, Rodbell M. Activation of adenylate cyclase in hepatic membranes involves interactions of the catalytic unit with multimeric complexes of regulatory proteins. J Biol Chem 254:5168–5176 (1979).
52. Schlegel W, Cooper DMF, Rodbell M. Inhibition and activation of fat cell adenylate cyclase by GTP is mediated by structures of different size. Arch Biochem Biophys 2011:678–682 (1980).
53. Kempner ES. Novel predictions from radiation target analysis. Trends Biochem Sci 18:236–239 (1993).
54. Codina J, Hildebrandt JD, Birnbaumer L, Sekura RD. Effects of guanine nucleotides and Mg on human erythrocyte Ni and Ns, the regulatory components of adenylyl cylase. J Biol Chem 259:11408–11418 (1984).
55. Northup JK, Sternweiss PC, Gilman AG. The subunits of the stimulatory regulatory component of adenylate cyclase. Resolution of the activiated 45,000-dalton (alpha). J Biol Chem 258:11369–11376 (1983).
56. Coulter S, Rodbell M. Heterotrimeric G proteins in synaptoneurosome membranes are crosslinked by p-phenylenedimaleimide, yielding structures comarable in size to crosslinked tubulin and F-actin. Proc Natl Acad Sci USA 89:5842–5846 (1992).
57. Sternweis PC, Northup JK, Smigel MD, Gilman AG. The regulatory component of adenylate cyclase. Purification and properties. J Biol Chem 256:11517–11526 (1981)
58. Jahangeer S, Rodbell M. The disaggregation theory of signal transduction revisited: further evidence that G proteins are multimeric and disaggregate to monomers when activated. Proc Natl Acad Sci USA 90:8782–8786 (1993).
59. Nakamura S, Rodbell M. Octyl glucoside extracts GTP-binding regulatory proteins from rat brain synaptoneurosomes as large, polydisperse structures devoid of beta gamma complexes and sensitive to disaggregation by guanine nucleotides. Proc Natl Acad Sci USA 87:6413–6417 (1990).
60. Nakamura S, Rodbell M. Glucagon induces disaggregation of polyner-like structures of the alpha subunit of the stimulatory G proteins in liver membranes. Natl Acad Sci 88:7150–7154 (1991).
61. Tang WJ, Gilman AG. Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. Science 254:1500–1503 (1991).
62. Rodbell M, Jahangeer S, Coulter S. In: GTPases in Biology (Dickey BF, Birnbaumer L, eds). Berlin:Springer-Verlag, 1993;3–14.
63. Rodbell M. The role of GTP-binding proteins in signal transduction: from the sublimely simple to the oncceptually complex. Curr Top Cell Regul 32:1–47 (1992).
64. Carlier MF. Actin polymerizaton and ATP hydrolysis. Adv Biophys 26:51–73 (1990).

Environmental and Dietary Estrogens and Human Health: Is There a Problem?

Stephen H. Safe

Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX 77843-4466 USA

It has been hypothesized that organochlorine pesticides and other environmental and dietary estrogens may be associated with the increased incidence of breast cancer in women and decreased sperm concentrations and reproductive problems in men. However, elevation of organochlorine compounds such as dichlorodipehenyldichloroethylene (DDE) and polychlorinated biphenyls (PCBs) in breast cancer patients is not consistently observed. Reanalysis of the data showing that male sperm counts decreased by over 40% during 1940 to 1990 indicated that inadequate statistical methods were used and that the data did not support a significant decline in sperm count. Humans are exposed to both natural and industrial chemicals which exhibit estrogenic and antiestrogenic activities. For example, bioflavonoids, which are widely distributed in foods, and several industrial compounds, including organochlorine pesticides and various phenolic chemicals, exhibit estrogenic activity. Humans are also exposed to chemicals which inhibit estrogen-induced responses such as the aryl hydrocarbon receptor (AhR) agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related chlorinated aromatics, polynuclear aromatic hydrocarbon combustion products, and indole-3-carbinol, which is found in cruciferous vegetables. Many of the weak estrogenic compounds, including bioflavonoids, are also antiestrogenic at some concentrations. A mass balance of dietary levels of industrial and natural estrogens, coupled with their estimated estrogenic potencies, indicates that the dietary contribution of estrogenic industrial compounds is 0.0000025% of the daily intake of estrogenic flavonoids in the diet. Moreover, dietary levels of antiestrogen equivalents (industrial or natural) are significantly higher than the estrogen equivalents of organochlorine pesticides. The suggestion that industrial estrogenic chemicals contribute to an increased incidence of breast cancer in women and male reproductive problems is not plausible. *Key words*: antiestrogens, dietary estrogens, estrogen equivalents, pesticides, polychlorinated biphenyls, TCDD. *Environ Health Perspect* 103: 346–351 (1995)

Recent reports have suggested that background levels of industrial chemicals and other environmental pollutants may play a role in development of breast cancer in women and decreased male reproductive success as well as the reproductive failures of some wildlife species (*1–6*). These suggestions have been supported by articles in the popular and scientific press (*7–13*) and by a television documentary (*14*) which have described the perils of exposure to endocrine-disrupting chemicals such as estrogenic organochlorine pesticides and pollutants. During the past two decades, environmental regulations regarding the manufacture, use, and disposal of chemicals have resulted in significantly reduced emissions of most industrial compounds and their by-products. Levels of the more environmentally stable organochlorine pesticides and pollutants are decreasing in most ecosystems including the industrialized areas around the Great Lakes in North America (*15–18*). Decreased levels of organochlorine compounds correlates with the improved reproductive success of highly susceptible fish-eating water birds in the Great Lakes region (*19*). This article reviews key papers that have been used to support the hypotheses that environmental estrogens play a role in the increased incidence of breast cancer in women and decreased sperm counts in males. Environmental/dietary estrogens and antiestrogens are identified and intakes of "estrogen equivalents" are estimated to compare the relative dietary impacts of various classes of estrogenic chemicals.

Role of Estrogens in Breast Cancer and Male Reproductive Problems

Concerns regarding the role of environmental and dietary estrogens as possible contributors to the increased incidence of breast cancer were fueled by several reports that showed elevated levels of organochlorine compounds in breast cancer patients (*20–24*). The results presented in Table 1 summarize some of these studies that compare levels of organochlorine compounds in breast tissue or serum from breast cancer patients and controls. Polychlorinated biphenyls (PCBs) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) are the two most abundant organochlorine pollutants identified in all human tissues with high frequencies. In one Scandinavian study, levels of DDE or PCBs in adipose tissue from breast samples were not significantly different in breast cancer patients compared to controls (*20*). In another study in Finland, β-hexachlorocyclohexane levels were elevated in breast cancer patients (*21*); however, this compound was not detected in adipose tissue of some individuals in the patient and control groups and has a relatively low frequency of detection in human tissue samples. Falck and co-workers reported that PCB levels were elevated in mammary adipose tissue samples from breast cancer patients in Connecticut (*22*). In contrast, serum levels of DDE (but not PCBs) were significantly elevated in breast cancer patients enrolled in the New York University Women's Health Study (*23*). DDE (but not PCB) levels were also elevated in estrogen receptor (ER)-positive but not ER-negative breast cancer patients from Quebec compared to levels in women with benign breast disease (*24*). It was initially concluded by Wolff and co-workers that "these findings suggest that environmental chemical contamination with organochlorine residues may be an important etiologic factor in breast cancer" (*22*). The correlations reported in the two U.S. studies (*22,23*) heightened public and scientific concern regarding the potential role of these compounds in development of breast cancer. These observations undoubtedly reinforced advocacy by some groups for a ban on the use of all chlorine-containing chemicals. However, the proposed linkage between PCBs and/or DDE and breast cancer is questionable for the following reasons:

- Most studies with PCBs indicate that these mixtures are not estrogenic, and the weak estrogenic activity observed for lower chlorinated PCB mixtures may be due to their derived hydroxylated metabolites;
- *p,p'*-DDE, the dominant persistent metabolite of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-DDT), is not estrogenic, and levels of *o,p'*-DDT, the estrogenic member of the DDT family, are low to nondetectable in most environmental samples;

I gratefully acknowledge financial support for research on antiestrogens from the National Institute of Environmental Health Sciences and for research on dietary/environmental estrogens from the Chemical Manufacturers Association.

Received 4 October 1994; accepted 13 December 1994.

Table 1. Organochlorine levels in breast cancer patients

Country	Organochlorine compound	Patient group (*n*)	Levels	Reference
Finland	β-Hexachlorocyclohexane levels elevated in breast cancer patients (breast tissue)	Breast cancer patients (24) Controls (16)	0.13 ± 0.06 ppm 0.08 ± 0.03 ppm	(*21*)
Norway	DDT and PCB levels comparable in patients and controls (breast tissue)	Breast cancer patients (18) Controls (35)	6.47 ± 2.35 ppm PCB 5.12 ± 2.38 ppm PCB; 1.97 ± 2.24 ppm DDT	(*20*)
USA (Connecticut)	PCB levels elevated in breast cancer patients (breast tissue)	Breast cancer patients (20) Controls (20)	1669 ± 894 ppm PCB 1105 ± 424 ppb PCB	(*23*)
USA (New York)	DDE levels elevated in breast cancer patients (serum)	Breast cancer patients (58) Controls (171)	11.0 ± 9.1 ng/mL 7.7 ± 6.8 ng/mL	(*22*)
USA (California)	PCB and DDE levels comparable in patients and controls (serum)	Breast cancer patients (150) Controls (150)	4.4 ± 1.8 ppb PCB; 43.3 ± 25.9 ppb DDE 4.8 ± 2.5 ppb PCB; 43.1 ± 23.7 ppb DDE	(*27*)
Canada (Quebec)	DDE levels increased in estrogen receptor-positive patients (breast tissue)	Breast cancer patients (9) Controls (17)	2132 ± 2050 ppm 765 ± 527 ppm	(*24*) (*24*)

- Epidemiology studies of individuals occupationally exposed to relatively high levels of DDT (*25*) or PCBs (*26*) do not show a higher incidence of breast cancer; and
- No single class of organochlorine compounds was elevated in all studies, suggesting that other factors may be critical for development of breast cancer.

Krieger and co-workers (*27*) recently reported results from a nested case–control study of women from the San Francisco area which showed that there were no differences in serum DDE or PCB levels between breast cancer patients and control subjects. The authors concluded that "the data do not support the hypothesis that exposure to DDE and PCBs increases risk of breast cancer" (*27*: p. 589). This was duly noted in *Time* magazine (*28*) by a three-line statement in "The Good News" section. Moreover, combined analysis of the 6 studies which report PCB and DDE levels in 301 breast cancer patients and 412 control patients showed that there were no significant increases in either DDE or PCB levels in breast cancer patients versus controls (*29*).

The second major link between environmental/dietary estrogens and human disease was precipitated by an article published in the *Lancet*, in which Sharpe and Skakkebaek (*5*) hypothesized that increased estrogen exposure may be responsible for falling sperm counts and disorders of the male reproductive tract. Unlike the proposed link between environmental estrogens and breast cancer, this hypothesis was not based on experimentally derived measurements of increased levels of any estrogenic compounds in males. Previous studies with diethylstilbestrol, a highly potent estrogenic drug, showed that *in utero* exposure results in adverse effects in male offspring (*30*), and the authors' hypothesized that *in utero* exposure to environmental/dietary estrogens may also result in adverse effects in male offspring. A critical experimental component supporting the authors' hypothesis was their analysis of data from several studies which indicated that male sperm counts had decreased by over 40% during the past 50 years (*31*). These observations, coupled with the hypothesis that environmental estrogens including organochlorine chemicals were possible etiologic agents, were reported with alarm in the popular and scientific press (*7–12*) and in a BBC television program entitled "Assault on the Male: a Horizon Special" (*14*). Subsequent and prior scientific studies have cast serious doubts on both the hypothesis (*5*) and the observed decrease in male sperm counts (*31*). In 1979, Macleod and Wang (*32*) reported that there had been no decline in sperm counts, and reanalysis of the data presented by Carlsen and co-workers showed that sperm counts had not decreased from 1960 to 1990 (*33*). Thus, during the time in which environmental levels of organochlorine compounds were maximal, there was not a corresponding decrease in sperm counts. Moreover, a reevaluation of the sperm concentration data was recently reported by Brownwich et al. (*34*) in the *British Medical Journal*, and their analysis suggested that the decline in sperm values in males was a function of the choice of the normal or reference value for sperm concentrations. The authors contend that their analysis of the data does "not support the hypothesis that the sperm count declined significantly between 1940 and 1990" (*34*: p. 19).

These results suggest that the increasing incidence of human breast cancer is not related to organochlorine environmental contaminants and that decreases in sperm counts is highly debatable. Nevertheless, human populations are continually exposed to a wide variety of environmental and dietary estrogens, and these compounds clearly fit into the category of "endocrine disrupters." The remainder of this article briefly describes the different structural classes of both environmental and dietary estrogens and quantitates human exposures to these compounds.

Synthetic Industrial Chemicals with Estrogenic Activity

The estrogenic activities of different structural classes of industrial chemicals were reported by several research groups in the late 1960s and 1970s in which *o,p′*-DDT and other diphenylmethane analogs (Fig. 1) and the insecticide kepone were characterized as estrogens (*35–38*). Subsequent studies have confirmed the estrogenic activity of *o,p*-DDT and related compounds (*39*) whereas the *p,p′*-substituted analogs were relatively inactive (*36,37*). In addition, *p,p′*-methoxychlor and its hydroxylated metabolites elicit estrogenic responses (*39,40*). Ecobichon and Comeau (*41*) investigated the estrogenic activities of commercial PCB mixtures (Aroclors) and individual congeners in the female rat uterus and reported estrogenic responses for some Aroclors and individual congeners. Studies in this laboratory showed that a number of commercial PCBs did not significantly increase secretion of procathepsin D, an estrogen-regulated gene product, in MCF-7 human breast cancer cells (*42*). It should be noted that several hydroxylated PCBs bind to the ER, and it is possible that *para*-hydroxylated PCB metabolites may be the active estrogenic compounds associated with lower chlorinated PCBs (*43*). A recent study reported that several additional organochlorine pesticides including endosulfan, toxaphene, and dieldrin exhibit estrogenlike activity and induce proliferation of MCF-7 human breast cancer cells (*44*).

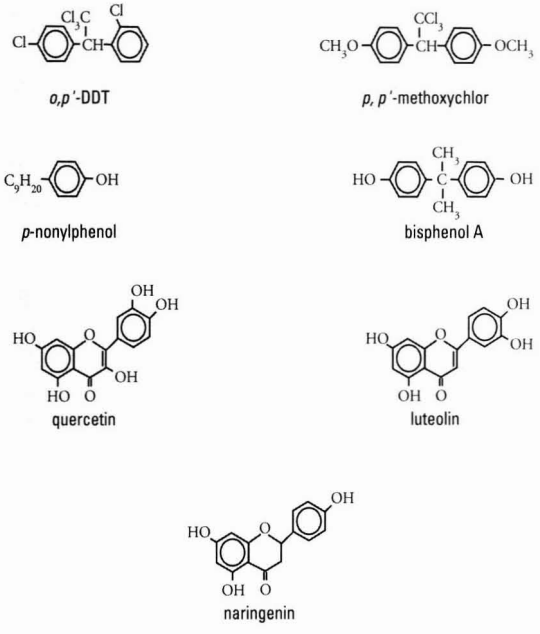


Figure 1. Structures of environmental estrogens (*o,p'*-DDT, *p,p'*-methoxychlor, *p*-nonylphenol, and bisphenol A) and estrogenic bioflavonoids (quercetin, naringenin, and luteolin).

Other industrial chemicals or intermediates that have been identified as estrogenic compounds include bisphenol-A (Fig. 1), a chemical used in the manufacture of polycarbonate-derived products (*45*); phenol red, a pH indicator used in cell culture media (*46*); and alkyl phenols and their derivatives, which are extensively used for preparation of polyethoxylates in detergents (*47,48*).

Natural Estrogenic Compounds

Human exposure to estrogenic chemicals is not confined to xenoestrogens derived from industrial compounds. Several different structural classes of naturally occurring estrogens have been identified, including plant bioflavonoids (Fig. 1) and various mycotoxins including zearalenone and related compounds (*49–52*). The plant bioflavonoids include different structural classes of compounds which contain a flavonoid backbone: flavones, flavanones, flavonols, isoflavones, and related condensation products (e.g., coumesterol). The estrogenic activities of diverse phytoestrogenic bioflavonoids and mycotoxins have been extensively investigated in *in vivo* models, *in vitro* cell culture systems, and in ER binding assays, and most of these compounds elicit multiple estrogenic responses in these assays. In addition, a number of plant foodstuffs contain 17β-estradiol (E_2) and estrone (*51,52*).

Environmental and Dietary Antiestrogens

Several different structural classes of chemicals found in the human diet also exhibit antiestrogenic activity (Fig. 2) (*13*). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related halogenated aromatics including polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and PCBs are also an important class of organochlorine pollutants that elicit a diverse spectrum of biochemical and toxic responses (*53*). These chemicals act through the aryl hydrocarbon receptor (AhR)-mediated signal transduction pathway, which is thought to play a role in most of the responses elicited by these compounds. AhR agonists such as TCDD have been characterized as antiestrogens using rodent and cell models similar to those used for determining the estrogenic activity of dietary and environmental chemicals. In the rodent model, TCDD and related compounds inhibit several estrogen-induced uterine responses including increased uterine wet weight, peroxidase activity, cytosolic and nuclear progesterone receptor (PR) and ER binding, epidermal growth factor (EGF) receptor binding, EGF receptor mRNA, and c-*fos* mRNA levels (*54–58*). In parallel studies, the antiestrogenic activities of TCDD and related compounds have also been investigated in several human breast cancer cell lines. For example, structurally diverse AhR agonists inhibit the following E_2-induced responses in MCF-7 human breast cancer cells: post-confluent focus production, secretion of tissue plasminogen activator activity, procathepsin D (52-kDa protein), cathepsin D (34-kDa protein), a 160-kDa protein, PR binding sites, glucose-to-lactate metabolism, pS2 protein levels, and PR, cathepsin D, ER, and pS2 gene expression (*42,59–65*). Moreover, TCDD inhibits formation and/or growth of mammary tumors in athymic nude mice and female Sprague-Dawley rats after long-term feeding studies or initiation with 7,12-dimethylbenzanthracene (*60,66,67*). A recent epidemiology study on women exposed to TCDD after an industrial accident in Seveso (*68*) reported that breast cancer incidence was decreased in areas with high levels of TCDD contamination (particularly in the age class 45 to 74) and among women living longest in an area of low TCDD contamination. Endometrial cancer showed a remarkable decrease, particularly in areas with medium and low TCDD contamination (*68*). Thus, TCDD and related compounds exhibit a broad spectrum of antiestrogenic activities and, not surprisingly, so do other AhR agonists such as the polynuclear aromatic hydrocarbons (PAHs), indole-3-carbinol (IC), and related compounds found in relatively high levels in foodstuffs (*69,70*). PAHs are found in cooked foods (*71,72*) and are ubiquitous environmental contaminants. IC is a major component of cruciferous vegetables (e.g., brussels sprouts, cauli-

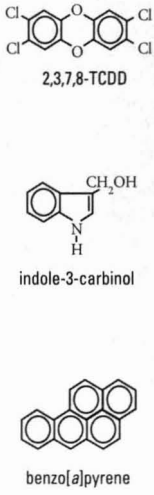


Figure 2. Structures of the environmental and dietary antiestrogens 2,3,7,8-TCDD, indole-3-carbinol, and benzo[*a*]pyrene.

flower) and exhibits antiestrogenic and anticancer (mammary) activities (*70,73*).

Bioflavonoids have been extensively characterized as weak estrogens and therefore may also be active as antiestrogens at lower concentrations. The interaction between estrogenic bioflavonoids and E_2 depends on their relative doses or concentrations, the experimental model, and the specific estrogen-induced endpoint. Markaverich and co-workers (*74*) reported that the estrogenic bioflavonoids quercetin and luteolin (Fig. 1) inhibited E_2-induced proliferation of MCF-7 human breast cancer cells and E_2-induced uterine wet weight increase in 21-day-old female rats. Similar results were also observed in this laboratory for quercetin, resperetin, and naringenin. For example, the bioflavonoid naringenin inhibited estrogen-induced uterine hyptertrophy in female rats and estrogen-induced luciferase activity in MCF-7 cells transfected with an E_2-responsive plasmid construct containing the 5'-promoter region of the pS2 gene and a luciferase reporter gene (unpublished results). In contrast, a recent study (*75*) reported that coumestrol, genistein, and zearalenone were not antiestrogenic in human breast cancer cells. The antiestrogenic activities of weak dietary and environmental estrogens require further investigation; however, it is clear that at subestrogenic doses, some of these compounds exhibit antiestrogenic activities in both *in vivo* and *in vitro* models.

Mass/Potency Balance

The uptake of environmental or dietary chemicals that elicit common biochemical/toxic responses can be estimated by using an equivalency factor approach in which estrogen equivalents (EQs) in any mixture are equal to the sum of the concentration of the individual compounds (EC_i) times their potency (EP_i) relative to an assigned standard such as diethylstilbestrol (DES) or E_2 (*51*). The total EQs in a mixture would be:

$$EQ = \sum ([EC_i] \times EP_i)$$

A similar approach is being used to determine the TCDD equivalents (TEQs) of various mixtures containing halogenated hydrocarbons (*76*). Verdeal and Ryan (*51*) have previously used this approach with DES equivalents assuming that the oral potency of E_2 is 15% that of DES. Winter (*77*) has estimated the dietary intake of pesticides based on FDA's total diet study, which includes estimates of food intakes and pesticide residue levels in these foods. The results presented in Table 2 summarize the estimated exposure of different groups to estrogenic pesticides. For example, 14- to 16-year-old males were exposed to a total of 0.0416 µg/kg/day of the estrogenic pesticides, DDT, dieldrin, endosulfan, and *p,p'*-methoxychlor (note: the DDT value represents *p,p'*-DDE and related metabolites, which are primarily nonestrogenic). Thus, the overall dietary intake of these compounds by this age group was 2.5 µg/day.

The relative potencies of dietary and xenoestrogens are highly variable. The results of *in vitro* cell culture studies suggest that estrogenic potencies of bioflavonoids relative to E_2 are 0.001 to 0.0001 (*75,78*) whereas Soto and co-workers (*44*) have assigned an estrogen potency factor of 0.000001 for the estrogenic pesticides. These relative estrogen potency factors for bioflavonoids and pesticides may be lower when derived from *in vivo* studies since pharmacokinetic factors and metabolism may decrease bioavailability. Thus, a more accurate assessment of dietary/environmental EQs requires further data from dietary feeding studies that evaluate these compounds using the same experimental protocols.

The results in Table 3 summarize human exposure to dietary and environmental estrogens and the estimated daily dose in terms of EQs. The relative estrogenic intakes for various hormonal drug therapies were previously estimated by Verdeal and Ryan (*51*); the average estimated daily intake of all flavonoids in food products was 1020 and 1070 mg/day, (winter and summer, respectively) (*52*). The results show that the estimated dietary EQ levels of estrogenic pesticides are 0.0000025 µg/day, whereas the corresponding dietary EQ levels for the bioflavonoids are 102 µg/day. Thus, the EQ values for the dietary intake of flavonoids was 4×10^7 times higher than the daily EQ intake of estrogenic pesticides, illustrating the minimal potential of these industrial estrogens to cause an adverse endocrine-related response in humans.

Previous studies have also shown that AhR agonists, such as TCDD and related compounds, PAHs, and IC and its most active derivative, indolo[3,2-*b*]carbazole (ICZ) all inhibit E_2-induced responses in MCF-7 cells (*59–65,69,70,79*). At a concentration of 10^{-9} M, TCDD inhibits 50–100% of most E_2-induced responses *in vitro* in which the concentration of E_2 is 10^{-9} M. Therefore, 1 TEQ is approximately equal to 1 EQ. The estimated daily intakes of TCDD and related compounds, PAHs, and ICZ (in 100 g brussels sprouts) are summarized in Table 3. The relative potencies of PAHs and ICZ as antiestrogens compared to TCDD are approximately 0.001 in MCF-7 cells (*69,79*). Thus, the TEQs or antiestrogen TEQs can be calculated for the dietary intakes of TCDD and related organochlorines and PAHs (in all foods) (*71,72*). The antiestrogen TEQs for the three classes of dietary AhR agonists are orders of magnitude higher than the estimated dietary intakes of estrogenic pesticide EQs. Thus, the major human intake of endocrine disrupters associated with the estrogen-induced response pathways are naturally occurring estrogens found in foods. Relatively high serum levels of estrogenic bioflavonoids have also been detected in a Japanese male population, whereas lower levels were observed in a Finnish group, and this is consistent with their dietary intakes of these estrogenic compounds (*80*). *p,p'*-DDE is present in

Table 2. Estimated dietary intake of estrogenic pesticides by different age groups based on food intakes and pesticide levels in these foodstuffs (*77*)

	Estimated exposure (µg/kg/day)		
Pesticide	6–11 months	14–16[a] years	60–65 years
DDT (total)	0.077	0.0260	0.0103
Dieldrin	0.0014	0.0016	0.0016
Endosulfan	0.0274	0.0135	0.0210
p,p'-Methoxychlor	0.0005	0.0005	0.0001

[a]Maximum exposure: 60 × 0.0416 = 2.5 µg/day.

Table 3. Estimated mass balance of human exposures to environmental and dietary estrogens and antiestrogens (*51,52,77,79*)

Source	Estrogen equivalents (µg/day)
Estrogens	
Morning after pill	333,500
Birth control pill	16,675
Post-menopausal therapy	3,350
Flavonoids in foods (1,020 mg/day × 0.0001)	102
Environmental organochlorine estrogens (2.5 × 0.000001)	0.0000025
Antiestrogens	**TCDD antiestrogen equivalents (µg/day)**
TCDD and organochlorines (80–120 pg/day)	0.000080–0.000120[a]
PAHs in food (1.2–5.0 × 106 pg/day; relative potency ~ 0.001)	0.001200–0.0050[b]
Indolo[3,2-*b*]carbazole in 100 g brussels sprouts (0.256–1.28 × 106 pg/day; relative potency ~ 0.001)	0.000250–0.00128[b]

[a]In most studies, 1 nM TCDD inhibits 50–100% of 1 nM E_2-induced responses in MCF-7 cells (*59–65*); therefore, 1 estrogen equivalent ≅ 1 antiestrogen equivalent.
[b]The antiestrogenic potencies of PAHs (*69*) and indolo[3,2-*b*]carbazole (*79*) compared to E_2 were approximately 0.001.

human serum; however, the estrogenic *o,p'*-DDE and *o,p'*-DDT analogs and other weakly estrogenic organochlorine compounds are not routinely detected in serum samples. A recent study identified several hydroxylated PCB congeners in human serum. All of the hydroxylated compounds were also substituted with chlorine groups at both adjacent meta positions (*81*). Based on results of previous structure–activity studies (*43*) for hydroxylated PCBs, these compounds would exhibit minimal estrogenic activity; however, further studies on the activity of hydroxylated PCBs are warranted.

Summary

The hypothesized linkage between dietary/environmental estrogens and the increased incidence of breast cancer is unproven; there is a lack of correlation between higher organochlorine levels in breast cancer patients compared to controls (Table 1) and the low levels of organochlorine EQs in the diet (Table 3). Higher levels of bioflavonoids are unlikely to contribute to increased breast cancer incidence because these compounds and the foods they are associated with tend to exhibit anticarcinogenic activity (*82,83*). The hypothesis that male reproductive problems and decreased sperm counts are related to increased exposure to environmental and dietary estrogens is also unproven. As noted above, dietary exposure to xenoestrogens derived from industrial chemical residues in foods is minimal compared to the daily intake of EQs from naturally occurring bioflavonoids. Moreover, there are serious questions regarding the decreased sperm counts reported by Carlsen and co-workers. Reanalysis of Carlsen et al.'s data suggests that there has not been a decrease in sperm counts in males over the past 30 years (*33*) and possibly over the past 50 years (*34*). Thus, in response to articles in the popular and scientific press such as "The Estrogen Complex" (*7*) and "Ecocancers: Do Environmental Factors Underlie a Breast Cancer Epidemic?" (*8*), the results would suggest that the linkage between dietary or environmental estrogenic compounds and breast cancer has not been made, and further research is required to determine the factors associated with the increasing incidence of this disease.

Note added in proof. A recent study (*84*) reported a 2.1% decrease in sperm concentrations in France from 1973 to 1979.

References

1. Hunter DJ, Kelsey KT. Pesticide residues and breast cancer: the harvest of a Silent Spring. J Natl Cancer Inst 85:598–599 (1993).
2. Colborn T, Vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378–384 (1993).
3. Thomas KB, Colborn T. Organochlorine endocrine disruptors in human tissue. In: Chemically induced alterations in sexual development: the wildlife/human connection (Colborn T, Clement C, eds). Princeton, NJ:Princeton Scientific Publishing, 1992;365–394.
4. El-Bayoumy K. Environmental carcinogens that may be involved in human breast cancer etiology. Chem Res Toxicol 5:585–590 (1993).
5. Sharpe RM, Skakkebaek NF. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract. Lancet 341:1392–1395 (1993).
6. Davis DL, Bradlow HL, Wolff M, Woodruff T, Hoel DG, Anton-Culver H. Medical hypothesis: xenoestrogens as preventable causes of breast cancer. Environ Health Perspect 101:372–377 (1993).
7. The estrogen complex. Newsweek March 21:76–77 (1994).
8. Raloff J. Ecocancers: do environmental factors underlie a breast cancer epidemic? Sci News 144:10–14 (1993).
9. Raloff J. That feminine touch. Sci News 145:56–58 (1994).
10. Raloff J. The gender benders. Sci News 145:24–27 (1994).
11. Hileman B. Environmental estrogens linked to reproductive abnormalities and cancer. Chem Eng News Jan 31:19–23 (1994).
12. Stone R. Environmental estrogens stir debate. Science 265:308–310 (1994).
13. Safe SH. Dietary and environmental estrogens and antiestrogens and their possible role in human disease. Environ Sci Pollut Res 1:29–33 (1994).
14. Assault on the male. Horizon, 31 October 1993. London:British Broadcasting Company.
15. Sole M, Porte C, Pastor D, Albaiges J. Long-term trends of polychlorinated biphenyls and organochlorinated pesticides in mussels from the western Mediterranean coast. Chemosphere 28:897–903 (1994).
16. Robinson PE, Mack GA, Remmers J, Levy R, Mohadjer L. Trends of PCB, hexachlorobenzene, and benzene hexachloride levels in the adipose tissue of the U.S. population. Environ Res 53:175–192 (1990).
17. Turle R, Norstrom RJ, Collins B. Comparison of PCB quantitation methods: re-analysis of archived specimens of herring gull eggs from the Great Lakes. Chemosphere 22:201–213 (1991).
18. Schmitt CJ, Zajicek JL, Peterman PH. National contaminant biomonitoring program: residues of organochlorine chemicals in U.S. freshwater fish, 1976–1984. Arch Environ Contam Toxicol 19:748–781 (1990).
19. Giesy JP, Ludwig JP, Tillitt DE. Deformities of birds in the Great Lakes region: assigning causality. Environ Sci Technol 28:128A–135A (1994).
20. Unger M, Kiaer H, Blichert-Toft M, Olsen J, Clausen J. Organochlorine compounds in human breast fat from deceased with and without breast cancer and in biopsy material from newly diagnosed patients undergoing breast surgery. Environ Res 34:24–28 (1984).
21. Mussalo-Rauhamaa H, Häsänen E, Pyysalo H, Antervo K, Kauppila R, Pantzar P. Occurrence of ß-hexachlorocyclohexane in breast cancer patients. Cancer 66:2124–2128 (1900).
22. Falck F, Ricci A, Wolff MS, Godbold J, Deckers P. Pesticides and polychlorinated biphenyl residues in human breast lipids and their relation to breast cancer. Arch Environ Health 47:143–146 (1992).
23. Wolff MS, Toniolo PG, Leel EW, Rivera M, Dubin N. Blood levels of organochlorine residues and risk of breast cancer. J Natl Cancer Inst 85:648–652 (1993).
24. Dewailly E, Dodin S, Verreault R, Ayotte P, Sauvé L, Morin J, Brisson J. High organochlorine body burden in women with estrogen receptor-positive breast cancer. J Natl Cancer Inst 86:232–234 (1994).
25. Higginson J. DDT epidemiologic evidence. IARC Scientific Publication no. 65. Lyon:International Agency for Research on Cancer, 1985;107–117.
26. Brown DP. Mortality of workers exposed to polychlorinated biphenyls—an update. Arch Environ Health 42:333–339 (1987).
27. Krieger N, Wolff MS, Hiatt RA, Rivera M, Vogelman J, Orentreich N. Breast cancer and serum organochlorines: a prospective study among white, black, and Asian women. J Natl Cancer Inst 86:589–599 (1994).
28. The good news. Time, 2 May 1994;20.
29. Key T, Reeves G. Organochlorines in the environment and breast cancer. Br Med J 308:1520–1521 (1994).
30. Stillman RJ. In utero exposure to diethylstilbestrol: adverse effects on the reproductive tract and reproductive performance in male and female offspring. Am J Obstet Gynecol 142:905–921 (1982).
31. Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for the decreasing quality of semen during the past 50 years. Br Med J 305:609–612 (1992).
32. Macleod J, Wang Y. Male fertility potential in terms of semen quality: a review of the past, a study of the present. Fertil Steril 31:103–116 (1979).
33. Ramilow M. In: The toxicology forum (proceedings of the annual winter meeting). Fairfax, UK:Caset Associates Ltd, 1994;79.
34. Bromwich P, Cohen J, Stewart I, Walker A. Decline in sperm counts: an artefact of changed reference range of normal. Br Med J 309:19–22 (1994).
35. Bitman J, Cecil HC, Harris SJ, Fries GF. Estrogenic activity of o,p'-DDT in the mammalian uterus and avian oviduct. Science 162:371–372 (1968).
36. Welch RM, Levin W, Conney AH. Estrogenic action of DDT and its analogs. Toxicol Appl Pharmacol 14:358–367 (1969).
37. Bitman J, Cecil HC. Estrogenic activity of DDT analogs and polychlorinated biphenyls. J Agric Food Chem 18:1108–1112 (1970).
38. Hammond B, Katzenellenbogen BS, Krauthammer N, McConnell J. Estrogenic activity of the insecticide chlordecone (Kepone) and interaction with uterine estrogen receptor. Proc Natl Acad Sci USA 76: 6641–6645 (1979).
39. Robinson AK, Mukku VT, Spalding DM, Stancel GM. The estrogenic activity of DDT: the in vitro induction of an estrogen-inducible protein by o,p-DDT. Toxicol Appl Pharmacol 76:537–543 (1984).
40. Tullner WW. Uterotrophic action of the insecticide methoxychlor. Science 133:647–648 (1961).
41. Ecobichon DJ, MacKenzie DO. The

uterotropic activity of commercial and isomerically-pure chlorobiphenyls in the rat. Res Commun Chem Pathol Pharmacol 9:85–95 (1974).
42. Krishnan V, Safe S. Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) as antiestrogens in MCF-7 human breast cancer cells: quantitative structure-activity relationships. Toxicol Appl Pharmacol 120:55–61 (1993).
43. Korach KS, Sarver P, Chae K, Mclachlan JA, McKinney JD. Estrogen receptor-binding activity of polychlorinated hydroxybiphenyls: conformationally restricted structural probes. Mol Pharmacol 33:120–126 (1988).
44. Soto AM, Chung KL, Sonnenschein C. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ Health Perspect 102: 380–383 (1994).
45. Krishnan AV, Starhis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132: 2279–2286 (1993).
46. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 83:2496–2500 (1986).
47. Soto AM, Justicia H, Wray JW, Sonnenschein C. p-Nonylphenol: an estrogenic xenobiotic released from "modified" polystyrene. Environ Health Perspect 92:167–173 (1991).
48. White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 135:175–182 (1993).
49. Whitten PL, Naftolin F. Dietary estrogens: a biologically active background for estrogen action. In: The new biology of steroid hormones (Hochberg RB, Naftolin F, eds). New York:Raven Press, 1991;155–167.
50. McLachlan JE. Estrogens in the Environment. New York:Elsevier, 1980.
51. Verdeal K, Ryan DS. Naturally-occurring estrogens in plant foodstuffs—a review. J Food Protection 42:577–583 (1979).
52. Kuhnau J. The flavonoids. A class of semi-essential food components: their role in human nutrition. World Rev Nutr Diet 24:117–191 (1976).
53. Goldstein JA, Safe S. Mechanism of action and structure-activity relationships for the chlorinated dibenzo-p-dioxins and related compounds. In: Halogenated biphenyls, naphthalenes, dibenzodioxins and related compounds (Kimbrough RD, Jensen AA, eds). Amsterdam:Elsevier, 1989;239–293.
54. Safe S, Astroff B, Harris M, Zacharewski T, Dickerson R, Romkes M, Biegel L. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and related compounds as antiestrogens: characterization and mechanism of action. Pharmacol Toxicol 69:400–409 (1991).
55. Astroff B, Safe S. 2,3,7,8-Tetrachlorodibenzo-p-dioxin as an antiestrogen: effect on rat uterine peroxidase activity. Biochem Pharmacol 39:485–488 (1990).
56. Astroff B, Rowlands C, Dickerson R, Safe S. 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibition of 17β-estradiol-induced increases in rat uterine EGF receptor binding activity and gene expression. Mol Cell Endocrinol 72:247–252 (1990).
57. Gallo MA, Hesse EJ, MacDonald GJ, Umbreit TH. Interactive effects of estradiol and 2,3,7,8-tetrachlorodibenzo-p-dioxin on hepatic cytochrome P-450 and mouse uterus. Toxicol Lett 32:123–132 (1986).
58. DeVito MJ, Thomas T, Martin E, Umbreit TH, Gallo MA. Antiestrogenic action of 2,3,7,8-tetrachlorodibenzo-p-dioxin: tissue-specific regulation of estrogen receptor in CD1 mice. Toxicol Appl Pharmacol 113:284–292 (1992).
59. Gierthy JF, Lincoln DW, Gillespie MB, Seeger JI, Martinez HL, Dickerman HW, Kumar SA. Suppression of estrogen-regulated extracellular plasminogen activator activity of MCF-7 cells by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Cancer Res 47:6198–6203 (1987).
60. Gierthy JF, Bennett JA, Bradley LM, Cutler DS. Correlation of in vitro and in vivo growth suppression of MCF-7 human breast cancer by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Cancer Res 53:3149–3153 (1993).
61. Gierthy JF, Lincoln DW. Inhibition of post-confluent focus production in cultures of MCF-7 breast cancer cells by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Breast Cancer Res 12:227–233 (1988).
62. Biegel L, Safe S. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on cell growth and the secretion of the estrogen-induced 34-, 52- and 160-kDa proteins in human breast cancer cells. J Steroid Biochem Mol Biol 37:725–732 (1990).
63. Krishnan V, Wang X, Ramamurthy P, Safe S. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in formation of estrogen-induced ER/Sp1 complexes on the cathepsin D promoter. Toxicologist 14:47 (1994).
64. Harper N, Wang X, Liu H, Safe S. Inhibition of estrogen-induced progesterone receptor in MCF-7 human breast cancer cells by aryl hydrocarbon (Ah) receptor agonists. Mol Cell Endocrinol 104:47–55 (1994).
65. Zacharewski T, Bondy K, McDonell P, Wu ZF. Antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on 17ß-estradiol-induced pS2 expression. Cancer Res 54:2707–2713 (1994).
66. Holcomb M, Safe S. Inhibition of 7,12-dimethylbenzanthracene-induced rat mammary tumor growth by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Cancer Lett 82:43–47 (1994).
67. Kociba RJ, Keyes DG, Beger JE, Carreon RM, Wade CE, Dittenber DA, Kalnins RP, Frauson LE, Park CL, Barnard SD, Hummel RA, Humiston CG. Results of a 2-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in rats. Toxicol Appl Pharmacol 46:279–303 (1978).
68. Bertazzi PA, Pesatori AC, Consonni D, Tironi A, Landi MT, Zocchetti C. Cancer incidence in a population accidentally exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Epidemiology 4:398–406 (1993).
69. Chaloupka K, Krishnan V, Safe S. Polynuclear aromatic hydrocarbon carcinogens as antiestrogens in MCF-7 human breast cancer cells. Role of the Ah receptor. Carcinogenesis 13:2223–2239 (1992).
70. Tiwari RK, Guo L, Bradlow HL, Telang NT, Osborne MP. Selective responsiveness of breast cancer cells to indole-3-carbinol, a chemopreventative agent. J Natl Cancer Inst 86:126–131 (1994).
71. Vaessen HAMG, Jekel AA, Wilbers AAMM. Dietary intake of polycyclic aromatic hydrocarbons. Toxicol Environ Chem 16:281–294 (1988).
72. Menzie CA, Potocki BB, Santodonato S. Exposure to carcinogenic PAHs in the environment. Environ Sci Technol 26:1278–1284 (1992).
73. Stoewsand GS, Anderson JL, Munson L. Protective effect of dietary brussels sprouts against mammary carcinogenesis in Sprague-Dawley rats. Cancer Lett 39:199–207 (1988).
74. Markaverich BM, Roberts RR, Alejandro MA, Johnson GA, Middleditch BS, Clark JH. Bioflavonoid interaction with rat uterine type II binding sites and cell growth inhibition. J Steroid Biochem 30:71–78 (1978).
75. Mäkelä S, Davis VL, Tally WC, Korkman J, Salo L, Vihko R, Santti R, Korach KS. Dietary estrogens act through estrogen receptor-mediated processes and show no antiestrogenicity in cultured breast cancer cells. Environ Health Perspect 102:572–578 (1994).
76. Safe S. Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). CRC Crit Rev Toxicol 21:51–88 (1990).
77. Winter CK. Dietary pesticide risk assessment. Rev Environ Contam Toxicol 127:23–67 (1992).
78. Miksicek RJ. Commonly occurring plant flavonoids have estrogenic activity. Mol Endocrinol 44:37–43 (1993).
79. Liu H, Wormke M, Safe S, Bjeldanes LF. Indolo[3,2-b]carbazole: a dietary factor which exhibits both antiestrogenic and estrogenic activity. J Natl Cancer Inst 86:1758–1765 (1994).
80. Aldercreutz H, Markkanen H, Watanabe S. Plasma concentrations of phyto-oestrogens in Japanese men. Nature 342:1209–1210 (1993).
81. Bergman A, Klasson-Wehler E, Kuroki, H. Selective retention of hydroxylated PCB metabolites in blood. Environ Health Perspect 102:464–469 (1994).
82. Verma AK, Johnson JA, Gould MN, Tanner MA. Inhibition of 7,12-dimethylbenz(a)-anthracene- and N-nitrosomethylurea-induced rat mammary cancer by dietary flavonol quercetin. Cancer Res 48:5754–5758 (1988).
83. Messina MJ, Persky V, Setchell KDR, Barnes S. Soy intake and cancer risk: a review of the in vitro and in vivo data. Nutr Cancer 21: 113–131 (1994).
84. Auger J, Kuntsmann JM, Czyglik F, Jouannet P. Decline in semen quality among fertile men in Paris during the past 20 years. N Engl J Med 332:281–285 (1985).

The Need for Water Quality Criteria for Frogs

Robin Boyer and Christian E. Grue

Washington Cooperative Fish and Wildlife Research Unit, School of Fisheries, University of Washington, Seattle, WA 98195 USA

Amphibians are considered reliable indicators of environmental quality. In the western United States, a general decline of frog populations parallels an apparent worldwide decline. The factors thought to be contributing to declines in frog populations include habitat loss, introduction of exotic species, overexploitation, disease, climate change, and decreasing water quality. With respect to water quality, agroecosystems use 80–90% of the water resources in the western United States, frequently resulting in highly eutrophic conditions. Recent investigations suggest that these eutrophic conditions (elevated pH, water temperature, and un-ionized ammonia) may be associated with frog embryo mortality or malformations. However, water quality criteria for frogs and other amphibians do not currently exist. Here, we briefly review data that support the need to develop water quality parameters for frogs in agroecosystems and other habitats. *Key words*: agroecosystems, amphibian populations, frogs, pollution, water quality. *Environ Health Perspect* 103:352–357 (1995)

Water contamination and poor water quality in general have escalated in recent years (*1,2*). Concerns about alterations in water quality have increased as the need to share water among different consumers, including wildlife, has risen. Water quality needs of wildlife have often been neglected; this neglect is particularly true for amphibians (*3*).

There are several reasons for this neglect. Amphibians are not generally viewed as "cute and cuddly," and therefore, they enjoy limited popularity with the public (*4*). A lack of public regard and economic significance has been paralleled by a lack of research funding. Furthermore, past ecological studies have often focused solely on terrestrial or aquatic organisms, neglecting amphibians, which frequently occupy both terrestrial and aquatic habitats (*5*). Amphibians are considered reliable indicators of environmental quality (*6*). The early life stages (egg and larval) of many species are restricted to the aquatic environment, and many adults respire through a moist skin (*7*). Consequently, all life stages of amphibians are susceptible to dermal absorption of toxicants in water. Ingestion of contaminated prey is also a potential pathway for toxicants to enter amphibians (*7*). Amphibians can be major contributors to biomass and biodiversity within ecosystems; many adults are predators, embryos are prey for other trophic levels, and larvae are both herbivorous grazers and prey (*5,8,9*). Recent data suggest that a worldwide decline of amphibians is occurring (*3,10–13*).

Factors Contributing to the Decline of Frog Populations

Several reasons for the perceived decline in frog populations have been suggested (*6,8,10,11,13–16*). The most widely recognized and documented is habitat loss (*6,9,14,15*). For example, the contiguous United States are thought to have contained approximately 60–75 million ha of wetlands (*17*). During the 1950s to 1970s, 185,000 ha/year or 8.5% of these wetlands were drained. Currently, an estimated 43.7 million ha or between 58 and 73% of wetlands remain in the continental United States (*17*). Nearly 120,000 ha of wetlands are lost per year (*18,19*).

Within the remaining wetland habitats, numerous factors may be contributing to the decline of frog populations, such as the introduction of exotic species that may outcompete indigenous species for food and breeding sites or that may prey upon the indigenous species (*20,21*). For example, Hayes and Jennings (*21*) hypothesize that exotic fish species introduced into the waters of California may have foraging behaviors that increased predation upon the eggs and tadpoles of native ranid frog species. They note that catfish (*Ictaluridae spp.*) and sunfish (*Centrarchidae spp.*) usually forage by stirring the sediments and aquatic plants, locations in which ranid tadpoles are often found. And these researchers observed that in areas in which the catfish and sunfish have been introduced, native ranid populations have declined (*21*). Bradford (*22*) found declines of native frog populations in lakes of the Sierra Nevada Mountains of California when rainbow trout (*Salmo gairdneri*) and possibly golden trout (*Salmo aguabonita*), along with brook charr (*Salvelinus fontinalis*), were introduced. In other regions of the country, the largemouth bass (*Micropterus salmoides)* has been introduced for recreational fishing. This omnivorous fish ingests amphibian eggs and larvae, as well as adults (*23*).

Disease is another factor suspected of contributing to declines of frog populations. Opportunistic pathogens may overwhelm native species in a short time, or noninfectious disease can enter frogs via their permeable skin (*24–28).* During the 1970s and early 1980s, the North American leopard frog (*Rana pipiens*) (Fig. 1) suffered dramatic declines in many locations, not only in the United States, but in Canada and Mexico as well (*24,25).* Although the reasons for the mortality remain unknown, a current hypothesis is that disease may have been responsible. A condition known as "red leg" may have spread from population to population across the continent. Red leg is a syndrome characterized by kidney failure, ulceration, and hemorrhaged blood vessels. The latter are particularly prevalent on the ventral surface of the hind limbs and give the syndrome its name. Red leg usually results from an infection caused by the gram-negative pathogen, *Aeromonas hydrophilia*; however, it may be caused by other pathogens as well *(25).*

Declines in other amphibian populations, for example, the boreal toad (*Bufo boreas boreas*), which was extirpated from Colorado during the early 1980s, may also have resulted from this opportunistic pathogen (*27*). And in Oregon, a population of the western toad (*Bufo boreas*) has suffered high egg mortality since 1989. A mold, *Saprolegnia ferax*, which is commonly found in fresh water throughout the world, has been identified as the pathogen responsible for the egg mortality (*28*). Research on the numerous other viruses and bacteria that may infect amphibians and contribute to declining populations is lacking (*25*).

The effects of global climate change on amphibian populations is currently under investigation (*29*; Hansen L, personal communication). Recent research has demonstrated decreased hatching success of eggs of some amphibian species associated with increased levels of ultraviolet (UV) radiation, specifically UV-B radiation (290–320 nm light), whereas other species seem to be less affected (*29*). Blaustein et al. (*29*) placed eggs of three species of amphibians in cages in the water of a lake in the Cascade Mountains of Oregon and found that one species, the pacific tree frog (*Hyla regilla*), had high levels of photolyase activity, which protects the eggs from the UV-B, while the other two species, the cascade frog (*Rana cascadae*) (Fig. 2) and western toad, had little photolyase activity and suffered higher mortality than the tree frog. The different

Address correspondence to R. Boyer, Washington Cooperative Fish and Wildlife Research Unit, School of Fisheries, WH-10, University of Washington, Seattle, WA 98195 USA.

This manuscript benefited from the comments of Greg Linder, Kelly McAllister, and two anonymous reviewers. We thank the Seattle Audubon Society for permission to reprint photographs from its publication *Amphibians of Washington and Oregon.*

Received 22 August 1994; accepted 12 December 1994.

responses observed among species of amphibians may explain why some species have declined in population size while other species have not, even though all reside within the same region.

Other human-related activities may also be contributing to worldwide declines of amphibians. For example, in parts of India, overexploitation of frog populations for export as gourmet food has directly reduced populations (*30,31*). The concurrent loss of amphibian predators of insect pests coincides with increased pest populations, increased use of pesticides, and a subsequent deterioration in water quality (*30*). And finally, deteriorating water quality per se may be resulting in amphibian population declines.

Alteration of Water Quality

Sensitivity of frogs to aquatic toxicants compared to other species. Historically, aquatic toxicology has focused on concerns for fishes and invertebrates (*32*). However, some studies have compared the responses of amphibians to other aquatic species and found that amphibians are as sensitive, and often more sensitive, than other species when exposed to aquatic contaminants (*3,33–35*). For example, Holcombe et al. (*34*) exposed tadpoles of the African clawed frog (*Xenopus laevis*) (Fig. 3) and seven other aquatic species, among them the daphnid *Daphnia magna*, rainbow trout, fathead minnow (*Pimephales promelas*), and a midge (*Tanytarsus dissimilis*). The tadpole was the most sensitive species for one of the four compounds to which it was exposed, less sensitive than the trout, but more sensitive than the fathead minnow to one of the other three compounds (*34*).

Standardized methods to assess impact of aquatic contaminants on frogs in the laboratory have only recently been developed. Birge et al. (*36,37*) were among the first to use frog embryos as bioassay organisms. And in the early 1980s, Dumont et al. (*38*) developed the frog embryo teratogenesis assay-*Xenopus* (FETAX) (Fig. 4). This bioassay was originally developed as an alternative to mammalian testing of pharmacological compounds to assess teratogenesis (*39*). During the mid 1980s, Bantle and co-workers (*40,41*) used the FETAX bioassay to assess aquatic contamination. In 1991, the FETAX bioassay became the first amphibian bioassay accepted by American Society for Testing and Materials (*42*). Currently, this bioassay is in the process of becoming the first standardized amphibian bioassay approved for assessment of amphibian responses to water-borne contaminants by the U.S. Environmental Protection Agency (*43,44*).

Acidification. Previous studies of water quality in relation to amphibians have focused on acidification (*6,45–48*). Beattie and Tyler-Jones (*49*) found that low pH inhibited fertilization and embryonic development of the common frog *(Rana temporaria)*. They found that acidic environments can alter the physiological ionic balance in amphibians and reduce their growth and survival. Other researchers have studied low pH in relation to the mobilization of aluminum from sediments and observed decreased embryo survival in numerous species of amphibians (*50*; Rowe-Krumdick S, unpublished data). In the central United States, researchers used FETAX to assess heavy metal contamination from mine tailings that entered acidic waters; many embryos died, and those that did survive were severely malformed (*51*). Research on the effects of acidification of lakes and other waterways has been conducted primarily in the eastern United States, where increasing acidification of ponds associated with acid rainfall may be contributing to declining amphibian populations (*6,48,50*). Although the western part of the country has been less of a focus for these studies, concern over acidification in high-altitude lakes is growing (*52–55*).

Figure 1. Adult northern leopard frog (*Rana pipiens*) of North America. During the 1970s, this species suffered an extensive decline that may have been caused by disease. [Photograph reproduced with permission from the Seattle Audubon Society (*99*).]

Figure 2. Adult cascade frog (*Rana cascadae*). Recent studies indicate susceptibility of this species to DNA damage in embryos from exposure to UV-B. [Photograph reproduced with permission from the Seattle Audubon Society (*99*).]

Contaminants in agroecosystems in the western United States. Agricultural needs consume 65% of the available fresh water worldwide (*56*). In the western part of the United States, 80–90% of the water resources are delegated to agricultural uses (*57,58*). Agricultural lands in the West are intensely managed ecosystems; the soils are often tilled and modified with fertilizers and pesticides. Irrigation is widespread, occurring in specific areas according to contract schedules that are purchased by participating farmers (*57*). The water may be shared and recycled among growers and then channelled away from the fields for disposal in a river, stream, or reservoir.

Figuure 3. Adult African clawed frog (*Xenopus laevis*). This nonindigenous species is often used in laboratory research (FETAX) and was used to evaluate water quality at the Klamath Basin National Wildlife Refuges.

The use of chemicals in agricultural production has escalated since the late-1940s (*59*). In 1960, synthetic organic pesticide production in the United States was 259 million kg (*60*). By 1986, 500 million kg of pesticides was applied to agricultural fields in the United States (*61*). Recently, 31 of the 50 states plus the Virgin Islands and Puerto Rico have reported concerns about groundwater contamination by pesticides to the EPA (*60*). Furthermore, the EPA has stated that agricultural runoff of pesticides and fertilizers contributes to the current nonpoint source pollution of fresh water in this country (*62*).

As noted previously, few studies on amphibian responses to pesticides have been conducted, and the results of these studies of single compounds have documented effects that range from temporary and reversible to delayed growth and death (*33,63*). Research on amphibians exposed to organochlorine compounds, which were widely applied to fields in the 1960s and early 1970s, indicated that these compounds were lethal to many amphibians (*64–66*). Today, organophosphates, carbamates, and synthetic pyrethroids are the insecticides predominately used in crop production. Thus far,

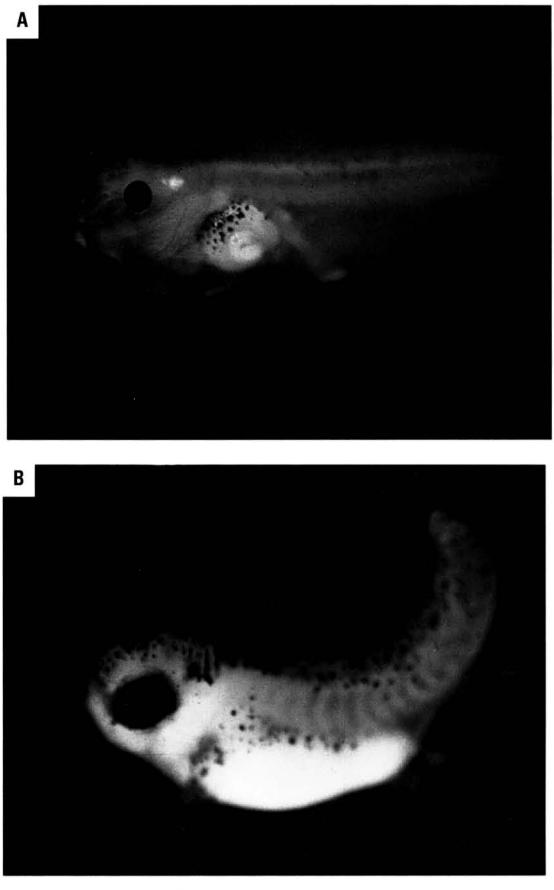


Figure 4. (A) Normally developed embryo at 96 hr (stage 46) and (B) severely malformed embryo of African clawed frog (*Xenopus laevis*) after 96 hr of exposure to agricultural drainwater from the Klamath Basin National Wildlife Refuges.

research on these compounds has demonstrated a range of effects, many of which are reversible or minor in whole organisms when exposed to concentrations of these compounds that are found in agroecosystems (*67–72*). However, researchers in Canada have recently used flow cytometry to assess frogs for effects from exposure to organophosphates. These researchers have observed increases in the coefficient of variation in the size of genomes in individual frogs as well as higher adult mortality and developmental malformations in the frog populations adjacent to the agricultural fields (Sharbel T, personal communication). Because information on the responses of amphibians to agrochemicals is limited, the need to assess amphibian responses to these compounds remains great (*3*).

Research on the effects of agricultural drainwater on biota has also been minimal (*73–75*) and was undertaken primarily because of concerns following the discovery of adverse effects of agricultural drainwater on the wildlife at the Kesterson National Wildlife Refuge in the San Joaquin Valley of California (*76*). After the discovery of selenium-related mortality and malformation among waterbirds at the refuge, reconnaissance studies of irrigation drainwater at 19 other western locations managed by the U. S. Department of Interior were conducted jointly by investigators from the U.S. Geological Survey, U.S. Fish and Wildlife Service, and the U.S. Bureau of Reclamation between 1986 and 1990.

Although the study of irrigation drainwater at Kesterson probably remains among the most publicized and extensive investigations to date, effects of the drainwater on amphibians were not assessed. In fact, none of the other 19 federal reconnaissance studies examined amphibian populations for adverse impacts from the agricultural drainwater. These studies included surveys at the Klamath Basin National Wildlife Refuges (*77,78*). The latter revealed that waters on the refuge have high temperatures, elevated pH, and low dissolved oxygen (*77,78*) (Fig. 5).

Subsequent evaluations of biological effects associated with water quality at the Klamath Refuges indicated that the irrigation drainwater was either lethal to, or caused significant malformation of, developing frog embryos (*79*). The agroecosystems surrounding the refuge use a variety of agrochemicals, including a number of herbicides and organophosphate and carbamate insecticides, for crop production. However, concentrations of these compounds in the water sampled were at or below detection limits (nanograms per liter). The study concluded that poor water quality (elevated pH and un-ionized ammonia) and/or pesticides may be contributing to the decline of indigenous frog populations (*79*). To our knowledge, this study was the first study of water quality and frogs in western agroecosystems.

Eutrophication. Elevated pH, low dissolved oxygen, high water temperatures, and elevated un-ionized ammonia levels characterize water in western agroecosystems and may singly or in combination have significant detrimental effect on the developing embryos of frogs. Elevated pH, ranging from 8.0 to 10.4, was recorded at Klamath Basin National Wildlife Refuges and has been recorded in waters at other wildlife refuges adjacent to agroecosystems in Colorado, Montana, and Wyoming (Osmundson B, Dickerson K, personal communications).

Ammonia is toxic to many aquatic organisms (*80*) and occurs in two forms in aqueous solution: the un-ionized form (NH_3) and the ionized form (NH_4^+). Ammonia equilibrium depends primarily on pH but also on temperature (*81*). As pH increases, the equilibrium moves toward the NH_3 form, and above pH 8.5, ammonia toxicity increases approximately 10-fold for each pH unit increase (*80*).

Water at the Klamath Basin National Wildlife Refuges contained NH_3 levels as high as 0.73 mg/L. Levels of this magnitude have killed fishes (*80–82*). Diamond et al. (*83*) reported an LC_{50} of 1.44 mg/L NH_3 for 96-hr acute tests with leopard frog (*Rana pipiens*) embryos at a pH of 7.14–8.21 and water temperature of 20°C. In the chronic test results, the leopard frog tadpoles were the most sensitive species with a no-observed-effect concentration of 0.27 mg/L (*83*).

In the case of salmonids, LC_{50} values for NH_3 range between 0.083 and 1.09 mg/L; for nonsalmonids, the range is 0.14–4.60 mg/L (*84*). The 19 species of invertebrates for which data were also reported have higher LC_{50} values (0.53 to 22.8 mg/L) than fishes (*84*). Further study of NH_3, pesticides, and other water-quality parameters in relation to frogs may elucidate the specific factors contributing to the

Figure 5. Agricultural fields border the waters of the Tule Lake National Wildlife Refuge on three sides and share water with the lake throughout the growing season. Many other wildlife refuges in the western United States receive irrigation drainwater as a primary source of water.

low frog populations on the Klamath Basin National Wildlife Refuges. These same factors may contribute to low frog populations in other western agroecosystems.

At Kesterson Refuge and in the Klamath Basin, the management of water within the agroecosystem upstream of the wildlife refuge has adversely affected water quality for fish and wildlife resources. From the beginning of agricultural production in this country, farmers have almost exclusively chosen monoculture crop production for short-term pecuniary gain (*85*). This singular focus has dramatically escalated during the 20th century and resulted in increased soil erosion, increased pesticide use, and water pollution (*56,57,60,86–88*).

For example, in a recent investigation, the U.S. Geological Survey studied water quality within the Pasco Basin of Washington State (*89*). Results indicated that the soils have become water logged and the groundwater levels have risen by 65 m during the past 35 years of crop irrigation. Many areas within the basin now require pumps to remove the groundwater from fields to protect crops from exposure to the water-logged soils. Simultaneously, nitrate concentrations in the basin water have increased as much as two orders of magnitude, and nitrogen fertilizers are the primary source of this change (*89*). Although these changes in water quantity and quality are now harming agricultural production in the basin, the impact on wildlife has not been studied.

Anthropogenic Determinants of Ecosystem Management and the Future of Frogs

Currently, no water quality criteria exist for amphibians in the United States. It is assumed that criteria for fishes and human health are adequate for protection of all aquatic species. This assumption, however, has not been tested. In view of the declines in the quantity and quality of water and amphibian populations in many parts of the United States, tolerance limits for amphibians need to be determined and compared with existing criteria for fishes and human health.

Postel (*56*) reminds us that we have been swift to claim water rights for our use, but slow to conserve quality or quantity of this vital resource for the needs of other species. Managers of metropolitan, agricultural, recreational, and industrial regions have largely aimed toward singular goals without consideration of the impacts of their activities on the larger landscape (*5,90–92*). Poor water quality is occurring on a global scale (*93,94*). Water is a resource that flows between and among ecosystems, permeating the larger landscape. Recently, Grumbine (*90*) suggested 10 specific themes for ecosystem management. Among them was a recommendation that managers depict boundaries at scales that are appropriate to the systems under management. Perhaps it is time for managers of specific regions or resources to consider the impact of their activities on the future of species other than humans. Grumbine also recommended interagency cooperation and organizational change to protect ecological integrity and biological diversity (*90*). Currently, the U.S Federal Wildlife Service and EPA are working together on water quality criteria for mammals and birds of the Great Lakes Basin. Perhaps amphibians will be next. In addition, the Declining Amphibian Populations Task Force (DAPTF) was established by the International Union for the Conservation of Nature in 1992. The DAPTF is an international network of researchers working to integrate specific information and studies of amphibian population declines.

Today, human populations control the resources on over half the land of the earth (*95*). All of the factors delineated above—habitat loss, introduction of exotic species, disease, overexploitation, global climate change, and water quality—are directly attributable to anthropogenic alteration of the landscape and climate resulting from the rapidly growing human population (*95*), a growth rate that is contributing to the increased rate of extinction of numerous species (*96*), including amphibians (*97*). If the human population continues to grow at its present rate, there will be even fewer resources available for all other species: less habitat, less water.

Protecting wetland habitat is critical, but alone it is insufficient for the survival and reproduction of wetland species. As stated previously, humans currently consume 65% of the available fresh water on the planet for agricultural use (*56*). As our population grows, we will claim even more of this vital resource for agricultural needs for food production. As the sharing and recycling of water increases, the quality of that water becomes vital to all consumers (*2,56,57,95*). Contamination by specific groups will affect all users. It seems both imperative and vital that protective water quality criteria be developed, acknowledged, and adhered to by all water users for the benefit and survival of all species that depend on this critical resource.

References

1. Smith RA, Alexander RB, Wolman MG. Water-quality trends in the nation's rivers. Science 235:1609–1615 (1987).
2. Becker CD, Neitzel DA, eds. Water quality in North American river systems. Columbus: Battelle Press, 1992.
3. Hall RJ, Henry PFP. Review: assessing effects of pesticides on amphibians and reptiles: status and needs. Herpetol J 2:65–71 (1992).
4. Chadwick DH. The biodiversity challenge. Special report. Washington DC:Defenders of Wildlife, 1990.
5. Bury RB. Habitat relationships and ecological importance of amphibians and reptiles in streamside management. In: Riparian wildlife and forestry interactions (Raedeke KJ, ed). Seattle:University of Washington Press, 1988; 61–76.
6. Dunson WA, Wyman RL, Corbett ES. A symposium on amphibian declines and habitat acidification. J Herpetol 26:349–342 (1992).
7. Duellman WE, Trueb L. Biology of amphibians. New York:McGraw-Hill Book Company, 1986.
8. Walls SC, Blaustein AR, Beatty JJ. Amphibian biodiversity of the Pacific Northwest with special reference to old-growth stands. Northwest Environ J 8:53–69 (1992).
9. Cohn JP. Salamanders slip-sliding away or too surreptious to count? Bioscience 44:219–223 (1994).
10. Barinaga M. Where have all the froggies gone? Science 247:1033–1034 (1990).
11. Wake DB. Declining amphibian populations. Science 253:860 (1991).
12. Crump ML, Hensley FR, Clark KL. Apparent decline of the golden toad: underground or extinct? Copeia 4:413–420 (1992).
13. Blaustein AR, Wake DB, Sousa WP. Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. Conserv Biol 8:60–71 (1994).
14. Allan JD, Flecker AS. Biodiversity conservation in running waters. Bioscience 43:32–43 (1993).
15. Johnson R. Habitat loss and declining amphibian populations. In: Declines in Canadian amphibian populations: designing a national monitoring strategy. Occasional paper no 76 (Bishop CA, Pettit KE, eds). Ottowa, Ontario: Canadian Wildlife Service, 1992;71–76.

16. Pounds JA, Crump ML. Amphibian declines and climate disturbance: the case of the golden toad and the harlequin frog. Conserv Biol 8:72–85 (1994).
17. Mitsch WJ, Gosselink JG. Wetlands. New York:Van Nostrand Reinhold, 1986.
18. Mitchell JG. Our disappearing wetlands. Natl Geogr Mag 182:2–45 (1992).
19. Associated Press. Solution is offered on wetlands protection. Everett Herald, Everett, WA, 17 October 1993.
20. Moyle, PB. Effects of introduced bullfrogs, *Rana catesbeiana*, on the native frogs of the San Joaquin Valley, California. Copeia 1:18–22 (1973).
21. Hayes MP, Jennings MR. Decline of ranid species in western North America: are bullfrogs (*Rana catesbeiana*) responsible? J Herpetol 20:490–509 (1986).
22. Bradford DF. Allotopic distribution of native frogs and introduced fishes in high Sierra Nevada lakes of California: implications of the negative effect of fish introductions. Copeia 3:775–778 (1989).
23. Lewis WM, Helms, DR. Vulnerability of forage organisms to largemouth bass. Trans Am Fish Soc 93:315 (1964)
24. Hine, RL, Les BL, Hellmich BF. Leopard frog populations and mortality in Wisconsin, 1974–76. Technical bulletin no 122. Madison, WI:Department of Natural Resources,1981.
25. Crawshaw GJ. 1992. The role of disease in amphibian decline. In: Declines in Canadian amphibian populations: designing a national monitoring strategy. Occasional paper no 76 (Bishop CA Pettit KE, eds). Ottowa, Ontario: Canadian Wildlife Service, 1992;60–62.
26. Scott NJ. Postmetamorphic death syndrome. Froglog 7:1–2 (1993).
27. Carey C. Hypothesis concerning the causes of the disappearance of boreal toads from the mountains of Colorado. Conserv Biol 7: 355–362 (1993).
28. Blaustein AR, Hokit DG, O'Hara RA. Pathogenic fungus contributes to amphibian losses in the Pacific Northwest Biol Conserv 67:251–254 (1994).
29. Blaustein AR, Hoffman PD, Hokit DG, Kiesecker JM, Walls SC, Hays JB. UV repair and resistance to solar UV-B in amphibian eggs: a link to population declines? Proc Natl Acad Sci USA 91:1791–1795 (1994).
30. Oza GM. Ecological effects of the frog's leg trade. Environmentalist 10:39–42(1990).
31. Phillips K. Tracking the vanishing frogs. New York:St. Martin's Press, 1994.
32. Stephen CE, Mount DI, Hansen DJ, Gentile JH, Chapman GA, Brungs WA. Guidelines for deriving numerical national water quality criteria for the protection of aquatic organisms and their uses. NTIS PB85-227049. Springfield, VA:National Technical Instutite Service, 1985.
33. Power T, Clark KL, Harfenist A, Peakall DB. A review and evaluation of the amphibian toxicological literature. Technical report no 61, Ottowa, Ontario:Canadian Wildlife Service, 1989.
34. Holcombe GW, Phipps GL, Sulaiman AH, Hoffman AD. Simultaneous multiple species testing:acute toxicity of 13 chemicals to 12 diverse freshwater amphibian, fish, and invertebrate families. Arch Environ Contam Toxicol 16:697–710 (1987).
35. Evaluation of aquatic pollutants using fish and amphibian eggs as bioassay organisms. In: Animals as monitors of environmental pollutants. Washington DC:National Academy Press, 1979;108–118.
36. Wesley J, Birge J, Black A, Westerman A. Short-term fish and amphibian embryo-larval test for determining the effects of toxicant stress on early life stages and estimating chronic values for single compounds and effluents. Environ Toxicol Chem 4:807–821 (1985).
37. Birge WJ, Black JA, Westerman AG, Ramey BA. Fish and amphibian embryos—a model system for evaluating teratogenesis. Fundam Appl Toxicol 3:237–242 (1983).
38. Dumont JN, Schultz TW, Buchanan MV, Kao GL. Frog embryo teratogenesis assay: *Xenopus* (FETAX)—a short-term assay applicable to complex environmental mixtures. In: Short-term bioassays in the analysis of complex environmental mixtures III (Waters MD, Sandhu SS, Lewtas J, Claxton L, Chernoff N, Newsow S, eds). New York:Plenum Publishing, 1983;393–405.
39. Goss LB, Sabourin TD. Utilization of alternative species for toxicity testing: an overview. J Appl Toxicol 5:193–217 (1985).
40. Bantle JA, Fort DJ, James BL. Identification of developmental toxicants using the frog embryo teratogenesis assay-*Xenopus* (FETAX). Hydrobiology 189:577–585 (1989).
41. Dawson DA, Fort DJ, Newell DL, Bantle JA. Developmental toxicity testing with FETAX: evaluation of five compounds. Drug Chem Toxicol 12:67–75 (1989).
42. ASTM. Standard guide for conducting the frog embryo teratogenesis assay-*Xenopus* (FETAX). Designation: E1439-91. In: Annual Book of ASTM Standards, vol 11.04. Philadelphia, PA:American Society for Testing and Materials, 1199–1209;1992.
43. Bantle JA, Burton TD, Dawson DA, Dumont JN, Finch RA, Fort DJ, Linder G, Rayburn JR, Buchwalter D, Gaudet-Hull AM, Maurice MA, Turley SD. FETAX interlaboratory validation study: phase II testing. Environ Toxicol Chem 13:1629–1637 (1994).
44. Bantle JA, Burton TD, Dawson DA, Dumont JN, Finch RA, Fort DJ, Linder G, Rayburn JR, Buchwalter D, Maurice MA, Turley SD. Initial interlaboratory validation study of FETAX: phase I testing. J Appl Toxicol 14:213–223 (1994).
45. Kutka F. Low pH effects on swimming activity of *Ambystoma* salamander larvae. Environ Toxicol Chem 13:1821–1824 (1994).
46. Freda J. The influence of acid pond water on amphibians: a review. Water Pollut 30: 439–450 (1986).
47. Pierce BA. Acid tolerance of amphibians. Bioscience 35:239–243 (1985).
48. Freda J, Sadinski WJ, Dunson WA. Long-term monitoring on amphibian populations with respect to the effects of acidic deposition. Water Air Soil Pollut 55:445–462 (1991).
49. Beattie RC, Tyler-Jones R. The effects of low pH and aluminum on breeding success in the frog *Rana temporaria*. J Herpetol 26: 353–360(1992).
50. Sadinski WJ, Dunson WA. A multilevel study of effects of low pH on amphibians on temporary ponds. J Herpetol 26:413–422 (1992).
51. Dawson DA, McCormick CA, Bantle JA. Detection of teratogenic substances in acidic mine water samples using the frog embryo teratogenesis assay-*Xenopus*(FETAX). J Appl Toxicol 5:234–244 (1985).
52. Eilers JM, Brakke DF, Landers DH, Overton WS. Chemistry of lakes in designated wilderness areas in the western United States. Environ Monit Assess 12:3–21 (1989).
53. Turk JT, Spahr NE. Rocky Mountains. In: Acidic deposition and aquatic ecosystems (Charles DF, ed). New York:Springer-Verlag, 1991;471–502.
54. Melack JM, Stoddard JL. Sierra Nevada, California. In: Acidic deposition and aquatic ecosystems (Charles DF, ed). New York: Springer-Verlag, 1991;503–530.
55. Nelson PO. Cascade mountains. In: Acidic deposition and aquatic ecosystems (Charles DF, ed). New York:Springer-Verlag, 1991; 531–563.
56. Postel S. Last oasis. New York:WW Norton and Company, 1992.
57. Reisner M. Cadillac desert. New York:Penguin Books, 1986.
58. Stegner W. Where the bluebird sings to the lemonade springs. New York:Random House, 1992.
59. Connell DW, Miller GJ. Chemistry and ecotoxicology of pollution. New York:John Wiley and Sons, 1984.
60. Van der Leeden F, Troise FL, Todd DK. The water encyclopedia. Second edition. Chelsea, MI:Lewis Publishers, 1990.
61. Pimental D. The dimensions of the pesticide question. In: Ecology, economics, ethics—the broken circle (Bormann FH, Keller SR, eds). New Haven:Yale University Press, 1991; 59–69.
62. Browner CM. The administration's proposals. EPA Journal 20:6–9 (1994).
63. Bishop CA. The effects of pesticides on amphibians and the implications for determining the causes of declines in amphibian populations. In: Declines in Canadian amphibian populations: designing a national monitoring strategy. occasional paper no 76. (Bishop CA, Pettit KE, eds). Ottowa, Ontario:Canadian Wildlife Service, 1992;60–62.
64. Cooke AS. Tadpoles as indicators of harmful levels of pollution in the field. Environ Pollut Ser A Ecol Biol 25:123–133 (1981).
65. Cooke AS. Effects of field applications of the herbicides diquat and dichlobenil on amphibians. Environ Pollut 12:43–50 (1977).
66. Sanders HO. Pesticide toxicities of the western chorus frog (*Pseudacris triseriata)* and Fowler's toad (*Bufo woodhousii fowleri)*. Copeia 2:246–251 (1970).
67. Berrill M, Bertram S, McGillvray L, Kolohon M, Pauli B. Effects of low concentrations of forest-use pesticides on frog embryos and tadpoles. Environ Toxicol Chem 13:657–664 (1994).
68. Snawder JE, Chambers J. Toxic and developmental effects of organophosphorus insecticides in embryos of the South African clawed frog. J Environ Sci Health B 24:205–218 (1989).
69. Birch WX, Prahlad KV. Effects of Nabam on developing *Xenopus laevis* embryos: minimum concentration, biological stability, and degradative products. Arch Environ Contam Toxicol 15:637–645(1986).
70. Mohanty-Hejmedi P, Dutta SK. Effects of some pesticides on the development of the Indian bullfrog *Rana Tigrina*. Environ Pollut Ser A Ecol Biol 24:145–161 (1981).
71. Marian MP, Arul V, Pandian TJ. Acute and chronic effects of carbaryl on survival, growth, and metamorphosis in the bullfrog *(Rana tigrina)*. Bull Environ Contam Toxicol 12: 271–275 (1983).
72. Johnson CR. The effects of five organophosphorus insecticides on thermal stress in tadpoles of the pacific tree frog, *Hyla regilla*. Zool J Linn Soc 59:143–147(1980).
73. Littleton TM. Water quality and fishes of the Lower Klamath and Tule Lake National Wildlife Refuges (MS thesis). Seattle, WA:

University of Washington, 1993.
74. Saiki MK, Jennings MR, Wiedmeyer RH. Toxicity of agricultural subsurface drainwater from the San Joaquin Valley, California, to juvenile chinook salmon and striped bass. Trans Amer Fish Soc 121:78–93 (1992).
75. Schroeder RA, Palawski DW, Skorupa JP. Reconnaissance investigation of water quality, bottom sediment, and biota associated with irrigation drainage in the Tulare lake bed area, southern San Joaquin Valley, California, 1986–87. Water-resources investigations report 88-4001. Sacramento, CA:U.S. Geological Survey, 1988.
76. Presser TA, Ohlendorf HM. Biogeochemical cycling of selenium in the San Joaquin Valley, California USA. Environ Manage 11:805–821 (1987).
77. Sorenson SK, Schwarzbach SE. Reconnaissance investigation of water quality, bottom sediment, and biota associated with irrigation drainage in the Klamath Basin, California and Oregon, 1988–89. Water-resources investigations report 90-4203. Sacramento, California: U.S. Geological Survey, 1991.
78. MacCoy, D. Physical, Chemical, and biological data for detailed study of irrigation drainage in the Klamath Basin, California and Oregon, 1990–1992. Open-file report 93–497. Sacramento, California:U.S. Geological Survey, 1994.
79. Boyer R. Evaluation of water quality in relation to frogs at Klamath Basin National Wildlife Refuges (MS thesis). Seattle:University of Washington, 1993.
80. Thurston RV, Chakoumakos C, Russo RC. Effect of fluctuating exposures in the acute toxicity of ammonia to rainbow trout *(Salmo gairdneri)* and cutthroat trout (*S. clarki*). Water Res 15:911–917 (1981).
81. Thurston RV, Russo RC, Vinofradov GA. Ammonia toxicity to fishes. Effect of pH on the toxicity of the un-ionized ammonia species. Environ Sci Toxicol 15:837–840 (1981).
82. Russo RC, Thurston RV. Toxicity of ammonia, nitrite and nitrate to fishes. In: Aquaculture and water quality. Advances in world aquaculture, vol 3 (Brune DE, Tomasso JR, eds). Baton Rouge, LA:World Aquaculture Society, 1991;58–89.
83. Diamond JM, Mackler DG, Rasnake WJ, Gruber D. Derivation of site-specific ammonia criteria for an effluent-dominated headwater stream. Environ Toxicol Chem 12:649–658 (1993).
84. U.S. EPA. Ambient water quality criteria for ammonia. EPA 440/5-85-001. Washington DC:Environmental Protection Agency, 1985.
85. Jackson W. Nature as the measure for a sustainable agriculture. In: Ecology, economics, ethics—the broken circle (Bormann FH, Keller SR, eds). New Haven, CT:Yale University Press, 1991;43–58.
86. Worster D. The wealth of nature. New York:Oxford University Press, 1993.
87. Jackson, W. At nature's pace. New York: Pantheon Books, 1993.
88. Pimental D, Acquay H, Biltonen M, Rice P, Silva M, Nelson J, Lipner V, Giordano S, Horowitz A, D'Amore M. Environmental and economic costs of pesticide use. Bioscience 42:750–760 (1992).
89. Drost BW, Ebbert JC, Cox SC. Long-term effects of irrigation with imported water on water levels and water quality. Water-Resources Investigations Report 93–4060. Tacoma, WA:U.S. Geological Survey,1993.
90. Grumbine RE. What is ecosystem management? Conserv Biol 8:27–38 (1994).
91. Botkin DB. Discordant harmonies. New York:Oxford University Press, 1990.
92. O'Neill RV, DeAngelis DL, Waide JB, Allen TFH. A hierarchical concept of ecosystems. Princeton, NJ:Princeton University Press, 1986.
93. Carpenter SR, Fisher SG, Grimm NB, Kitchell JF. Global change and freshwater systems. Annual Rev Ecol System 23:119–140 (1992).
94. Vighi M, Chiaudani G. Eutrophication in Europe: the role of agricultural activities. In: Reviews in environmental toxicology 3 (Hodgson E, ed). Amsterdam:Elsevier, 1987; 213–258.
95. Vitousek PM. Beyond global warming: ecology and global change. Ecology 75:1861–1876 (1994).
96. Wilson EO. The diversity of life. New York:Norton, 1992.
97. U.S. Fish and Wildlife Service. Endangered and threatened wildlife and plants. Title 50 code of Federal Regulations, part 17, subpart B, 1993.
98. Leonard WP, Brown HA, Jones LCL, McAllister KR, Storm RM. Amphibians of Washington and Oregon. Seattle, WA:Seattle Audubon Society, 1993.

Alkyl Ethoxylated and Alkylphenol Ethoxylated Nonionic Surfactants: Interaction with Bioactive Compounds and Biological Effects

Tibor Cserháti

Central Research Institute, Hungarian Academy of Sciences,1525 Budapest, Hungary

This review deals with recent advances in the study of interactions of nonionic surfactants with proteins, peptides, amino acids, membrane phospholipids, and organisms. The effect of surfactants on the structure and biological activity of the interacting biomolecules and organisms is discussed, with emphasis on the impact of hydrophobic and hydrophilic molecular substructures on biological efficiency. *Key words*: alkyl ethoxylated nonionic surfactants, alkylphenol ethoxylated nonionic surfactants, phospholipids, proteins, surfactants. *Environ Health Perspect* 103:358–364 (1995)

Nonionic surfactants are amphipathic molecules consisting of a hydrophobic (alkylated phenol derivatives, fatty acids, long-chain linear alcohols, etc.) and a hydrophilic part (generally ethylene oxide chains of various length). Although not as important commercially, tertiary amine and various sugar surfactants are also nonionic surfactants. Due to their favorable physicochemical properties, nonionic surfactants are extensively used in many fields of technology and research. The application of nonionic surfactants in various biotechnological processes has been recently reviewed (*1*). Surfactants have been successfully used to decrease the foaming of fermentation broths during solvent extraction (*2*), increase the conversion of linoleic acid to its hydroperoxide (*3*), and enhance the rate of cellulose hydrolysis (*4*). Nonionic surfactants are an integral part of the majority of pesticide formulations (*5*). They increase the leaf retention of spray solutions (*6*), enhance adhesional forces of aqueous droplets on crop leaf surfaces (*7*), and generally improve the effectiveness of active ingredients (*8,9*). However, not only do surfactants influence the performance of pesticides, but the pesticides exert some effects on the fate of surfactants; for example, pesticides promote or inhibit the photolytic degradation of nonionic surfactants (*10*).

Nonionic surfactants are also used in pharmaceuticals to increase their stability (*11*) and to enhance the dissolution rate of active ingredients from suppositories (*12*) and solid dispersions (*13*), for example. The pharmaceutical industry also uses nonionic surfactants to facilitate solubilization (*14*) and to increase the stability of drug-carrier emulsions (*15*). Surfactants markedly modify the particle size of precipitated drugs, too (*16,17*). Due to strict regulations, nonionic surfactants have only limited application in the food industry, where they are employed to change the stability of various emulsions (*18*) and to decrease the retrogradation of amylopectin (*19*). Nonionic surfactants also have been used in analytical chemistry to increase the fluorescence of dansylated amino acids (*20*), improve protein separation in capillary zone electrophoresis (*21*), and mask side effects in spectrophotometry (*22*).

This review presents a critical evaluation of recent results of studies on the interaction of alkyl ethoxylated and alkylphenol ethoxylated nonionic surfactants with various bioactive macromolecules and with organisms. The fate of surfactants in various ecological systems has been extensively studied. Nonionic surfactants are generally easily degradable; however, in some cases the persistence of intermediates has been observed. Due to the limited scope of this review, investigations of intermediates will not be discussed in detail.

Interaction with Bioactive Macromolecules

The mode of action of nonionic surfactants and the hydrophilic (electrostatic) or hydrophobic character of their interaction with bioactive molecules, organs, and organisms have been extensively discussed. The results are sometimes contradictory, and the character of interaction depends considerably on the interactive molecular species.

Proteins, peptides, and amino acids. Many studies have indicated that nonionic surfactants readily bind to various proteins. This phenomenon has been frequently exploited to extract and solubilize sparingly soluble proteins such as membrane proteins (*23*). Nonionic surfactants derived from tris(hydroxymethyl)-aminomethane perform well in the solubilization of subcellular proteins of rat hepatocytes and membrane antigens from tumor cells (*24*). Nonionic surfactants are generally less effective than ionic surfactants; for example, Tween 80 and polyoxyethylene-9-laurylether have a negligible effect on the dissociation, γ-chymotryptic degradation, and enteral absorption of insulin hexamers (*25*). Surfactants also modify the adsorption capacity of proteins and peptides: Tween 80, Triton X-100, and PEG 6000 decrease the adsorption of urokinase on glass surfaces; however, they were less effective than gelatin (*26*). The adsorption of fibrinogen was markedly lower on polyoxyethylene-polyoxypropylene-coated polystyrene latex (*27*), and the adsorption on self-assembled monolayers of fibrinogen, lysozyme, pyruvate kinase, and RNAse A was inhibited by oligoethyleneoxides (*28*).

Surfactants exert a protective effect on proteins. At a 2% concentration, Tween 20 completely prevented the denaturation of rabbit skeletal myosin by freezing and thawing, and glycerol enhanced synergistically the protective effect (*29*).

The majority of research on protein–surfactant interaction has focused on the binding of surfactants to enzymes and the effect of surfactant binding on enzyme activity. These results will be discussed later. Because the molecular basis of the binding of surfactants to proteins has not been elucidated in detail, some investigators have tried to pinpoint individual amino acids accounting for the binding. Charge-transfer chromatographic methods indicate that nonylphenyl hexaethoxylate only interacts with some amino acids, with the order of relative strength of interaction Tyr> Glu>Phe>Hyp>Gln>Cys>Gly. A significant linear relationship has been found between the interactive strength and the hydrophobicity of amino acids. The authors concluded that the interaction of individual amino acids with the surfactant is fairly low and does not explain the strong interaction of surfactant with proteins observed in many studies (*30*). It was assumed that the long surfactant molecule lies parallel with the protein surface, contacting more than one amino acid residue. The strength of interaction varies according to the amino acid sequences, and hydrophobic forces are probably involved in the interaction (*30*). A similar study dealing with the interaction of amino acids with ethoxylated stearic acid surfactants found that surfactants interact with free amino acids in the following order: Cys>Phe>Tyr>Asn>Met>Nle>Leu> Gln>Lys>Ser>Trp. In this case the electronic parameters of surfactants had a significant impact on the strength of interaction (*31*).

The forces involved in the binding of nonionic surfactants to proteins are being characterized. The results indicate that the hydrophobic moiety of surfactants can bind to the apolar amino acids, whereas the hydrophilic ethyleneoxide chain can inter-

Address correspondence to T. Cserháti, Central Research Institute, Hungarian Academy of Sciences, PO Box 17, 1525 Budapest, Hungary.

This work was supported by a grant for Cooperation in Science and Technology with Central and Eastern European Countries: "Enhanced removal and prevention of environmental pollution by attachment and immobilization of bacteria at surfaces."

Received 2 May 1994; accepted 16 November 1994.

act with the peptide bond and with one or more polar amino acid residues, probably by electrostatic forces and hydrogen bonding.

Membrane phospholipids. Results of many studies indicate that nonionic surfactants interact not only with proteins but also with membrane phospholipids by modifying their structure and permeability. As phospholipids are chemically simple compounds, the principles of various surfactant–phospholipid interactions and the character of forces involved are fairly well known.

Surfactants generally increase the permeability of phospholipid membranes and vesicles, causing leakage of compounds with low molecular mass. The loss of ions, amino acids, etc., may result in cell damage or cell death. It is generally accepted that the increased permeability is the result of membrane disruption. Supramolecular surfactants (polyethylene glycol + dicarboxylic acid esters) as well as Triton X-100 readily disrupt egg yolk phosphatidylcholine membranes (*32*). An increase in permeability has been observed in many model systems: Triton X-100 and some new synthetic surfactants caused leakage from palmitoyloleoyl phosphatidylcholine/cholesterol large unilamellar vesicles (*33*). The concentration and aggregation state of surfactants also exert a considerable effect on their membrane-damaging capacity: monomeric Triton X-100 causes leakage of dipalmitoyl-phosphatidylcholine vesicles, whereas micellar solutions result in the catastrophic rupture of membrane (*34*). Some new surfactants, $(HO(C_2H_4O)_6CO(CH_2)_{14}CO_2C_2H_4O)_6H$ and $HO(C_2H_4O)_6CO(CH_2)_6CH{=}CH(CH_2)_6CO_2C_2H_4O)_6H$ and their polymeric counterpart, were synthesized and their capacity to disrupt egg yolk phosphatidylcholine and palmitoyloleoyl phosphatidylcholine bilayers determined at various cholesterol concentrations in the bilayer. It was established that the effect of new synthetic surfactants depends on the cholesterol concentration in the bilayer, whereas the effect of Triton X-100 is not affected by the cholesterol concentration (*35*). Unfortunately, the cause of the damaging behavior of the new surfactants was not explained in detail. The same surfactants caused leakage or rupture of palmitoyloleoyl phosphatidylcholine vesicles depending on the membrane packing (*36*). The condensation product of hexaethyleneglycol and various dicarboxylic acids considerably increased the release of 5(6)-carboxyfluorescein from the large, unilamellar vesicles of palmitoyloleoyl phosphatidylcholine (*37,38*).

The interaction of surfactants with artificial membranes modifies many physicochemical parameters of the phospholipids: A fluorescence depolarization study indicated that alkanoyl-*N*-methylglucamide surfactants decrease the fluidity of dipalmitoyl phosphatidylcholine membranes (*39*). Nonionic surfactants decreased the phase transition temperature of negatively charged dilauroylphosphatidic acid membrane. The interaction between surfactant molecules incorporated in the lipid membrane was also observed (*40*).

The effect of surfactants on natural membranes has also been observed. Surfactant can disrupt not only artificial membranes but also modify the physicochemical characteristics of natural membranes. Nonionic surfactants were able to increase the permeability of sarcoplasmic reticulum vesicles (*41*), and Pluronic L81, a hydrophobic surfactant, markedly influenced the cholesterol homeostasis of intestinal mucosa; however, it was not specified whether this effect was due to the direct surfactant–cholesterol interaction or due to the result of other, not well known biochemical or biophysical processes (*42*).

The number of studies dealing with the elucidation of the relationship between surfactant structure and membrane-damaging activity is surprisingly low. Adiabatic differential-scanning calorimetric measurements indicated that [2-(alkoxy)-phenyl]-2-(1-piperidinyl)ethyl esters of carbamic acid interact with dipalmitoyl phosphatidylglycerol model membranes, and the effect depends on the length of ethyleneoxide chain (*43*). The effect of polyoxyethylene cetyl ethers on the vesicle to micelle transitions of egg yolk phosphatidylcholine liposomes also markedly depends on the length of polar ethyleneoxide chain (*44*). It has been found that polyoxyethylene-polyoxypropylene block copolymer molecules are intercalated with phosphatidylcholine monolayers (*45*).

Although the binding of surfactants to proteins and phospholipids seem to be two independent procedures, a comparative study suggested that there is a strong relationship between the skin irritation potential of surfactants and their capacity to increase dye leakage from egg yolk phosphatidylcholine unilamellar liposomes (*46*).

It can be concluded that the interaction of nonionic surfactants with membrane phospholipids involves the insertion of the hydrophobic moiety of surfactants into the apolar fatty acid domain of phospholipids. However, this insertion is not enough to disturb the membrane organization. Linear substructures (fatty acids, long-chain alcohols) are well accomodated and do not disturb the membrane organization. Bulky hydrophobic moieties (alkylated phenols) cause severe disturbances between the apolar fatty acid chains, resulting in increased permeability and leakage. The hydrophilic ethyleneoxide chain probably has two functions: it regulates the insertion depth of hydrophobic moiety (longer ethyleneoxide chain draws the hydrophobic moiety toward the aqueous outer phase), indirectly influencing its membrane damaging effect, or it binds to the polar head group of phospholipids. As the long ethyleneoxide chain can contact more than one head group, it can stabilize the membrane organization. The effects observed are the result of the interplay of the interactions outlined above.

Proteins and membrane phospholipids simultaneously occur in many living cells. In these instances surfactants can bind both to the proteins and phospholipids. The preference of surfactants either for proteins or for phospholipids in a complicated living system has never been studied in detail.

Biological Effects

Stimulation and inhibition of enzymes. Nonionic surfactants readily bind to various proteins, and the binding modifies protein solubility and structure. These changes may also result in the stimulation or inhibition of the biological activity of enzymes. Unfortunately, most studies dealing with the effect of surfactants on enzyme activity are limited to determining the degree of stimulation or inhibition and do not elucidate the underlying molecular mechanism.

An *N*-acetyl-D-glucosaminyltransferase detected in human carcinoma Colo 205 cells showed optimum activity in the presence of the nonionic detergent Triton CF-54 (*47*). Glycolipid glucuronyltransferase isolated from embryonic chicken brain shows optimum activity in the presence of neutral detergents such as Triton CF-54, Triton DF-12, and Nonidet P-40 (*48*). Triton X-100 activated lecithin:cholesterol acyltransferase (*49*), stimulated the activity of rat liver mitochondrial phosphatidylserine decarboxylase (*50*), and, together with other nonionic surfactants (Myrj 52, Myrj 59, Tween 20, Tween 80, etc.) at 0.1% (w/v), increased the activity of human leukocyte proteinase elastase and cathepsin G (*51*). Nonionic surfactants having a polyoxyethylene chain have been shown to effectively increase the activity of *Chromobacterium viscosum* lipase in aerosol bis(2-ethylhexyl)sodium sulfosuccinate reverse micelles (*52*). Octaethylene glycol dodecyl ether induced the dissociation of membrane-bound Na^+/K^+-ATPase purified from dog kidney (*53*). An interesting study indicated that the effect of surfactant strongly depends on its concentration: Triton X-100 stimulated the activity of the ATPase-active P-glycoprotein at low concentrations and inhibited it at higher concentrations (*54*).

The hydrophobic or hydrophilic character of surfactant–enzyme interactions has been established only in a few instances. Nonhomologous series of nonionic surfac-

tants increased the activity of papain and modified its structure as determined by differential scanning calorimetry. Both the hydrophobic and hydrophilic molecular characteristics of surfactants influenced their effect on the activity and structure of papain (*55*). In contrast, similar surfactants markedly inhibited the activity of horseradish peroxidase. Also in this instance both the hydrophobic and hydrophilic molecular characteristics of surfactants influenced their effect on the activity of the enzyme (*56*). Triton X-100 activated the plasma membrane ATPase. This effect was tentatively explained by the alteration of the hydrophobic environment around the enzyme (*57*). Reduced lysozyme at pH 2.5 bound polyoxyethylene alkylethers (C10E6, C12E6, and C12E8 surfactants); the maximum bond reached 0.5–0.7 mol/mol amino acid residue (*58*). It was further established that the interaction most likely takes place between the hydrocarbon tail of the surfactant and the hydrophobic domain of reduced lysozyme (*59*).

Many results prove that nonionic surfactants can considerably modify the activity of various enzymes. This effect can be both beneficial (biotechnological processes) or harmful (toxicity toward humans, animals, plants, etc.). It is currently impossible to predict the behavior of surfactant–enzyme systems. We need much more data on the molecular basis of mode of surfactant binding to proteins for the rational design of surfactants with optimal biological efficiency and with minimal toxic side effects.

Microorganisms and insects. Due to their capacity to interact with proteins and phospholipids, nonionic surfactants exert many biological effects on microorganisms and insects. These effects have been successfully exploited in some biotechnological and immunological processes. Tween 80 enhanced the ligninase production and growth of the fungi *Phanerochaete chrysosporium* (*60*). Polyethylene glycol 600 increased the γ-amylase production of *Bacillus subtilis,* whereas polyethylene glycol 3350, Triton X-100, and Tween 80 were ineffective, proving again that the character of surfactant has a marked influence on its biological efficiency (*61*). Tween 80 modified invertase secretion by *Neurospora crassa* and the cell-wall-less slime secreted by an *N. crassa* mutant(*62*). Polyethylene oxide-polypropylene oxide block-polymers up to 7.90 Da molecular mass stimulated the secretion of antibodies against *Streptococcus pneumoniae*-derived hexasaccharide–protein conjugates (*63*). The same block-polymers enhanced the avidity of antibodies in polyclonal antisera against *Streptococcus pneumoniae* type 3 in normal and Xid mice (*64*). Nonionic surfactants such as hexa-, octa-, and decaethylene glycol monohexadecyl ether in combination with alkyl phosphates inhibit the adherence of *Streptococcus mutans* on a hydroxyapatite surface (*65*).

Nonionic surfactants have toxic effects too. They increased cell fusion caused by polyethylene glycol (*66*). Triton X-100 and Triton XR suppressed spore germination and germ tube growth of *Mucor mucedo* on tomatoes in storage (*67*). Triton X-100 caused the cell death of *Bacillus subtilis* by inducing cell autolysis (*68*). It has been suggested that the surfactant interacts with the regulatory system of autolysis and thus affects the activation of autolysis in *B. subtilis* (*69*). Three to four orders of magnitude differences were found between the sensitivity of various algae species and surfactant toxicity (*70*). Two types of nonylphenol ethylene oxide-acetate did not influence the growth of *Acinetobacter calcoaceticus, Photobacterium phosphoreum,* or *Serratia marinorubra,* but inhibited the growth of marine heterotrophic flagellates (*71*). Nonionic surfactants (Activator N.F. and Ortho X-77) were moderately toxic to larvae of the midge *Chironomus riparius* (*72*).

The fate of nonionic surfactants in soils and surface waters has been vigorously studied. It was established that they decompose relatively easily; however, the results depend slightly on the characterisitcs of the ecological system under investigation. Polyethoxylated linear alcohol derivatives were mineralized without lag periods by rhizosphere microbial communities in surface soils (*73*). The microflora of aquatic plants decompose about 30–40% on nonionic surfactants in 30 days (*74*). According to another study, the half-life of linear ethoxylated surfactants was 8.4 days as decomposed by the microbiota of submerged plant detritus (*75*). The effect of surfactants on the biodegradation of other xenobiotics has not been determined unambiguously. One study found that nonionic surfactants inhibit the mineralization of phenanthrene in soil, probably by interacting with the membrane of soil microflora (*76*), whereas another study reported that the nonionic surfactant $(CH_2)_{12\text{-}14}(OCH_2CH_2)_{5.6}OH$ added to the soil surface promoted the biodegradation of phenanthrene and biphenyl in Lima silt loam (*77*). This discrepancy may be due to the different microbial populations of soils and the different stability of surfactants against microbial decomposition.

The relationship between the microbiological effect of surfactants and their chemical structure has been studied only in a few instances. Tween compounds induced hydrogen production in aqueous suspensions of *Anabaena variabilis* in the order Tween 85>Tween 80>Tween 60. Tween 20 was ineffective (*78*). This finding indirectly proves that the effect of surfactants depends both on the character of the hydrophobic moiety and the length of the polar ethylene oxide chain. Polyalkylene glycols improved cell growth, viability, and alcohol production of *Saccharomyces cerevisiae.* The effect depended on the number of ethylene oxide groups in the surfactant molecule (*79*). Surfactants with more ethylene oxide groups showed lower toxicity toward *Mysidopsis bahia* (*80*).

Nonionic surfactants can stimulate or inhibit the growth of a wide variety of microorganisms. These effects have a marked impact on human health care, biotechnology, environmental protection, and agrochemistry. A better understanding of the underlying biochemical and biophysical processes would be of considerable interest for the safer application of nonionic surfactants.

Plants. The direct effect of nonionic surfactants on plant species has rarely been studied because surfactants generally contact plants in combination with various pesticides. It has been found that nonionic surfactants cause phytotoxic symptoms in tobacco (*Nicotiana tabacum*), sugar beets (*Beta vulgaris*), and spiderwort (*Tradescantia albiflora).* Surfactants with low and high numbers of ethylene oxide groups were less effective (*81*). It has been shown that the more hydrophilic surfactants (fewer ethylene oxide groups) had the smallest effect both on ethylene evolution and leaf growth in *Phaseolus vulgaris* (*82*). Nonionic surfactants considerably decreased the net potassium influx in roots of wheat seedlings; their effect depended on the number of ethylene oxide groups and on the overall hydrophobicity of the surfactant (*83*). The pH of the solution did not significantly influence the sorption of octylphenoxy surfactants on isolated tomato fruit cuticles, indicating that ionic interactions have a negligible effect on sorption (*84*). The toxicity of these surfactants to cowpea leaves was found to be inversely related to the length of the ethylene oxide chain (*85*).

These data suggest that the physicochemical parameters of surfactants play a considerable role in the extent of phytotoxic activity. Similar results have been found when surfactants were used in combination with pesticides. Octylphenoxy surfactants increased the foliar uptake of DDT and atrazine. The effect was inversely related to the hydrophile:lipophile balance of surfactants (*86*). Complex stability between 2-(1-naphthyl)acetic acid and surfactant micelles decreased with the logarithm of the length of ethyleneoxide chain for

Triton X surfactants. Nondissociated forms of the plant growth hormone formed more stable complexes (*87*).

Animals and animal models. The widespread use of nonionic surfactants makes it probable that organisms may absorb a great quantity of surfactants. To elucidate their toxic effects, a variety of animal models have been used.

In rats, surfactant can enhance the toxic effects of xenobiotics when administered simultaneously. Surfactants increased the absorption of xenobiotics in rat colon (*88*). Tween 80 enhanced the intestinal absorption of the anthelminthic drug albendazole in rat gut (*89*), whereas polysorbate 80 increased the absorption of phenylalkylcarboxylic acids in rat colon (*90*).

Nonionic surfactants themselves show toxic effects. Hexaethoxylated linear primary alcohol ($C_{9\text{-}11}$) is moderately toxic by the oral route in rats. By the dermal route, it does not produce skin irritation or systemic or reproductive toxicity at concentrations used in formulated cleaning products (*91*). Lubrol PX 0.8% (v/v) (pH 6.98–0.02) and Triton X-100 0.5% (v/v) (pH 7.41–0.03) significantly increased the pH of mucosal surface of rat proximal jejunum (control pH 6.23–0.02) (*92*). Emulgen 913 (polyoxyethylene glycol nonylphenyl ether) decreased liver weight and the cytochrome P450, cytochrome b_5, and microsomal heme content in rats, whereas heme oxygenase activity was greatly enhanced (*93,94*). The nonionic surfactant nonoxynol-9 changes vaginal permeability in ovariectomized rats as determined by nigrosin staining and measurement of bioelectronic parameters, whereas Tween 80 was ineffective (*95,96*).

In mice, polysorbates (Tween 20, 21, 80, and 81) as well as poloxamer and poloxamine surfactants had only a slight influence on the permeability of methanol through a full thickness mouse skin; however, the permeability of lipophilic octanol decreased (*97,98*).

In rabbits, nonionic surfactants enhanced the systemic absorption of α-melanocyte-stimulating hormone via the ocular route in rabbits (*99*). The cytotoxicity order of surfactants on rabbit corneal epithelial cells was cationic>anionic= amphoteric>nonionic; however, Triton X-100 had a ranking similar to anionic surfactants (*100*). Poloxalene (30% polyethyleneoxide and 70% polypropylene oxide, MW 3000) inhibited neutral fat and cholesterol absorption in rabbits (*101*). The study of the uptake of neutral red by rabbit corneal cells revealed that nonionic surfactants have a lower toxic effect than cationic, anionic, and amphoteric ones (*102*).

Triton X-100 at concentrations over the critical micellar concentration induced lysis of isolated gill epithelial cells in *Oncorhynchus mykiss* (*103*); however, Triton X-100 showed a lower effect than ionic surfactants (*104*). Emulgen 913 (polyoxyethylene glycol nonylphenyl ether) significantly decreased the concentration of metal-binding proteins in the hepatopancreas and lessened the heme-oxygenase activity in the kidney of red carp (*105*). The adsorption of salicylic acid on hamster cheek pouch decreased in the presence of the nonionic surfactant polysorbate 80, while ionic surfactants enhanced adsorption (*106*).

The results discussed above clearly show that nonionic surfactants influence many biological processes, and the effect is general noxious to the living organisms. However, it has been found that Tween 20 was as efficient as natural surfactant in improving gas exchange and compliance in preterm lambs with respiratory failure (*107*).

The structure and physicochemical parameters of surfactants exert a marked impact on their biological activities. The effect of nonylphenol-polyethoxylates on the bioelectric properties of the vagina of rats showed a nonlinear relationship with the number of ethylene oxide groups per molecule (*108*). Surfactants having a linear alkyl chain greater than 8 carbons and an ethylene oxide chain length of less than 18 caused significant increases in the flux of methyl nicotinate across hairless mouse skin. Surfactants having branched alkyl chain or aromatic moieties in the hydrophobic portion were ineffective (*109*). The toxicity of polyoxyethylene alkyl ethers decreased by increasing length of the alkyl chain and increased by the length of the polyoxyethylene headgroup (*110*).

These data draw attention to the fact that the appropriate selection of surfactants and the synthesis of new surfactants with less toxic side effects may result in lower environmental pollution without losing the advantages of surfactant application.

Human aspects. Human skin has the highest probability of being in contact with surfactants. The cytotoxicity of 17 surfactants on cultured human skin fibroblasts were determined, and it was found that Brij 35, 58, and 99 are a highly cytotoxic. Addition of fetal calf serum decreased the toxicity, probably by binding the surfactants and lowering the concentration of free surfactants (*111*). Brij 78, Brij 99, and Triton X-100 were more toxic than Tween 40 and 80 (*112*). It has been stated that the method used is suitable for predicting irritation potential of surfactant *in vivo*.

Not only can surfactants cause skin irritation, they can also exert beneficial effects, such as promoting the transport of drugs accross the skin. Brij-36 increased the transport of methyl nicotinate and hexyl nicotinate across the skin, whereas sodium dodecyl sulfate was ineffective (*113,114*). Surfactants can effectively increase the transdermal permeation of therapeutic peptides and proteins (*115*). Polysorbate 80 and polyoxyl 40 markedly influenced the transepithelial permeability in monolayers of human intestinal epithelial cells (*116*). The capacity of surfactants to increase the transport of many drugs across the skin may be due to the interaction of surfactants either with the drug or with the skin: sorbitane mono-oleate and polyoxyethylene-*n*-lauryl ether can interact with both the drugs and the skin in degrees dependent on the polarity of the surfactant and the drug (*117*). Diethylene glycol laurylether increased the penetration of theophylline and adenosine into excised human skin by a factor of 2.2–2.7, respectively (*118*). Anionic and cationic surfactants exert a marked effect on the permeability of human skin, whereas the effect of Tween 60 was negligible (*119*).

Surfactants can modify the permeability of blood cells when they enter the organism. Triton X-100 caused a rapid release of ATP from human red blood cells, while the presence of Brij 58 retarded the mobilization of the intracellular ATP (*120*). A study comparing two cytotoxicity tests for predicting ocular irritancy established that the red blood cell lysis test was predictive. Surfactants caused membrane disruption; anionic and cationic surfactants were more toxic than nonionic ones (*121*). Polyethoxylated nonionic surfactants inhibit the transport of 2,4-dinitrophenyl glutathione out of intact human erythrocytes. Surfactants may modify the arrangement of integral membrane proteins such as P glycoprotein and presumably the glutathione transporters (*122*).

Nonionic surfactants show considerable therapeutic effects by synergistically increasing the efficiency of drugs. The nonionic block-polymer surfactants L101 and 31R1 stimulated the induction of delayed-type hypersensitivity on the murine humoral and cellular immune response to a synthetic peptide composed of amino acid residues 9–21 of herpes simplex virus type 1 glycoprotein D (*123*). The neuroleptic activity of haloperidol increased in the presence of the nonionic surfactant poly(55)oxypropylene/dipoly (8)oxyethylene (*124*). Parental P388 murine leukemia cell lines sensitive to adriamycin, a subline of P388 resistant to adriamycin; sarcolemma-180; and Ehrlich ascites tumor were used to study the influence of nonionic surfactants on the activity of adriamycin. An enhanced biosynthesis inhibition by adriamycin was observed when used in combination with Brij 30 or Brij 35 in all the murine tumor models.

The increase in adriamycin cytotoxicity was due to an increased accumulation of adriamycin in the tumor models (*125*). Polyethoxylated nonionic surfactants with no similarities in the hydrophobic moiety are able to reverse multidrug resistance in a human leukemia cell line (*126*). Triton X-100 prevented the net uptake of vinblastin in inside-out membrane vesicles prepared from multidrug-resistant human leukemia cells (*127*).

It can be established that nonionic surfactants are moderately toxic to humans, and they probably can synergistically increase the toxicity of other xenobiotics. However, the beneficial effect of surfactants (promotion of penetration of drugs across the skin, increase of the effect drugs) probably overshadows their eventual noxious effects, and these compounds can be a useful tool for the improvement of human health care in the future.

Conclusions

Nonionic surfactants are widely used in many fields and exert both beneficial and toxic effects. They bind to proteins as well as to phospholipids influencing (stimulating or inhibiting) enzyme activity and membrane permeability. Hydrophilic and hydrophobic forces are simultaneously involved in the binding, and the effects observed are the result of the interplay of the various interacting forces. As recent research indicates, the biological effects strongly depend on the structure of surfactants. We need additional data for the more profound elucidation of the relationship between molecular structure and biological efficiency. With the exact knowledge of this relationship, it will be possible to select for each purpose a surfactant with minimal toxicity and maximal benefits.

References

1. Galaev IY, Mattiasson B. Thermoreactive water-soluble polymers, nonionic surfactants, and hydrogels as reagents in biotechnology. Enzyme Microb Technol 15:354–366 (1993).
2. Lennie S, Halling PJ, Bell G. Causes of emulsion formation during solvent extraction of fermentation broths and its reduction by surfactants. Biotech Bioeng 35:948–950 (1990).
3. Piazza GJ. Lipoxygenase catalyzed hydroperoxide formation in microemulsions containing nonionic surfactants. Biotechnol Lett 14:1153–1158 (1992).
4. Helle SS, Duff SJB, Cooper DG. Effect of surfactants on cellulose hydrolysis. Biotechnol Bioeng 42:611–617 (1993).
5. Seaman D. Trends in the formulation of pesticides—an overview. Pestic Sci 29:437–449 (1990).
6. De Ruiter H, Uffing AJM, Meinen E, Prins A. Influence of surfactants and plant species on leaf retention of spray solutions. Weed Sci 38:567–572 (1990).
7. Watanabe T, Yamaguchi I. The specific adhesional forces of aqueous droplets on crop leaf surfaces and factors influencing them. J Pestic Sci 18:99–107 (1993).
8. Nalewaja JD, Palczinski J, Manthey FA. Imazetapyr efficacy with adjuvants and environments. Weed Technol 4:765–770 (1990).
9. Nalewaja JD, Woznica Z, Manthey FA. DPX-V9360 efficacy with adjuvants and environment. Weed Technol 5:92–96 (1991).
10. Tanaka FS, Wien RG, Zaylskie RG. Photolytic degradation of a homogeneous Triton X nonionic surfactant:nonaethoxylated *p*-(1,1,3,3-tetramethylbutyl)phenol. J Agr Food Chem 39:2046–2052 (1991).
11. Siebenbrodt I, Keipert S. Versuche zur Entwickelung und Characterisierung ophtalmologisch verwendbarer tensidhaltiger Mehrkomponentsysteme. Pharmazie 46: 435–438 (1991).
12. Fontan JE, Arnaud P, Chaumel JC. Enhancing properties of surfactants on the release of carbazepine from suppositories. Int J Pharm 73:17–21 (1991).
13. Sjökvist E, Nyström C, Alden M, Caram-Lelham N. Physico-chemical aspects of drug release. XIV. The effect of some ionic and nonionic surfactants on properties of a sparingly soluble drug in solid dispersions. Int J Pharm 79:123–133 (1992).
14. Fahelelbom KMS, Timoney RF, Corrigan OI. Micellar solubilization of clofazimine analogues in aqueous solutions of ionic and nonionic surfactants. Pharm Res 10:631–634 (1993).
15. Lundberg B. Preparation of drug-carrier emulsions stabilized with phosphatidylcholine-surfactant mixtures. J Pharm Sci 83:72–75 (1994).
16. Sjöström B, Kronberg B, Carlfors J. A method for the preparation of submicron particles of sparingly water-soluble drugs by precipitation in oil-in-water emulsions. I: Influence of emulsification and surfactant concentration. J Pharm Sci 82: 579–583 (1993).
17. Sjöström B, Bergenstahl B, Kronberg B. A method for the preparation of submicron particles of sparingly water-soluble drugs by precipitation in oil-in-water emulsions. II: Influence of the emulsifier, the solvent, and the drug substance. J Pharm Sci 82:584–589 (1993).
18. Dickinson E, Tanai S. Protein displacement from emulsion droplet surface by oil-soluble and water-soluble surfactants. J Agric Food Chem 40:179–183 (1992).
19. Gudmundsson M, Eliasson AC. Retrogradation of amylopectin and the effects of amylose and added surfactants/emulsifiers. Carbohydr Polym 13:295–315 (1990).
20. Baeyens W, Lin B, Corbisier V. Surfactant and cyclodextrin fluorescence enhancement of dansylamino acids and of thiolammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate derivatives. Analyst 115:359–363.
21. Towns JK, Regnier FE. Capillary electrophoretic separations of proteins using nonionic surfactant coatings. Anal Chem 63:1126–1132 (1991).
22. Miura J-I. Masking agents in the spectrophotometric determination of metal ions with 2-(5-bromo-2-pyridazo)-5-diethylaminophenol and non-ionic surfactants. Analyst 114:1323–1329 (1989).
23. Brenner-Henaff C, Valdor J-F, Plusquellec D, Wroblewski H. Synthesis and characterization of N-octanoyl-ß-D-glucosyl-amine, a new surfactant for membrane studies. Anal Biochem 212:117–127 (1993).
24. Maurizis JC, Pavia AA, Pucci B. Efficiency of nonionic telomeric surfactants for the solubilization of subcellular fractions proteins. Bioorg Med Chem Lett 3:161–164 (1993).
25. Shao Z, Li Y, Krishnamoorthy R, Chermak T, Mitra AK. Differential effects of anionic, cationic, nonionic, and physiologic surfactants on the dissociation, α-chymotryptic degradation, and enteral absorption of insulin hexamers. Pharm Res 10:243–251 (1993).
26. Kurzhals P, Larsen C, Johansen M. On the design of urokinase labile prodrugs. Effect of surfactants on the surface adsorption of urokinase and comparison of methods for the determination of K_M and k_{cat}. Acta Pharm Suec 25:15–26 (1988).
27. O'Mullane JE, Davison CJ, Petrak K, Tomlinson E. Adsorption of fibrinogen onto polystyrene latex coated with the nonionic surfactant, poloxamer 338. Biomaterials 9:203–204 (1988).
28. Prime KL, Whitesides GM. Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide): a model system using self-assembled monolayers. J Am Chem Soc 115:10714–10721 (1993)
29. Watanabe T, Kitabatake N, Doi E. Protective effects of non-ionic surfactants against denaturation of rabbit skeletal myosin by freezing and thawing. Agric Biol Chem 52:2517–2523 (1988).
30. Forgács E. Interaction of amino acids with the nonionic surfactant nonylphenyl hexaethoxylate. Biochem Mol Biol Int 30:1–11 (1993).
31. Cserháti T. Charge-transfer chromatographic study on the interaction of amino acids with ethoxylated stearic acid surfactants. Biomed Chromatogr 8:45–48 (1994).
32. Regen SL, Jayasuriya N, Fabianowski W. Supramolecular surfactants: amphiphilic polymers designed to disrupt lipid membranes. Biochem Biophys Res Commun 159: 566–571 (1989).
33. Naka KA, Sadownik ASL, Regen SL. Molecular harpoons. Membrane-disruptive surfactants that can recognize osmotic stress in phospholipid bilayers. J Am Chem Soc 115:2278–2286 (1993).
34. Liu Y, Regen SL. Control over vesicle rupture and leakage by membrane packing and by the aggregation state of an attacking surfactant. J Am Chem Soc 115:708–713 (1993).
35. Nagawa Y, Regen SL. Membrane disrupting surfactants that are highly selective toward lipid bilayers of varying cholesterol content. J Am Chem Soc 113:7237–7240 (1991).
36. Nagawa Y, Regen SL. Surfactant-induced release from phosphatidylcholine vesicles. Regulation of rupture and leakage pathways by membrane packing. J Am Chem Soc 114:1668–1672 (1992).
37. Jayasuriya N, Bosak S, Regen SL. Design, synthesis, and activity of membrane-disrupting bolaphiles. J Am Chem Soc 112:5844–5850 (1990).
38. Jayasuriya N, Bosak S, Regen SL. Supramolecular surfactants polymerized bolaphiles exhibiting extraordinary high membrane-disrupting activity. J Am Chem Soc 112:5851–5854 (1990).
39. Inoue T, Muraoka Y, Fukushima K, Shimozawa R. Interaction of surfactants with vesicle membrane of dipalmitoylphosphatidylcholine: a fluorescence depolarization study. Chem Phys Lipids 46:107–115 (1988).

40. Inoue T, Iwanaga T, Fukushima K, Shomozawa R, Suezaki Y. Interaction of surfactants with bilayer of negatively charged lipid: effect on gel-to-liquid crystalline phase transition of dilauroylphosphatidic acid vesicle membrane. Chem Phys Lipids 48:189–196 (1988).
41. Teruel JA, Soler F, Gomez-Fernandez JC. On the effect of lysophosphatidylcholine, platelet activating factor and other surfactants on calcium permeability in sarcoplasmic reticulum vesicles. Chem Phys Lipids 59:1–7 (1991).
42. Pool C, Nutting DF, Simmonds WJ, Tso P. Effect of pluronic L81, a hydrophobic surfactant, on intestinal mucosal cholesterol homeostasis. Am J Physiol 261:G256–G262 (1991).
43. Gallova J, Bagelova J, Balgavy P, Cizmarik J. Interaction of [2-(alkoxy)-phenyl]-2-(1-piperidinyl)ethyl esters of carbamic acid with dipalmitoylphosphatidylglycerol model membranes: a calorimetric study. Gen Physiol Biophys 12:357–370 (1993).
44. Kim J-G, Kim J-D. Vesicle to micelle transitions of egg phosphatidylcholine liposomes induced by nonionic surfactants, poly(oxyethylene) cetyl ethers. J Biochem 110:436–442 (1991).
45. Weingarten C, Magelhaes SNS, Baszkin A, Benita S, Seiller M. Interaction of a nonionic ABA copolymer surfactant with phospholipid monolayers: possible relevance to emulsion stabilization. Int J Pharm 75:171–179 (1991).
46. Charaf UK, Hart GL. Phospholipid liposomes/surfactant interactions as predictors of skin irritation. J Soc Cosmet Chem 42:71–85 (1991).
47. Basu M, Khan FA, Das KK, Zhang B. Biosynthesis in vitro of core lacto-series glycosphingiolipids by N-acetyl-D-glucoaminyl-transferases from human colon carcinoma cells, Colo 205. Carbohydr Res 209:261–277.
48. Das KK, Basu M, Li Z, Basu S, Jungalwala F. Characterization of solubilized GlcAT-1(UDP-GlcA:nLcOse4Cerß1-3-glucuro-nyl transferase) activity from embryoinc chicken brain and its inhibition by D-eryhtro-sphingosine. Indian J Biochem Biophys 27:396–401 (1990).
49. Bonelli FA, Jonas A. Reaction of lecithin:cholesterol acyltransferase with a water soluble substrate: effects of surfactants. Biochim Biophys Acta 1166:92–98 (1993).
50. Dygas A, Zborowski J. Effect of Triton X-100 on the activity and solubilization of rat liver mitochondrial phosphatidylserine decarboxylase. Acta Biochim Pol 36:131–141 (1989).
51. Wenzel HR, Feldmann A, Engelbrecht S, Tschesche H. Activation of the human leukocyte proteinases elastase and cathepsin G by various surfactants. Biol Chem Hoppe-Seyler 371:721–724 (1990).
52. Yamada Y, Kuboi R, Komasawa I. Increased activity of Chromobacterium viscosum lipase in aerosol OT reverse micelles in the presence of noionic surfactants. Biotechnol Prog 9:468–472 (1993).
53. Mimura K, Matsui H, Takagi T, Hayashi Y. Change in oligomeric structure of solubilized Na^+/K^+-ATPase by octaethylene glycol dodecyl ether, phosphatidylserine and ATP. Biochim Biophys Acta 1145:63–74 (1993).
54. Doige CA, Yu X, Sharom FJ. The effects of lipids and detergents on ATPase-active P-glycoprotein. Biochim Biophys Acta 1146:65–72 (1993).
55. Szögyi M, Cserháti T. Nonionic tensides modify papain structure and proteolytic activity. Acta Biotechnol 10:85–92 (1990).
56. Gullner G, Cserháti T. Structural requirement for the inhibition of horseradish peroxidase activity by non-homologous series of nonionic tensides. Die Nahrung 33:889–894 (1989).
57. Sandstrom RP, Cleland RE. Selective delipidation of the plasma membrane by surfactants enrichment of sterols and activation of atphase. Plant Physiol 90:1524–1531 (1989).
58. Nishiyama H, Maeda H. Reduced lysozyme in solution and its interaction with nonionic surfactants. Biophys Chem 44:199–208 (1992).
59. Tsuji E, Maeda H. Interaction of unfolded lysozyme with hexa(oxyethylene) dodecylether. Coll Polym Sci 270:894–900 (1992).
60. Lestan D, Strancar A, Perdih A. Influence of some oils and surfactants on ligninolytic activity, growth and lipid fatty acids of Phanerochaete chrysosporium. Appl Microbiol Biotechnol 34:426–428 (1990).
61. Ramgren M, Andersson E, Hahn-Hagerdahl B. α-amylase production with *Bacillus subtilis* in the presence of PEG and surfactants. Appl Microbiol Biotechnol 29:337–340 (1988).
62. Buzzi M, Felipe MSS, Azevedo MO, Caldas RA. Membrane lipid composition and invertase secretion on *Neurospora crassa* and its wall-less mutant slime: effects of temperature and the surfactant Tween 80. J Gen Microbiol 139:1885–1889 (1993).
63. Zigterman GJWJ, Schotanus K, Ernste EBHW, Van Dam GJ, Jansze M, Snippe H, Willers JMN. Nonionic block polymer surfactants modulate the humoral immune response against Streptococcus pneumoniae-derived hexasaccharide-protein conjugates. Infect Immun 57:2712–2718 (1989).
64. Van Dam GJ, Verheul AFM, Zigterman GJWJ, De Reuver MJ, Snippe H. Nonionic block polymers surfactants enhance the avidity of antibodies in polyclonal antisera against Streptococcus pneumoniae type 3 in normal and Xid mice. J Immunol 143:3049–3053 (1989).
65. Olsson J, Carlen A, Holmberg K. Inhibition of Streptococcus mutans adherence to hydroxyapatite with combinations of alkyl phosphates and nonionic surfactants. Caries Res 25:51–57 (1991).
66. Prado A, Partearroyo MA, Mencia M, Goni M, Brabara-Guillem E. Surfactant enhancement of polyethyleneglycol-induced cell fusion. FEBS Lett 259:149–152 (1989).
67. Reyes AA. Comparative effects of an antitranspirant, surfactants and fungicides on Mucor rot of tomatoes in storage. Microbios 71:235–241 (1992).
68. Cho H-Y, Tsuchido T, Ono H, Takano M. Cell death of Bacillus subtilis caused by surfactants at low concentrations results from induced cell autolysis. J Ferment Bioeng 70:11–14 (1990).
69. Tsuchido T, Svarachorn A, Soga H, Takano M. Lysis and aberrant morphology of Bacillus subtilis cells caused by surfactants and their relation to autolysin activity. Antimicrob Agents Chemother 34:781–785 (1990).
70. Lewis MA. Chronic toxicities of surfactants and detergent builders to algae: a review and risk assesment. Ecotoxicol Environ Saf 20:123–140 (1990).
71. Poremba K, Gunkel W, Lang S, Wagner F. Marine biosurfactants. III. Toxicity testing with marine microorganisms and comparison with synthetic surfactants. Z Naturforsch 46c:210–216 (1991).
72. Buhl KJ, Faerber NL. Acute toxicity of selected herbicides and surfactants to larvae of the midge Chironomus riparius. Arch Environ Contam Toxicol 18:530–536 (1989).
73. Knaebel B, Vestal Jr. Effects of intact rhizosphere microbial communities on the mineralization of surfactants in surface soils. Can J Microbiol 38:643–653 (1992).
74. Federle T, Schwab B. Mineralization of surfactants by microbiota of aquatic plants. Appl Environ Microbiol 55:2092–2094 (1989).
75. Federle TW, Ventullo RM. Mineralization of surfactants by the microbiota of submerged plant detritus. Appl Environ Microbiol 56:333–339 (1990).
76. Laha S, Luthy RG. Effects of nonionic surfactants on the solubilization and mineralization of phenanthrene in soil-water system. Biotechnol Bioeng 40:1367–1380 (1992).
77. Aronstein BN, Alexander M. Effect of a nonionic surfactant added to the soil surface on the biodegradation of aromatic hydrocarbons within the soil. Appl Microbiol Biotechnol 39:386–390 (1993).
78. Famiglietti M, Hochkoeppler A, Luisi PL. Surfactant-induced hydrogen production in Cyanobacteria. Biotechnol Bioeng 42:1014–1018 (1993).
79. Benchekroun K, Bonaly R. Physiological properties and plasma membrane composition of Saccharomyces cerevisiae grown in sequented batch culture and in the presence of surfactants. Appl Microbiol Biotechnol 36:673–678 (1992).
80. Hall WS, Patoczka JB, Mirenda RJ, Porter BA, Miller E. Acute toxicity of industrial surfactants to Mysidopsis bahia. Arch Environ Contam Toxicol 18:765–772 (1989).
81. Oros G, Cserháti T, Szejtli J. Cyclodextrins decrease the phytotoxicity of nonionic tensides. Acta Agron Hung 38:211–217 (1989).
82. Knoche M, Noga GJ. Effect of noionic surfactants on ethylene release and leaf growth of Phaeolus vulgaris L. Sci Hortic 46:1–11 (1991).
83. Bujtás C, Cserháti T, Szigeti Z. Effect of some nonionic tenzides on potassium influx in roots of wheat seedlings. Biochem Physiol Pflanz 183:277–318 (1988).
84. Schafer WE, Bukovac MJ. Studies on octylphenoxy surfactants. III. Sorption of Triton X-100 by isolated tomato fruit cuticles. Plant Physiol 85:965–970 (1987).
85. Lownds NK, Bukovac MJ. Studies on octylphenoxy surfactants: V. Toxicity to cowpea leaves and effects of spray application parameters. J Am Soc Hortic Sci 113: 205–210 (1988).
86. Stevens PJG, Bukovac MJ. Studies on octylphenoxy surfactants. Part 2: Effects on foliar uptake and translocation. Pestic Sci 20:37–52 (1987).
87. Heredia A, Bukovac MJ. Interaction between 2-(1-naphtyl)acetic acid and micelles of nonionic surfactants in aqueous solution. J Agric Food Chem 40:2290–2293 (1992).
88. Martinez-Coscollá A, Miralles-Loyola E, Garrigues TM, Sirvent MD, Salianas E, Casabó VG. Studies on the reliability of a novel absorption-lipophilicity approach to interpret the effects of the synthetic surfactants on drug and xenobiotic absorption. Arzneim Forsch 43:699–705 (1993).
89. Del Estal JL, Alvarez AI, Villaverde C,

Coronel P, Fabra S, Prieto JG. Effect of surfactants on Albendazole absorption. J Pharm Biomed Anal 9:1161–1164 (1991).

90. Bermejo MV, Perez-Verona AT, Segura-Bono MJ, Martin-Villodre A, Pla-Delfina JM, Garrigues TM. Compared effects of synthetic and natural bile acid surfactants on xenobiotic absorption. I. Studies with polysorbate and taurocholate in rat colon. Int J Pharm 69:221–231 (1991).
91. Gingell R, Lu CC. Acute, subchronic, and reproductive toxicity of a linear alcohol ethoxylate surfactant in the rat. J Am Coll Toxicol 10:477–486 (1991).
92. McKie AT, Stewart W, Lucas ML. The effect of sodium deoxycholate and other surfactants on the mucosal surface pH in proximal jejunum of rat. Naunyn-Schmiedeberg's Arch Pharmacol 343:659–664 (1991).
93. Ariyoshi T, Hasegawa H, Nanri Y, Arizono K. Profile of hemoproteins and heme-metabolizing enzymes in rats treated with surfactants. Bull Environ Contam Toxicol 44:369–376 (1990).
94. Ariyoshi T, Hasegawa H, Matsumoto H, Arizono K. Effects of surfactants on the contents of metallothionein, heme, and hemoproteins and on the activities of heme oxygenase and drug-metabolizing enzymes in rats pretreated with phenobarbital or ß-naphtoflavone. Bull Environ Contam Toxicol 46:120–127 (1991).
95. Levin RJ, Parker A. Changes in the bioelectrical parameters and dye (nigrosin) staining as quantitative indices of the acute action of surfactants on the vagina of ovariectomized rats. J Physiol 378:5P (1986).
96. Levin RJ. Bioelectric activity as a quantitative index of acute spermicide (nonoxynol-9 actions on rat vaginal epithelial function during the ostrous cycle. Pharmacol Toxicol 60:175–178 (1987).
97. Cappel MJ, Kreuter J. Effect of nonionic surfactants on transdermal drug delivery. I. Polysorbates. Int J Pharm 69:143–153 (1991).
98. Cappel MJ, Kreuter J. Effect of nonionic surfactants on transdermal drug delivery. II. Poloxamer and poloxamine surfactants. Int J Pharm 69:155–167 (1991).
99. Chiou GCY, Shen ZF, Zheng YQ, Chen J. Enhancement of systemic delivery of peptide drugs via ocular route with surfactants. Drug Dev Res 27:177–183 (1992).
100. Grant RL, Yao C, Gabaldon D, Acosta D. Evaluation of surfactant cytotoxicity potential by primary cultures of ocular tissues: I. Characterization of rabbit corneal epithelial cells and initial injury and delayed toxicity studies. Toxicology 76:153–176 (1992).
101. Rodgers JB, Tang G, Bochenek WJ. Hydrophobic surfactant inhibits hypercholesterolemia in pair-fed rabbits on a cholesterol-free, low-fat diet. Amer J Med Sci 296:177–181 (1989).
102. Roguet R, Dossou KG, Rougier A. Prediction of eye irritation potential of surfactants using the SIRC-NRU cytotoxicity test. ATLA 20:451–456 (1992).
103. Partearroyo MA, Pilling SJ, Jones MN. The lysis of isolated fish (*Oncorhynchus mykiss*) gill epithelial cells by surfactants. Comp Biochem Physiol 100:381–388 (1991).
104. Partearroyo MA, Pilling SJ, Jones MN. The effects of surfactants on the permeability of isolated perfused fish gills to urea. Comp Biochem Physiol 101A:653–659 (1992).
105. Ariyoshi T, Shiiba S, Hasegawa H, Arizono K. Profile of metal-binding proteins and heme oxigenase in red carp treated with heavy metals, pesticides and surfactants. Bull Environ Contam Toxicol 44:643–649 (1990).
106. Kurosaki Y, Hisaichi SI, Hamada C, Nakayama T, Kimura T. Effects of surfactants on the adsorption of salicylic acid from hamster cheek pouch as a model of keratinized oral mucosa. Int J Pharm 47:13–19 (1988).
107. Gladstone IM, Ray AO, Salafia CM, Perez-Fontan J, Mercurio MR, Jacobs HC. Effect of artificial surfactant on pulmonary function in preterm and full-term lambs. J Appl Physiol 69:465–472 (1990).
108. Levin RJ. Structure/activity relationships of a homologous series of surfactants (nonyl-phenoxypolyethoxyethanols) on rat vaginal bioelectric activity and the ostrous cycle. Pharmacol Toxicol 62:131–134 (1988).
109. Walters KA, Walker M, Olejnik O. Nonionic surfactant effects on hairless mouse skin permeability characteristics. J Pharm Pharmacol 40:525–529 (1988).
110. Hofland HEJ, Bowstra JA, Verhoef JC, Buckton G, Chowdry BZ, Ponec M, Junginger HE. Safety aspects on nonionic surfactant vesicles: a toxicity study related to the physicochemical characteristics of nonionic surfactants. J Pharm Pharmacol 44: 287–294 (1992).
111. Cornelis M, Dupont C, Wepierre J. In vitro cytotoxicity test on cultured human skin fibroblasts to predict the irritation potential of surfactants. ATLA 19:324–336 (1991).
112. Cornelis M, Dupont C, Wepierre J. Prediction of eye irritating potential of surfactants by cytotoxicity tests in vitro on cultures of human skin fibtoblasts and keratinocytosis. Toxic in Vitro 6:119–128 (1992).
113. Ashton P, Hadgraft J, Brain KR, Miller TA. Walters KA. Surfactant effects in topical drug availability. Int J Pharm 41:189–195 (1988).
114. Ashton P, Walters KA, Brain KR, Hadgraft J. Surfactants effect in percutaneous absorption. I. Effects on transdermal flux of methyl nicotinate. Int J Pharm 87:261–264 (1992).
115. Banga AK, Chein YW. Systemic delivery of therapeutic peptides and proteins. Int J Pharm 48:15–50 (1988).
116. Anderberg EK, Nystrom C, Artursson P. Epithelial transport of drugs in cell culture. VII: Effects of pharmaceutical surfactant excipients and bile acids on transepithelial permeability in monolayers of human intestinal epithelial (Caco-2) cells. J Pharm Sci 81:879–887 (1992).
117. Di Golo G, Giannessi C, Nannipieri E, Serafini MF, Vitale D. Influence of drug-surfactant and skin-surfactant interactions on percutaneous absorption of two model compounds from ointment basis in vitro. Int J Pharm 50:27–34 (1989).
118. Kadir R, Stempler D, Liron Z, Cohen S. Penetration of theophylline and adenosine into excised human skin from binary and ternary vehicles. Effect of a nonionic surfactant. J Pharm Sci 78:149–153 (1989).
119. Kompaore F, Marty JP. Dupont CH. Modifications de la per meabilitè cutanèe in vivo chez l'homme après application de surfactifs. Therapie 46:79–82 (1990).
120. Köszegi T, Kellermayer M, Kövecs T, Jobst K. Bioluminescent monitoring of ATP release from human red blood cells treated with nonionic detergent. J Clin Chem Clin Biochem 26:559–604.
121. Lewis RW, McCall JC, Botham PA. A comparison of two cytotoxicity tests for predicting the ocular irritancy of surfactants. Toxic in Vitro 7:155–158 (1993).
122. Board PG. Inhibition of erythrocyte glutathione conjugate transport by polyethoxylated surfactants. FEBS Lett 315:298–300 (1993).
123. Geerligs HJ, Weijer WJ, Welling GW, Welling-Wester S. The influence of different adjuvants on the immune response to a synthetic peptide comprising amino acid residues 9–21 of herpes simplex virus type 1 glycoprotein D. J Immunol Meth 124:95–102 (1989).
124. Kabanov AV, Chekhonin VP, Alakhov VY, Batrakova EV, Lebedev AS, Melik-Nubarov NS, Arzhakov SA, Levashov AV, Morosov GV, Severin ES, Kabanov VA. The neuroleptic activity of haloperidol increases after its solubilization in surfactant micelles. FEBS Lett 258:343–345 (1989).
125. Parekh HK, Chitnis MP. Effect of alterations in permeability by nonionic surfactants on adriamycin cytotoxicity in murine tumor models in vitro. Oncology 47:501–507 (1990).
126. Woodcock DM, Linsenmeyer ME, Chojnowski G, Kriegler AB, Nink V, Webster LK, Sawyer WH. Reversal of multidrug resistance by surfactants. Br J Cancer 66:62–68 (1992).
127. Syed SK, Christopherson RI, Roufogalis BD. Vinblastine transport by membrane vesicles from human multidrug-resistant CCRF-CEM leukemia cells: inhibition by taxol and membrane permeabilizing agents. Biochem Molec Biol Int 30:743–753 (1993).

2nd

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 induction/adaptation)
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 and/or screening be routinely done on exposed workers
5. Future of human population monitoring

For Further Information Contact:

Dr. R.J. Šrám
Laboratory of Genetic Ecotoxicology, Prague Institute of Advanced Studies
U Michelského lesa 366
140 00 Prague Czech Republic
tel.: 422 - 472 47 56
fax: 422 - 472 47 57

Dioxin Activates HIV-1 Gene Expression by an Oxidative Stress Pathway Requiring a Functional Cytochrome P450 CYP1A1 Enzyme

Yan Yao, Amy Hoffer, Ching-yi Chang, and Alvaro Puga

Center for Environmental Genetics and Department of Environmental Health, University of Cincinnati Medical Center, Cincinnati, OH 45267-0056 USA

We have studied the effect of several environmental chemicals on the transient expression of a chloramphenicol acetyltransferase (*cat*) reporter gene linked to the promoter sequences in the long terminal repeat (LTR) of the human immunodeficiency virus type 1 (HIV-1). Aflatoxin B_1, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) and benzo[*a*]pyrene cause a significant increases in CAT expression in mouse hepatoma Hepa-1 cells. The induction of CAT after TCDD treatment is abolished by administration of N-acetyl-L-cysteine or 2-mercaptoethanol and does not take place in a mutant cell line that lacks CYP1A1 enzymatic activity. Linker-scanning mutational analysis of transcription factor binding sites in the promoter revealed that both the NFκB and an adjacent aromatic hydrocarbon response element (AhRE) are required for TCDD-dependent CAT expression. In addition, mutation of the NFAT/AP-1 binding sites in the negative regulatory region of the promoter increases the magnitude of the TCDD effect. We conclude that induction of a functional CYP1A1 monooxygenase by TCDD stimulates a pathway that generates thiol-sensitive reactive oxygen intermediates which, in turn, are responsible for the TCDD-dependent activation of genes linked to the LTR. These data might provide an explanation for findings that TCDD increases infectious HIV-1 titers in experimental systems and for epidemiologic reports suggesting that exposure to aromatic hydrocarbons, such as found in cigarette smoke, is associated with an acceleration in AIDS progression. *Key words*: benzo[*a*]pyrene, chloramphenicol acetyltransferase, c37, CYP1A1, dioxin, Hepa-1 cells, HIV-1, TCDD. *Environ Health Perspect* 103:366–371 (1995)

Halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) cause a profusion of apparently unrelated toxic effects in which the single common denominator is the aromatic hydrocarbon receptor-mediated transcriptional activation of the cytochrome P450 *CYP1A1* gene (*1–6*). In humans, exposure to dioxin and various other chlorinated phenolic agents causes chloracne, a long-lasting skin disease characterized by the hyperkeratinization of follicular sebocytes (*7,8*). In addition, recent long-term epidemiological studies have established a link between exposure to high doses of TCDD and certain types of cancers (*9,10*). Dioxin is one of the strongest tumor promoters ever tested in animal model systems; it causes an elevated incidence of hepatic carcinoma and pulmonary and skin tumors (*11–13*) and promotes tumor formation at one-hundredth the dose of the classical tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the skin of hairless mice (*14–16*). During rodent embryogenesis, TCDD administration also causes craniofacial abnormalities such as cleft palate and hydronephrosis (*17–20*). Characteristic events of secondary palate formation, such as osteoblast differentiation and synthesis and mineralization of extracellular matrix, are inhibited by TCDD (*21*). Unlike in whole animal studies, TCDD has no toxic effect in tissue culture cells, although it causes a large elevation of intracellular calcium, which induces decreased ß-adrenergic responsiveness in cardiac myocytes (*22,23*), and causes apoptosis of immature thymocytes (*24,25*). In this regard, the developing immune system is a particularly sensitive target for TCDD, with thymic atrophy being the most common pathological consequence of exposure (*26*).

Work from our laboratory has shown that treatment of mouse hepatoma cells with polycyclic or halogenated aromatic hydrocarbons such as TCDD and benzo[*a*]pyrene (BaP) causes an increase in the steady-state mRNA levels of the proto-oncogenes c-*fos*, c-*jun*, *jun-B*, and *jun-D* and the concomitant increase of the DNA-binding activity of the transcription factor AP-1 (*27*). These results suggested the possibility that other transcription factors might also be activated by TCDD treatment and that genes which contain binding sites for these transcription factors in their regulatory domains might respond to TCDD or BaP treatment. We tested this hypothesis in mouse hepatoma cells by analyzing the effect of TCDD treatment on the activation of a chloramphenicol acetyltransferase (*cat*) reporter gene fused to the long terminal repeat (LTR) sequences of the human immunodeficiency virus-1 (HIV-1).

Materials and Methods

Aflatoxin B_1 was a gift of Howard G. Shertzer, and TCDD was a gift of the Dow Chemical Company; all polycyclic aromatic hydrocarbons used were purchased from the National Cancer Institute Chemical Carcinogen Repository. The mouse cell lines used in these studies were the wild-type Hepa-1 hepatoma line (*28*) and its CYP1A1 metabolism-deficient derivative, c37 (*29–31*), a variant that carries two missense mutations in the *Cyp1a1* gene, rendering the resulting enzyme nonfunctional (*31*). These cells were grown in α-minimal essential media supplemented with 5% fetal bovine serum.

The bacterial chloramphenicol acetyltransferase (*cat*) gene was used as a reporter in transient transfection experiments. The chimeric plasmid pBennCAT, carrying a fusion of the *cat* gene sequences to the HIV-1 U3 LTR was obtained from the National Institutes of Health AIDS Research and Reference Program. This plasmid contains approximately 500 base pairs of uncharacterized human DNA sequences (*32*), which were removed by standard recombinant DNA techniques, giving rise to plasmid pHIVLTRCAT. Several plasmid constructs carrying mutations in the transcription factor binding sites in the LTR were derived from the wild-type pHIVLTRCAT by linker-scanning mutagenesis (*33*). The sequence of the relevant portion of the U3 LTR is shown in Figure 1; the sequences that were replaced indicated by a single overline. In all cases, 10 or 30 nucleotide residues (1 or 3 helical turns) were replaced by 10 residues (one helical turn), thus preserving the relative position of the unaffected binding sites on the DNA helix. Mutagenesis was carried out by polymerase

Address correspondence to A. Puga, Center for Environmental Genetics and Department of Environmental Health, University of Cincinnati Medical Center, PO Box 670056, Cincinnati, OH 45267-0056 USA. Y. Yao is currently at the Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109-0626 USA.

We thank Howard G. Shertzer for a gift of aflatoxin B_1, Oliver Hankinson for a gift of the c37 cell line, and Daniel W. Nebert for many helpful discussions and suggestions and for a critical reading of the manuscript. The experiments in Figure 2 were presented at the 8th International Conference on Cytochrome P450 Biochemistry and Molecular Biology held in Lisbon, Portugal, in October 1993. This work was supported by NIEHS grants 1R01 ES06273 and P30 ES06096.

Received 13 October 1994; accepted 16 December 1994.

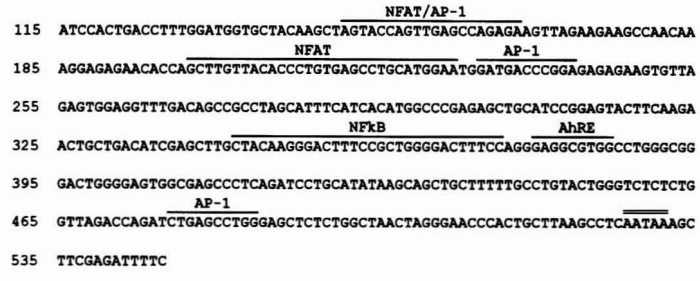


Figure 1. Transcription factor binding sites in the human immunodeficiency virus-1 long-terminal repeat. The sequence shown is from GenBank locus *hivhxb2cg*. Transcription factor binding sites, indicated by the overline, were determined using the transcription factor site database (TFSITES) in combination with the MAP algorithm of the DNA Analysis Package (Genetics Computer Group, Madison, Wisconsin). The double overline indicates the TATA box; transcription initiation is at residue 547. The three contiguous Sp1 binding sites located between residues 376 and 416 are not highlighted.

chain reaction (PCR) of the wild-type plasmid using primers complementary to the flanking sequences of the site to be deleted and containing a *Not*I restriction enzyme site sequence at their 5′ end. The PCR products were digested with *Not*I, ligated, and used to transfect electrocompetent *E. coli* DH5α. In mutant pLS1, the site replaced was the composite NFAT/AP-1 binding site located between residues 146 and 165. Mutants pLS2 (NFAT site at 200–230), pLS3 (AP-1 site at 233–243), and pLS4n (two NFκB sites at 344–374) were constructed in a similar manner. An *Xho*I site was used instead of *Not*I for mutant pLS4a (AhRE site at 378–388), and an *Xba*I site was used for mutant pLS5 (AP-1 site at 477–487). Double and triple mutants were constructed from the single mutants by *in vitro* recombinant techniques using recombination of fragments generated by appropriate restriction enzymes. All constructs were confirmed by restriction enzyme analysis and DNA sequencing.

Approximately 10 μg of the appropriate plasmid was transfected by standard calcium phosphate techniques (*34,35*) into semiconfluent Hepa-1 or c37 cells. In some experiments, to determine the effect of the viral transactivator Tat protein (*36,37*), we co-transfected the cells with plasmid pCV-1 (obtained from the NIH AIDS Research and Reference Program) that expresses HIV-1 Tat under the control of the SV40 early promoter and enhancer. We found that Tat expression increased basal CAT levels and induced CAT levels by approximately the same magnitude; all the experiments reported here were done in the absence of Tat expression. As a negative control we used pSV0CAT, containing a promoterless *cat* gene, and as a positive control we used pSV2CAT, carrying the *cat* gene under the control of the SV40 early promoter and enhancer sequences. In initial experiments, we also used TPA treatment as a positive control for HIV LTR-dependent expression. To control for variations due to differences in transfection efficiency, all cultures were co-transfected with plasmid pCMVßgal (CloneTech, Palo Alto, California), which expresses the bacterial ß-galactosidase gene under the control of the cytomegalovirus immediate-early enhancer and promoter. Expression of ß-galactosidase under regulation by this enhancer is independent of treatment to the cells. Twelve to 16 hr after transfection, the cells were fed with low serum (0.1%) α-minimal essential media to deplete preexisting transcription factors that respond to stimulation by serum, and 24–48 hr later the cells were treated with TCDD or other compounds or with an equivalent amount of dimethylsulfoxide vehicle. We prepared cell extracts 18 hr later by three cycles of freeze–thawing, and expression of CAT and ß-galactosidase activities was determined. We measured CAT activity by the phase extraction method (*38*) using 0.2 μCi of [^{14}C]chloramphenicol (Amersham, Arlington Heights, Illinois) as the substrate. Chloramphenicol conversion to acetylated forms was 1–25%, well within the linear range [0–50% (*38*)] of the assay. We determined ß-galactosidase activity using a kit from Promega BioTech (Madison, Wisconsin). Data were normalized for differences in transfection efficiency by determination of the relative amount of chloramphenicol converted to acetylated forms per unit of ß-galactosidase. Experiments were repeated at least three times, and the values shown are the means ± SEM.

Results

We first tested the effect of aflatoxin B_1, TCDD, and several polycyclic aromatic hydrocarbons on transient CAT expression directed by the wild-type HIV-1 LTR. Mouse hepatoma Hepa-1 cells were transfected with pHIVLTRCAT; 24 hr after transfection, the cells were treated with the compounds indicated in Figure 2. Two of these compounds, aflatoxin B_1 and BaP, caused approximately a fivefold increase in CAT activity over the dimethylsulfoxide control. Four others, anthracene, benzo[*a*]anthracene, TCDD, and 3-methylcholanthrene, were next in potency, causing a two- to threefold increase in CAT activity. The effects of the last two, dimethylbenzanthracene (DMBA) and dibenzo[*a,h*]anthracene (DBA), were not significantly different from the control (Fig. 2). In initial control experiments, we estimated that the effect of BaP on CAT expression was approximately 50% of maximal activation obtained with TPA (data not shown).

The HIV-1 LTR is a regulatory domain approximately 500 bases long that contains several sequence motifs recognized by cellular transcription factors. These recognition motifs include binding sites for AP-1, AP-2, AP-3, AP-4, NFAT-1, USF, URS, NFκB (*39,40*), as well as the TAR sequence, recognized by the viral Tat protein (*37*). The initial rate of proviral transcription is determined by interactions between these transcription factors and their cognate sequences in the LTR (*36,37,39–42*). Since TCDD induces AP-1 activity (*27*), one possible explanation for the stimulation of CAT activity by TCDD was that it resulted from TCDD-induced increases in AP-1. Alternatively, CAT stimulation could be due to activation of transcription factors other than AP-1. To address this question and to analyze which transcription factor binding sites were responsible for CAT induction by TCDD, we prepared a collection of single, double, and triple mutant derivatives of the reporter plasmid and measured CAT expression directed by these mutants in transient expression assays. Mutation of the proximal AP-1 in LS5 had no effect on basal expression levels but caused an increase in TCDD-dependent CAT expression, whereas mutation of the distal NFAT and AP-1 sites in LS1, LS2, and LS3 had a negligible effect on both basal and TCDD-induced CAT expression (Fig. 3). Mutation of the NFκB sites in LS4n or of the cryptic AhRE site in LS4a almost completely abolished basal and TCDD-stimulated CAT expression (Fig. 3). Double mutations caused diverse effects: LS12 abolished TCDD induction and increased basal expression slightly, whereas LS15 and LS35 showed both elevated basal expression levels and absence of TCDD stimulation; LS23 and LS25 had low basal levels and a TCDD stimulation factor of 6- to 7-fold (Fig. 3 and Table 1). The LS123 triple mutant exhibited extremely elevated basal as well as TCDD-stimulated

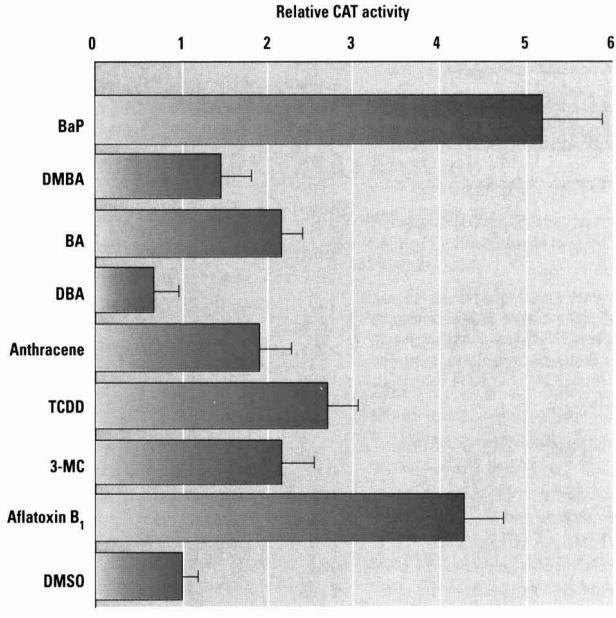


Figure 2. Activation of pHIVLTRCAT expression by various foreign chemicals. Forty-eight hours after transfection, cells were treated for 24 hr with the following compounds: BaP: 10 μM benzo[*a*]pyrene; DMBA: 20 μM 7,12-dimethylbenzo[*a*]anthracene; BA: 20 μM benzo[*a*]anthracene; DBA: 20 μM dibenzo[*a*,*h*]anthracene; anthracene: 20 μM anthracene; TCDD: 15 nM TCDD; 3-MC: 30 μM 3-methylcholanthrene; aflatoxin B_1: 100 μM aflatoxin B_1; DMSO: dimethylsulfoxide vehicle control at a final concentration of 0.1%. Stocks of all compounds were prepared as 1000-fold concentrated solutions in DMSO to ensure that in all cases the DMSO concentration in the cultures did not exceed 0.1%. The extent of chloramphenicol conversion ranged between 3 and 25% and was normalized to ß-galactosidase activity. The values shown are relative to those of the DMSO control.

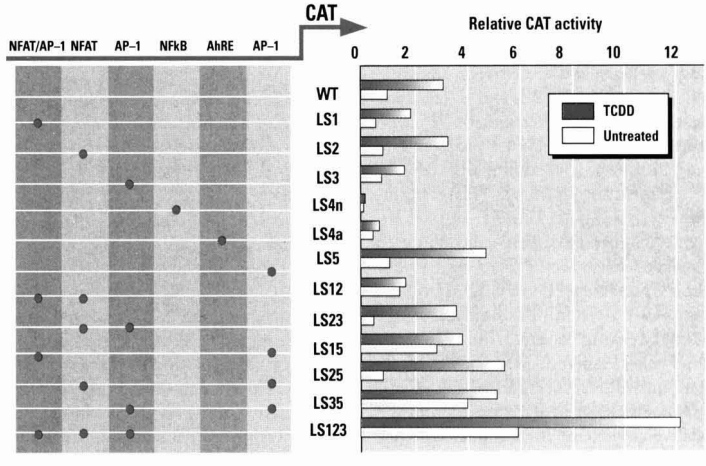


Figure 3. Effect of TCDD on CAT expression directed by linker-scanning mutants. The diagram on the left shows the approximate position of the mutated sites, with the individual mutations denoted by a blue circle (see Figure 1 for the actual coordinates and the text for a complete description of each mutated site). Relative CAT activity values were determined as indicated in Figure 2.

levels, but the extent of induction was not significantly different than in the wild-type (Fig. 3 and Table 1). These results indicate that the NFκB sites mutated in LS4n are required for basal and TCDD-stimulated CAT expression. Furthermore, a previously unrecognized Ah receptor binding site, the canonical GCGTG AhRE site at position 380 mutated in LS4a, was also found to be essential for expression in hepatoma cells. In addition, the region between residues 115 and 255 that contains the NFAT and AP-1 sites appears to dampen the stimulation by TCDD because mutation of these sites resulted in increased levels of CAT activity after TCDD treatment. This region is known to contain negative regulatory elements for HIV-1 expression (*39*,*40*). The proximal AP-1 site mutated in LS5 seems to behave in a similar fashion because its absence increases the effect of TCDD. The values for the fold induction by TCDD for the different mutants tested are shown in Table 1 and are discussed in more detail in the next section.

Table 1. Induction by TCDD of CAT activity directed by HIV long-terminal repeat linker-scanning mutants

Mutant	Fold induction[a]
WT	3.2
LS1	2.8
LS2	3.7
LS3	2.3
LS4n	2.0
LS4a	1.5
LS5	4.3
LS12	1.2
LS23	6.6
LS15	1.3
LS25	6.1
LS35	1.3
LS123	2.1

[a]The values shown are calculated from the data in Figure 3.

The involvement of an AhRE site on CAT expression suggested the possibility that the Ah receptor and a TCDD-inducible cytochrome P450 CYP1A1 enzyme might participate in the stimulation of CAT expression observed after TCDD treatment. We tested this hypothesis by comparing the transient expression of CAT activity directed by the pHIVLTRCAT plasmid in wild-type Hepa-1 cells and in the c37 derivative that lacks CYP1A1 enzymatic activity. If CYP1A1 activity were involved in stimulation of CAT expression by TCDD, this stimulation would not take place in cells lacking the CYP1A1 enzyme. As shown in Figure 4, this expectation was correct; stimulation of CAT activity was found at normal 2- to 2.5-fold levels in Hepa-1 cells, but was absent in the c37 derivative.

These results hinted at the possibility that oxidative stress mediated by TCDD-inducible CYP1A1 activity could be responsible for the effect of TCDD on LTR-directed CAT expression. To determine whether thiol-sensitive reactive oxygen species were involved in this effect, pHIVLTRCAT-transfected cells were grown in the presence of various concentrations of *N*-acetyl-L-cysteine (NAC) or 2-mercaptoethanol prior to treatment with TCDD for 16 hr and determination of CAT activity. We observed a clear decrease of TCDD stimulation of CAT activity

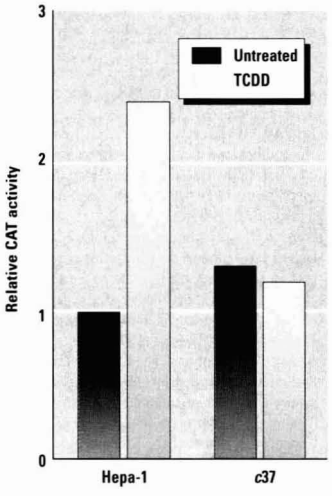


Figure 4. TCDD-dependent CAT expression in wild-type and CYP1A1-deficient cells. Forty-eight hours after transfection, wild-type Hepa-1 cells and their CYP1A1-deficient variant, c37, were treated with TCDD or left untreated. The activities of CAT and ß-galactosidase were measured 24 hr later. The values shown are relative to the ratio of CAT/ß-galactosidase activities in untreated Hepa-1 cells.

with increasing doses of either compound. At a dose of 1 mM NAC or 20 µM 2-mercaptoethanol, the effect of TCDD was completely abolished (Fig. 5), suggesting that, indeed, CYP1A1-dependent oxidative stress might be responsible for the effect of TCDD on LTR-directed CAT expression.

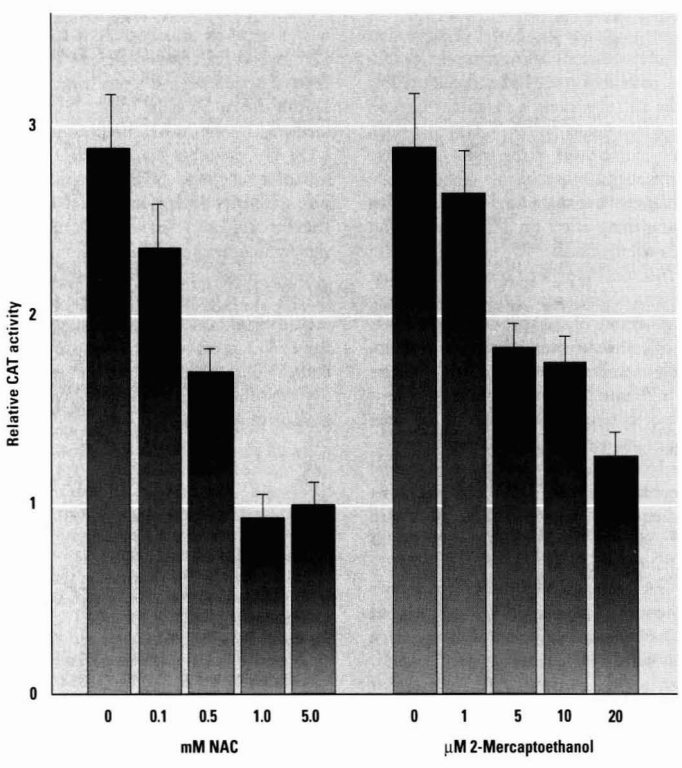


Figure 5. Inhibition of TCDD-dependent CAT activation by *N*-acetyl-L-cysteine (NAC) and 2-mercaptoethanol. Thirty to 60 min before TCDD treatment, NAC or 2-mercaptoethanol was added to the medium at the concentrations indicated and maintained throughout the duration of the TCDD treatment. The values shown are relative to the CAT activity in transfected cells not treated with TCDD.

Discussion

The results that we present in this article indicate that TCDD, aflatoxin B_1, and several polycyclic aromatic hydrocarbons (PAHs) can significantly activate the expression of genes linked to the LTR sequences of HIV-1. The magnitude of the activation appears to be different for the various compounds tested. In the case of TCDD, stimulated values are significantly higher than control values, although they do not usually exceed them by more than 2.5- to 3-fold. This stimulation is in agreement with observations by others that TCDD can cause an increase of infectious HIV-1 titers in experimental systems (*43,44*). As for PAHs, the highest levels of CAT activation that we observed were a result of BaP treatment, a finding that may provide a possible molecular explanation for the observation that cigarette smoking accelerates the progression of AIDS (*45–48*). It is, of course, likely that the effect of cigarette smoke on AIDS progression results from a combination of many different causes, of which gene activation by BaP is only one. Surprisingly, DBA had no effect on CAT expression, a finding that we cannot explain at present.

Using linker-scanning mutational analysis, we have identified several domains of the HIV-1 LTR responsible for basal as well as TCDD-stimulated CAT expression. We find that expression directed by the HIV-1 LTR is high in mouse hepatoma cells, in agreement with previous observations in human hepatoma cell lines (*49–51*), this suggests that the liver may be a primary virus reservoir. Mutation of the NFκB binding sites eliminates CAT expression, confirming the absolute requirement for NFκB. NFκB, however, is not the only transcription factor necessary for expression; we have uncovered an Ah receptor response element containing the canonical AhRE sequence GCGTG, which is also essential for basal expression. In addition, this site participates in the effect of TCDD on CAT activation because its mutation in pLS4a reduces drastically the fold induction by TCDD (Table 1). This AhRE site is embedded within the first of three adjacent Sp1 sites, which have been shown to interact cooperatively with NFκB in HIV enhancer activation (*52*). It could be argued that Sp1, and not the Ah receptor, was the transcription factor responsible for the loss of activity of pLS4a because both binding sites would be equally affected by the mutation. This possibility is unlikely because mutations in just one of the three Sp1 binding sites have little or no effect on HIV enhancer expression (*40*); this suggests that the Ah receptor, and not Sp1, is the relevant transcription factor whose binding and subsequent activity are affected by the LS4a mutation. As shown by the mutagenesis analysis, both NFκB and Ah receptor binding sites are responsible for the basal expression levels, and it is possible that both transcription factors function in synergy.

The NFAT and AP-1 binding sites in mutants LS1, LS2, and LS3, clustered in the negative regulatory region of the LTR, do not show a major effect on expression when altered individually. In double mutants that include LS1, as well as in the triple mutant LS123, basal level of expression is elevated, suggesting that the NFAT/AP-1 site at 146–165 is responsible for downregulating the basal level of expression. This is in agreement with earlier findings that this region of HIV-1 contains negative regulatory elements (*39,40*). As shown in Table 1, these double mutants and the triple mutant LS123 also show a low level of induction by

TCDD, suggesting that the same sites that downregulate the basal level of expression may respond to TCDD activation. In contrast, mutations at the LS2 site, and possibly at the LS5 site, cause a significant increase in the fold induction by TCDD that reach levels double those of the wild-type (Table 1); this suggests that the role of the LS2 site is antagonistic to that of the LS1 site, having a dampening effect on TCDD induction when not modified.

Interpretation of these results must take into account recent findings regarding the properties of transcription factor AP-1: 1) different combinations of *fos* and *jun* family members have very different effects on the same promoter (*53*); 2) different promoters respond differently to the same combination of *fos* and *jun* family members (*53*); and 3) *fos* and *jun* are integral components of the NFAT complex (*54*). Consequently, sites LS1, LS2, LS3, and LS5 may have antagonistic roles due to conflicting effects of free AP-1 and of AP-1 in NFAT complexes on different promoter sequences. The overall transcriptional effect of this combination of antagonistic sites would be rather unpredictable. Within this context, the outcome of TCDD exposure is likely to result from a combination of two opposing effects; on one hand, activation of expression may take place by means of the Ah receptor, the AhRE site, and a particular set of *fos/jun* members with positive effects on LTR expression. On the other hand, dampening of this induction of expression may occur by activation of other *fos/jun* members with negative regulatory functions.

TCDD toxicity has been proposed to result from epoxides and other derivatives of arachidonic acid metabolism catalyzed by TCDD-induced cytochrome P450 enzymes (*23,55,56*). In agreement with this hypothesis, we find that stimulation of CAT expression by TCDD is absent in the variant cell line c37 that lacks cytochrome P450 CYP1A1 activity, strongly suggesting that the effect of TCDD is mediated by the monooxygenase activity of CYP1A1. This activity might generate arachidonate metabolites responsible for the elevation of the pro-oxidant status of the cell, and indeed several cytochromes P450, including the TCDD-inducible CYP1A1 and CYP1A2 enzymes (*57–59*) and others (*60,61*), possess arachidonic acid epoxygenase activity. Our experiments, although not directly aimed at the identification of possible mediators, show that NAC and 2-mercaptoethanol eliminate the effect of TCDD, indicating that, as shown for NFκB activation (*62–64*), oxidative stress caused by thiol-sensitive reactive oxygen species is likely to be involved in the TCDD-dependent activation events.

In conclusion, our data are consistent with a signal transduction mechanism that includes at least two different TCDD-dependent pathways. On one hand, activation of the Ah receptor triggers expression mediated by the AhRE site present in the LTR. On the other hand, TCDD induction of a functional CYP1A1 monooxygenase stimulates generation of thiol-sensitive reactive oxygen species, which in turn activate transcription factors operative in LTR-directed expression. We find that, in addition to TCDD, several other toxic environmental chemicals can activate expression of the HIV-1 promoter-enhancer sequences, underscoring the importance that exposure to these compounds might have in the progression of AIDS.

References

1. Nebert DW, Gonzalez FJ. P450 genes. Structure, evolution and regulation. Annu Rev Biochem 56:945–993 (1987).
2. Whitlock JP Jr. The regulation of cytochrome P-450 gene expression. Pharmacol Rev 39:147–161 (1987).
3. Nebert DW. The *Ah* locus: genetic differences in toxicity, cancer, mutation, and birth defects. Crit Rev Toxicol 20:153–174 (1989).
4. Landers JP, Bunce NJ. The *Ah* receptor and the mechanism of dioxin toxicity. Biochem J 276:273–287 (1991).
5. Whitlock JP Jr. Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. Annu Rev Pharmacol Toxicol 30:251–277 (1991).
6. Swanson HI, Bradfield CA. The AH-receptor: genetics, structure and function. Pharmacogenetics 3:213–230 (1993).
7. Suskind RR. Chloracne, the hallmark of dioxin intoxication. Scand J Work Environ Health 11:165–171 (1985).
8. Zugerman C. Chloracne. Clinical manifestations and etiology. Dermatol Clin 8:209–213 (1990).
9. Fingerhut MA, Halperin WE, Marlow DA, Piacitelli LA, Honchar PA, Sweeney MH, Greife AL, Dill PA, Steenland K, Suruda AJ. Cancer mortality in workers exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. N Engl J Med 324:212–218 (1991).
10. Manz A, Berger J, Dwyer JH, Flesch-Janys D, Nagel S, Waltsgott H. Cancer mortality among workers in chemical plant contaminated with dioxin. Lancet 338:959–964 (1991).
11. Flodström S, Busk L, Kronevi T, Ahlborg UG. Modulation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and phenobarbital-induced promotion of hepatocarcinogenesis in rats by the type of diet and vitamin A deficiency. Fundam Appl Toxicol 16:375–391 (1991).
12. Kociba RJ, Keyes DG, Beyer JE, Carreon RM, Wade CE, Dittenber DA, Kalnins RP, Frauson LE, Park CN, Barnard SD, Hummel RA, Humiston CG. Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats. Toxicol Appl Pharmacol 46:279–303 (1978).
13. Schüller HM. The signal transduction model of carcinogenesis. Biochem Pharmacol 42:1511–1523 (1991).
14. Knutson JC, Poland A. Response of murine epidermis to 2,3,7,8,-tetrachlorodibenzo-p-dioxin: interaction of the *Ah* and *hr* loci. Cell 30:225–234 (1982).
15. Poland A, Palen D, Glover E. Tumour promotion by TCDD in skin of HRS/J hairless mice. Nature 300:271–273 (1982).
16. Poland A, Knutson JC. 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanisms of toxicity. Annu Rev Pharmacol Toxicol 22:517–554 (1982).
17. Abbott BD, Birnbaum LS. TCDD-induced altered expression of growth factors may have a role in producing cleft palate and enhancing the incidence of clefts after coadministration of retinoic acid and TCDD. Toxicol Appl Pharmacol 106:418–432 (1990).
18. Abbott BD, Birnbaum LS. Cellular alterations and enhanced induction of cleft palate after coadministration of retinoic acid and TCDD. Toxicol Appl Pharmacol 99:287–301 (1989).
19. Couture LA, Abbott BD, Birnbaum LS. A critical review of the developmental toxicity and teratogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: recent advances toward understanding the mechanism. Teratology 42:619–627 (1990).
20. Abbott BD, Harris MW, Birnbaum LS. Comparisons of the effects of TCDD and hydrocortisone on growth factor expression provide insight into their interaction in the embryonic mouse palate. Teratology 45:35–53 (1992).
21. van der Meulen JC, Vaandrager JM. Facial clefts. World J Surg 13:373–383 (1989).
22. Canga L, Levi R, Rifkind AB. Heart as a target organ in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity: decreased ß-adrenergic responsiveness and evidence of increased intracellular calcium. Proc Natl Acad Sci USA 85:905–909 (1988).
23. Canga L, Paroli L, Blanck TJ, Silver RB, Rifkind AB. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin increases cardiac myocyte intracellular calcium and progressively impairs ventricular contractile responses to isoproterenol and to calcium in chick embryo hearts. Mol Pharmacol 44:1142–1151 (1993).
24. McConkey DJ, Hartzell P, Duddy SK, Hakansson H, Orrenius S. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin kills immature thymocytes by $Ca2^+$-mediated endonuclease activation. Science 242:256–259 (1988).
25. McConkey DJ, Orrenius S. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin kills glucocorticoid-sensitive thymocytes *in vivo*. Biochem Biophys Res Comm 160:1003–1008 (1989).
26. Greenlee WF, Neal RA, The *Ah* receptor: a biochemical and biological perspective. In: The receptors, vol 2. (Conn PM, ed). New York:Academic Press, 1985;89–129.
27. Puga A, Nebert DW, Carrier F. Dioxin induces expression of c-*fos* and c-*jun* proto-oncogenes and a large increase in transcription factor AP-1. DNA Cell Biol 11:269–281 (1992).
28. Bernard HP, Darlington GJ, Ruddle FH. Expression of liver phenotypes in cultured mouse hepatoma cells: synthesis and secretion of serum albumin. Dev Biol 35:83–96 (1973).
29. Hankinson O. Dominant and recessive aryl hydrocarbon hydroxylase-deficient mutants of mouse hepatoma line Hepa-1 and assignment of recessive mutants to three complementation groups. Somat Cell Genet 9:497–514 (1983).
30. Hankinson O, Andersen RD, Birren B, Sander F, Negishi M, Nebert DW. Mutations affecting the regulation of transcription of the

cytochrome P_1-450 gene in the mouse Hepa-1 cell line. J Biol Chem 260:1790–1795 (1985).
31. Kimura S, Smith HH, Hankinson O, Nebert DW. Analysis of two benzo[*a*]pyrene-resistant mutants of the mouse hepatoma Hepa-1 P_1-450 via cDNA expression in yeast. EMBO J 6:1929–1933 (1987).
32. Gendelman HE, Phelps W, Feigenbaum L, Ostrove JM, Adachi A, Howley PM, Khoury G, Ginsberg H, Martin MA. Trans-activation of the human immunodeficiency virus long terminal repeat sequence by DNA viruses. Proc Natl Acad Sci USA 83:9759–9763 (1986).
33. McKnight SL, Kingsbury R. Transcriptional control signals of a eukaryotic protein-coding gene. Science 217:316–324 (1982).
34. Graham FL, Van der Erb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456–467 (1973).
35. Puga A, RayChaudhuri B, Salata K, Zhang Y-H, Nebert DW. Stable expression of mouse *Cyp1A-1* and human *CYP1A-2* cDNAs transfected into mouse hepatoma cells lacking detectable P450 enzyme activity. DNA Cell Biol 9 425–436 (1990).
36. Southgate C, Green MR. The HIV-1 Tat protein activates transcription from an upstream DNA-binding site: implications for Tat function. Genes Dev 5:2496–2507 (1991).
37. Jones KA, Peterlin MB. Control of RNA initiation and elongation at the HIV-1 promoter. Annu Rev Biochem 63:717–743 (1994).
38. Seed B, Sheen J-Y. A simple phase extraction assay for chloramphenicol acetyltransferase activity. Gene 67:271–277 (1988).
39. Lu YC, Touzjian N, Stenzel M, Dorfman T, Sodroski JG, Haseltine WA. Identification of cis-acting repressive sequences within the negative regulatory element of human immunodeficiency virus type 1. J Virol 64:5226–5229 (1990).
40. Gaynor R. Cellular transcription factors involved in the regulation of HIV-1 gene expression. AIDS 6:347–363 (1992).
41. Lu YC, Touzjian N, Stenzel M, Dorfman T, Sodroski JG, Haseltine WA. The NF kappa B independent cis-acting sequences in HIV-1 LTR responsive to T-cell activation. J Acquir Immune Defic Syndr 4:173–177 (1991).
42. Tong-Starksen SE, Welsh TM, Peterlin BM. Differences in transcriptional enhancers of HIV-1 and HIV-2. Response to T cell activation signals. J Immunol 145:4348–4354 (1990).
43. Tsyrlov IB, Pokrovsky A. Stimulatory effect of the CYP1A1 inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the reproduction of HIV-1 in human lymphoid cell culture. Xenobiotica 23:457–467 (1993).
44. Pokrovsky AG, Cherykh AI, Yastrebova ON, Tsyrlov IB. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin as a possible activator of HIV infection. Biochem Biophys Res Commun 179:46–51 (1991).
45. Boulos R, Halsey NA, Holt E, Ruff A, Brutus JR, Quinn TC, Adrien M, Boulos C. HIV-1 in Haitian women 1982–1988. The Cite Soleil/JHU AIDS project team. J Acquir Immune Defic Syndr 3:721–728 (1990).
46. Nieman RB, Fleming J, Coker RJ, Harris JR, Mitchell DM. The effect of cigarette smoking on the development of AIDS in HIV-1-seropositive individuals. AIDS 7:705–710 (1993).
47. Clarke JR, Taylor IK, Fleming J, Nukuna A, Williamson JD, Mitchell DM. The epidemiology of HIV-1 infection of the lung in AIDS patients. AIDS 7:555–560 (1993).
48. Burns DN, Kramer A, Yellin F, Fuchs D, Wachter H, DiGioia RA, Sanchez WC, Grossman RJ, Gordin FM, Biggar RJ. Cigarette smoking: a modifier of human immunodeficiency virus type 1 infection? J Acquired Immune Defic Syndr 4:76–83 (1991).
49. Chang KS, Hsu ML, Josephs SF. Regulation of HIV-1 LTR trans-activating activities in two different human hepatocellular carcinoma cell lines. Cancer Lett 74:75–83 (1993).
50. Pizzella T, Banerjee R. Identification of a human immunodeficiency virus type 1 TAR binding protein in human hepatoblastoma HepG2 cells that trans-activates HIV-1 LTR-directed gene expression. DNA Cell Biol 13:67–74 (1994).
51. Wang Z, Goldberg MA, Scadden DT. HIV-1 suppresses erythropoietin production in vitro. Exp Hematol 21:683–688 (1993).
52. Perkins ND, Edwards NL, Duckett CS, Agranoff AB, Schmid RM, Nabel GJ. A cooperative interaction between NFκB and Sp1 is required for HIV-1 enhancer activation. EMBO J 12:3551–3558 (1993).
53. Hsu JC, Bravo R, Taub, R. Interactions among LRF-1, JunB, c-Jun, and c-Fos define a regulatory program in the G_1 phase of liver regeneration. Mol Cell Biol 12:4654–4665 (1992).
54. Northrop JP, Ullman KS, Crabtree GR. Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells (NF-AT) complex. J Biol Chem 268:2917–2923 (1993).
55. Lentnek M, Griffith OW, Rifkind AB. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin increases reliance on fats as a fuel source independently of diet: evidence that diminished carbohydrate supply contributes to dioxin lethality. Biochem Biophys Res Commun 174:1267–1271 (1991).
56. Rifkind AB, Gannon M, Gross SS. Arachidonic acid metabolism by dioxin-induced cytochrome P-450: a new hypothesis on the role of P-450 in dioxin toxicity. Biochem Biophys Res Comm 172:1180–1188 (1990).
57. Capdevila J, Falck JR, Estabrook RW. Cytochrome P450 and the arachidonate cascade. FASEB J 6:731–736 (1992).
58. Capdevila J, Karara A, Waxman DJ, Martin MV, Falck JR, Guengerich FP. Cytochrome P-450 enzyme-specific control of the regio and enantiofacial selectivity of the microsomal arachidonic acid epoxygenase. J Biol Chem 265:10865–10871 (1990).
59. Capdevila J, Gil L, Orellana M, Marnett LJ, Mason JI, Yadagiri P, Falck JR. Inhibitors of cytochrome P-450-dependent arachidonic acid metabolism. Arch Biochem Biophys 261:257–263 (1988).
60. Kanetoshi A, Ward AM, May BK, Rifkind AB. Immunochemical identity of the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and ß-naphthoflavone-induced cytochrome P-450 arachidonic acid epoxygenases in chick embryo liver: distinction from the Ω-hydroxylase and the phenobarbital-induced epoxygenase. Mol Pharmacol 42:1020–1026 (1992).
61. Nakai K, Ward AM, Gannon M, Rifkind AB. ß-Naphthoflavone induction of a cytochrome P-450 arachidonic acid epoxygenase in chick embryo liver distinct from the aryl hydrocarbon hydroxylase and from phenobarbital-induced arachidonate epoxygenase. J Biol Chem 267:19503–19512 (1992).
62. Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. EMBO J 10:2247–2258 (1991).
63. Staal FJT, Roederer M, Herzenberg LA. Intracellular thiols regulate activation of nuclear factor kB and transcription of human immunodeficiency virus. Proc Natl Acad Sci USA 87:9943–9947 (1990).
64. Roederer M, Staal FJT, Raju PA, Ela SW, Herzenberg LA. Cytokine-stimulated human immunodeficiency virus replication is inhibited by N-acetyl-L-cysteine. Proc Natl Acad Sci USA 87:4884–4888 (1990).

Effect of Nitrous Acid on Lung Function in Asthmatics: A Chamber Study

William S. Beckett, Mark B. Russi, Alan D. Haber, Robin M. Rivkin, James R. Sullivan, Zeynep Tameroglu, Vahid Mohsenin, and Brian P. Leaderer

John B. Pierce Laboratory and Environmental Health Sciences Division, Yale University School of Medicine, New Haven, CT 06510 USA

Nitrous acid, a component of photochemical smog and a common indoor air pollutant, may reach levels of 100 ppb where gas stoves and unvented portable kerosene heaters are used. Nitrous acid is a primary product of combustion and may also be a secondary product by reaction of nitrogen dioxide with water. Because the usual assays for nitrogen dioxide measure several oxides of nitrogen (including nitrous acid) together, previous studies of indoor nitrogen dioxide may have included exposure to and health effects of nitrous acid. To assess the respiratory effects of nitrous acid exposure alone, we carried out a double-blinded crossover chamber exposure study with 11 mildly asthmatic adult subjects. Each underwent 3-hr exposures to 650 ppb nitrous acid and to filtered room air with three 20-min periods of moderate cycle exercise. Symptoms, respiratory parameters during exercise, and spirometry after exercise were measured. A statistically significant decrease in forced vital capacity was seen on days when subjects were exposed to nitrous acid. This effect was most marked at 25 min and 85 min after exposure began. Aggregate respiratory and mucous membrane symptoms were also significantly higher with nitrous acid. We conclude that this concentration and duration of exposure to nitrous acid alters lung mechanics slightly, does not induce significant airflow obstruction, and produces mild irritant symptoms in asthmatics. *Key words*: asthma, indoor air pollution, nitrogen dioxide, nitrous acid. *Environ Health Perspect* 103:372–375 (1995)

Nitrous acid (HNO_2) is the major gas-phase acid in environmental tobacco smoke (*1*) and in its vapor phase is found in automobile emissions. Although outdoor ambient concentrations are less than those of sulfuric acid (H_2SO_4) and nitric acid (HNO_3), up to 8 ppb HNO_2 has been measured in ambient air in California during an air pollution episode (*2*). In homes with combustion sources, elevated HNO_2 levels may be associated with direct emissions from the source as well as with reactions of emitted NO_2 with water vapor in air. Indoor concentrations of HNO_2 are higher than outdoor concentrations, even when indoor concentrations of NO_2 do not exceed outdoor levels. Peak levels of HNO_2 may exceed 50 ppb and persist for several hours (*3,4*). Nitrous acid may also be a secondary reaction product of NO_2 with water on indoor surfaces and, under experimental conditions, has been found to make up as much as 10% of oxides of nitrogen after an interval of reaction (*5*). Conventional assays of NO_2 measure several oxides of nitrogen together, including HNO_2. For this reason, previous studies of respiratory effects of indoor NO_2 may have included exposures to HNO_2 without independent measurement of exposure and effect (*6*).

Based on *in vitro* studies, it has been postulated that at environmental concentrations HNO_2 is formed within the respiratory system predominantly by hydrogen abstraction (*7*), with subsequent conversion of HNO_2, at physiologic pH, to H^+, and NO_2^- (*7*). It has been proposed that HNO_2 formed in this way may contribute to the bronchoconstricting effects of NO_2 seen in normal subjects and asthmatics. Studies of the direct effects of HNO_2 on the human respiratory system are thus of interest because exposures may occur from primary indoor and outdoor sources or from reaction products of NO_2 formed within the human respiratory system. A need for more information on the health effects of HNO_2 has recently been identified (*8*).

We performed a chamber exposure study to determine whether there is an effect on respiratory symptoms or lung mechanics in a group of patients (mild asthmatics) who have been demonstrated in some but not all studies to be sensitive to other acid species (*9–12*). We used a concentration of HNO_2 higher than that usually measured in homes with unvented combustion sources (*4*), but the duration of exposure was shorter than may occur in such homes.

Methods

Subjects. The protocol was approved by the Yale University School of Medicine Human Investigations Committee, and all subjects gave informed consent to participate. The 11 subjects were recruited by advertisements and were selected using the following inclusion criteria: age between 18 and 40 years, nonsmoking, and in good general health other than mild asthma (as defined by a physician's diagnosis with typical symptoms and occasional but not regular use of bronchodilator medications). In addition to these criteria, all subjects had baseline forced expiratory volume in 1 sec (FEV_1) and forced vital capacity (FVC) within the normal range for age, sex, and height (*13*) and had methacholine reactivity within the asthmatic range [a provocative concentration (PC) of methacholine less than 8 mg/mL causing a 20% fall in FEV_1 excepting subject 2, whose PC_{20} was 26 mg/mL]. Exclusion criteria included regular or current use of bronchodilator medications, current active asthma symptoms, presence of wheezing on physical exam, or inability to comfortably perform moderate cycle exercise for 20 min. Subjects did not need or use asthma medications during the days before or during the chamber studies. Once accepted into the study, subjects had a training session with spirometry and a cycle ergometer exercise session during which a workload tolerable for 20 min was determined.

Protocol. Each subject underwent two 3-hr intermittent exercise chamber exposures which differed only in that one was conducted with continuous HNO_2 exposure at a target level of 700 ppm, while the other was conducted with filtered, clean air. Air temperature was maintained at 18°C during exposure to provide a comfortable ambient environment for sustained moderate exercise. The 3-hr chamber exercise periods were performed in a balanced, randomized double-blinded crossover design, so that six subjects were exposed to HNO_2 on their first test day and five to clean air. Exposures were separated by a 1- to 2-week washout period. Subjects and investigators (who entered the chamber with subjects to perform measurements of exercise responses and resting lung mechanics) were blinded as to whether exposure was to HNO_2 or to clean air. The effectiveness of the blinding procedure was assessed by asking subjects and investigators to indicate, at the end of each exposure session, whether they believed exposure had been to HNO_2 or clean air.

During each 3-hr exposure, subjects completed a baseline symptom questionnaire and spirometry immediately on entering the chamber, and then 20 min of cycle ergometer exercise at their predetermined constant workload at the start of each hour. Heart rate (HR) (Polar Electro Inc., Heartland, Wisconsin), minute ventilation (V_E), and tidal volume (V_T) (5410 volume meter, Ohmeda, Englewood

Address correspondence to W. S. Beckett, Occupational/Environmental Medicine Program, Yale University School of Medicine, 3rd Floor, 135 College Street, New Haven, CT 06510 USA

This work was supported in part by NIEHS grant ES05401 and the National Heart, Lung, and Blood Institute, Division of Lung Diseases Preventive Pulmonary Academic Award.

Received 28 June 1994; accepted 12 December 1994.

Colorado, calibrated with a Tissot spirometer) were measured at 5, 10, and 15 min of each exercise period. Immediately after exercise, subjects completed the symptom questionnaire, and then performed spirometry within the chamber 5 min later (Eagle II Stead Wells survey spirometer, W.E. Collins Inc., Braintree, Massachusetts). American Thoracic Society criteria for standardization of spirometry were applied (*14*). Subjects then rested in a seated position within the chamber until the next exercise session. A final spirometry and questionnaire were completed at the end of 180 min, just before leaving the chamber.

Symptoms. Subjects completed the same symptom questionnaire five times over each exposure session. They rated each symptom by placing a mark on a 10-cm continuous line representing a score of "absent" through "the most severe ever experienced." Four respiratory symptoms (shortness of breath, wheeze, cough, chest tightness), six sensory irritant questions (skin irritation, eye irritation, eye tearing, throat irritation, nasal stuffiness, nasal dryness), and one negative control question (headache) were included.

Generation of HNO_2. Nitrous acid was generated by a reaction of sodium nitrite with sulfuric acid using the method of Taira and Kanda (*15*). A solution of 0.08 M sulfuric acid and a solution of 0.06 M sodium nitrite were prepared with distilled deionized water. A peristaltic pump added each of the solutions at 2 ml/min onto a circular piece of fritted glass, which was located near the base of the reaction chamber. Ambient air was filtered through a system of Purafil (potassium permanganate-coated aluminum) and activated charcoal and passed into the reaction chamber below the glass frit at 20 L/min. The cleaned air passed through the glass frit and bubbled through the reagent mixture, removing HNO_2 from the solution. The mixture was passed through a condensing chamber to remove excess water vapor. This HNO_2-containing gas was then fed into the exposure chamber. Excess reagent was removed from the reaction chamber by three tubes located 8 mm above the reagent inlet, and connected to a vacuum flask and a vacuum pump.

Measurement of HNO_2. We monitored the nitrous acid concentration in the chamber air by a continuous method using a chemiluminescent NO_x analyzer with a system of filters and a valve. Two filter packs were set up in parallel to the NO_x analyzer inlet, with a valve switching from one filter pack to the other every 2 min. One filter pack contained a glass-fiber filter coated with sodium carbonate and glycerol, and the other contained an uncoated filter. The coated filter removed the HNO_2 from the air passing through it, while allowing the NO and NO_2 to pass through. The uncoated filter did not remove any of these gases. The NO_x analyzer measured HNO_2 as the difference in signal with and without the coated filter.

We also used a noncontinuous method of measuring HNO_2 employing the Harvard EPA Annular Denuder System (*16*) with two denuders in series sampling at 4 L/min. In this system the sample air passed through the space between concentric glass tubes (the annular denuder), which was coated with sodium carbonate and glycerol. Nitrous acid diffused to the coated walls and was trapped, while nonacidic gases passed through uncollected. The denuders were extracted with ultra-pure water after the sampling period. The concentration of nitrite ion in the denuder extract was measured by ion chromatography to allow determination of the total amount of nitrous acid that passed through the denuder system. The chamber HNO_2 concentration was then calculated from this value. The chamber size was 18 m^3 (624 ft^3), and the ventilation rate averaged approximately 30 ft^3/min, or three air changes per hour.

Statistical analysis. To ensure comparable baseline pulmonary function at the start of each session, we compared the two pre-exercise spirometry readings using a paired *t*-test. The effects of HNO_2 exposure, time, and their interactive influence upon exercise and spirometry measurements were evaluated with repeated measures analysis of variance. We analyzed spirometry results with actual values and expressed them as percent predicted (*13*). Symptom scores for each symptom of the four questionnaires completed 20, 80, 140, and 180 min after entering the chamber were combined as a mean score, and results from the exposure day compared with clean air by the Wilcoxon signed-rank sum test (*17*). For all analyses, a two-sided significance level was chosen at $p < 0.05$. Due to equipment failure, minute ventilation, tidal volume, and respiratory rate were not obtained for one subject at a single time point. For purposes of statistical analysis, this subject's data were deleted at all time points. Data were analyzed using Systat statistical software (Systat Inc., Evanston, Illinois).

Results

All 11 subjects selected for inclusion in the study completed the protocol. Subject characteristics are listed in Table 1, along with the ergometer workload and minute ventilation established during the practice session. The mean HNO_2 concentration and standard deviation within the chamber on exposure days was 648 ± 41 ppb. (Measurements of HNO_2 were not made in the homes of subjects).

Subjects were successfully blinded as to exposure conditions, in that they correctly identified the chamber exposure conditions of only 27% of the sessions. The investigators were also successfully blinded, identifying exposure correctly in 60% of the sessions (which was not significantly different from an expected 50%; 95% CI, 39–81%).

Results of serial spirometry during HNO_2 control and exposure days are shown in Figure 1. Baseline values were not different at the beginning of control and exposure days, but there was a statistically significant decrease in FVC during HNO_2 exposure which was most marked at 25 min after the beginning of exposure and persisted throughout the 3-hr exposure ($p = 0.017$ when vital capacity expressed as absolute value; $p = 0.020$ when vital capacity expressed as percent predicted). The

Table 1. Characteristics of mildly asthmatic subjects and workload set during exercise

Subject no.	Age (years)	Sex	Height	FEV_1 (%)	FVC (%)[a]	PC_{20}[b]	Work (kpm/min)	Minute ventilation (L/min)
1	28	F	160	3.10 (104)	3.60 (97)	0.6	360	35
2	24	F	169	3.79 (110)	4.64 (108)	26	180	23
3	23	M	173	3.71 (87)	4.53 (86)	4	540	34
4	38	F	171	3.35 (109)	4.25 (108)	8	270	32
5	26	F	156	2.79 (98)	3.09 (87)	2	360	31
6	28	M	172	3.34 (81)	4.73 (92)	1	450	48
7	27	F	157	2.94 (101)	3.59 (99)	2	450	33
8	35	M	164	3.92 (109)	4.65 (103)	2	630	39
9	39	F	164	2.91 (101)	3.16 (86)	0.2	540	25
10	30	F	157	2.60 (92)	3.26 (92)	0.7	360	36
11	18	M	167	3.75 (88)	4.00 (79)	3	540	30
Mean	29		165	3.29 (98)	3.95 (94)	4.5	425	33
SD	7		6	0.45 (9)	0.64 (10)	7.5	134	7
SE	2		2	0.14 (3)	0.19 (3)	2.25	40	10

Abbreviations: FEV_1, forced expiratory volume in 1 sec; FVC, forced vital capacity; kpm, kilopond-meters.
[a]Predicted normal values from Morris et al. (*13*).
[b]PC_{20} FEV_1 methacholine: the provocative concentration of inhaled methacholine chloride, causing a 20% decline from the baseline FEV_1.

difference between control and exposure FVC was small: at 25 min the mean difference was 108 mL, representing a mean difference of approximately 3% between exposure and control conditions. Mean values of FEV_1 were also not different at the beginning of control or exposure sessions, and no effect of HNO_2 exposure was seen on expiratory airflow at the beginning of expiration (FEV_1) or during midflow (MMEF, maximal mid-expiratory flow rate). Although the controlled design of this study permitted evaluation of exercise-induced bronchospasm independently of any effect of HNO_2 exposure, little exercise-induced bronchospasm was seen in this group of mild asthmatics. The maximum mean FEV_1 decline from pre-exercise baseline was 32 mL on the control days and 39 mL on the exposure days, representing an approximately 1% post-exercise decline. Because spirometry was performed 5 min after exercise, delayed exercise-induced bronchospasm, which may be maximal more than 5 min after the end of exercise, may not have been detected.

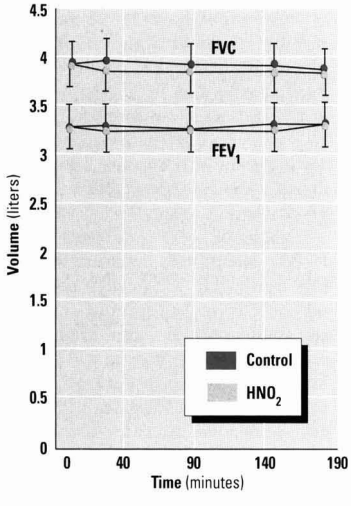


Figure 1. Mean forced vital capacity (FVC) and forced expiratory volume in the first second of expiration (FEV_1) for 11 subjects just before the start of the chamber sessions and at 25, 85, 145, and 180 min of exposure (bars represent SE of the mean). Vital capacity on HNO_2 exposure days was significantly lower than on control days.

Serial measures of heart rate, minute ventilation, tidal volume, and breathing frequency on control and exposure days are shown in Table 2. Comparison showed no statistically significant effects of HNO_2 exposure on these responses to exercise. The comparable values of HR and V_E under the two exposure conditions indicate comparable exercise workloads and cardiovascular and ventilatory responses on control and exposure days.

Mean symptom scores reported during exposure were low under both control and exposure conditions, ranging from 0.0 to 1.0 on a 10-point scale (Table 3). As reflected in the successful blinding, the subjects were uncertain as to whether exposure sessions were to HNO_2 or clean air. However, the aggregate score of all 10 test symptoms was higher on the HNO_2 than control days. This difference was small but statistically significant ($p = 0.038$). The difference between the mean score on exposure and control days for the negative control symptom (headache) was lower than for seven other symptoms and identical to that for wheeze, cough, and nasal stuffiness.

Discussion

Nitrous acid at 650 ppb over a 3-hr exposure period is a weak sensory irritant, as demonstrated by the failure of subjects to distinguish exposure from control days. Nonetheless, a physiologic effect of exposure was detectable with this concentration and duration, as seen in the alteration of static lung mechanics in these mildly asthmatic subjects. The small, statistically significant effect on FVC in the absence of an effect on FEV_1 or MMEF suggests that the exposure–duration combination used in this study is above, but not far, from the threshold for effects on lung mechanics. The primary response at this dose, a reduction in vital capacity, may be due either to inhibition of maximal inspiratory effort, a reduction in respiratory system compliance, or closure of airways at higher lung volumes. However, because of the solubility of HNO_2 in airway mucosal water and the relatively low concentration tested, it is likely that most of the vapor is absorbed in the respiratory mucosa before reaching terminal bronchioles and alveoli. For this reason, we speculate that the mechanism for this effect is inhibition of maximal inspiration due to effects on sensory afferent nerves. This mechanism has been demonstrated for ozone, a potent respiratory irritant, at 500 ppb (*18*). However, because ozone is an aqueous, insoluble gas which is poorly absorbed in the upper airways and HNO_2 would be expected to be well absorbed in the upper airways, these data raise the possibility that the effect seen was due to stimulation of upper airway receptors, having the effect of inhibiting maximal inspiration.

Asthmatic subjects were chosen for this study as a potentially more sensitive clinical group. Because only asthmatics were studied, we do not know whether nonasthmatic subjects are less susceptible, or more severe asthmatics are more susceptible, to this concentration and duration of exposure.

Nitrous acid is of interest as an environmental exposure due to its presence in emissions from automobiles, natural gas and kerosene-burning appliances, and environmental tobacco smoke. It may also be a reaction product of inhaled NO or NO_2 within the respiratory system. Still, little information is available on respiratory system effects of nitrous acid alone. An *in vitro* study has demonstrated that HNO_2 is capable of functionally inactivating human plasma α-1 proteinase inhibitor in a 0.05 M sodium acetate buffer solution when incubated for 15 min at 25°C, pH 4.0. (*19*). Two studies have suggested, on the basis of *in vitro* simulations and studies in isolated perfused rat lungs, that inhaled NO_2 undergoes nonsaturable uptake or transformation in the lung, forming low molecular weight soluble reaction products, the predominant one being HNO_2. Using cyclo-hexane to simulate lung lipid with *in*

Table 2. Exercise parameters (means ± SEM) during control and HNO_2 exposure days[a]

		Time (min)								
		5	10	15	65	70	75	125	130	135
HR	Control	126 ± 4	130 ± 5	135 ± 5	125 ± 5	126 ± 4	132 ± 5	128 ± 5	133 ± 5	135 ± 6
	HNO_2	131 ± 4	133 ± 5	135 ± 4	127 ± 4	132 ± 4	136 ± 4	132 ± 5	134 ± 3	136 ± 4
V_E (L/min)	Control	33 ± 2	34 ± 2	34 ± 2	31 ± 2	32 ± 2	33 ± 2	32 ± 2	34 ± 2	35 ± 2
	HNO_2	33 ± 2	35 ± 3	34 ± 2	31 ± 2	32 ± 2	34 ± 2	35 ± 2	36 ± 2	35 ± 2
V_t (L)	Control	1.70 ± 0.18	1.58 ± 0.12	1.80 ± 0.20	1.73 ± 0.18	1.65 ± 0.17	1.64 ± 0.14	1.63 ± 0.13	1.70 ± 0.16	1.74 ± 0.16
	HNO_2	1.69 ± 0.16	1.64 ± 0.15	1.62 ± 0.13	1.67 ± 0.13	1.53 ± 0.10	1.61 ± 0.12	1.68 ± 0.14	1.78 ± 0.12	1.61 ± 0.13
f (breaths/min)	Control	20 ± 2	22 ± 1	21 ± 1	19 ± 1	21 ± 2	22 ± 2	23 ± 3	21 ± 2	21 ± 1
	HNO_2	21 ± 2	22 ± 2	22 ± 2	20 ± 1	23 ± 1	22 ± 1	22 ± 1	21 ± 2	23 ± 2

Abbreviations: HR, heart rate; V_E, minute ventilation, V_t, tidal volume, f, breathing rate.
[a]There were no statistically significant differences between control and HNO_2 measurements for any parameter.

Table 3. Cumulative symptom responses (10-point scale) for all subjects with control air and HNO_2 chamber exposures[a]

Question	Exposure status	Mean value
Shortness of breath	Air	0.625
	HNO_2	1.075
Wheeze	Air	0.1
	HNO_2	0.125
Cough	Air	0.25
	HNO_2	0.275
Chest tightness	Air	0.25
	HNO_2	0.325
Skin irritation	Air	0.25
	HNO_2	0.375
Eye irritation	Air	0.60
	HNO_2	0.65
Eye tearing	Air	0.225
	HNO_2	0.225
Throat irritation	Air	0.150
	HNO_2	0.4
Nasal stuffiness	Air	0.625
	HNO_2	0.75
Nasal dryness	Air	0.70
	HNO_2	0.725

[a]The aggregate score of all 10 test symptoms was significantly higher on the HNO_2 exposure than the control days. Each symptom was assessed just before, during, and immediately after exposure. (Headache was included in the questionnaire as a negative control symptom and was not included in this analysis.)

vitro conditions simulating low exposure to nitrogen dioxide (less than 100 ppm), Pryor and Lightsey (*20*), proposed that the conversion of NO_2 to HNO_2 is initiated according to the following reaction:

$$NO_2 + \text{–HC=CH–CH}_2\text{–} \rightarrow \text{HNO}_2 + \text{–CH–CH–CH–}$$

which is similar to the mechanism for formation of HNO_2 from NO_2 postulated on the basis of experimental observations in airways (*7,21*).

The rate of tissue absorption of a vapor as it is inhaled in the respiratory system is determined by its concentration, the solubility of the vapor in water, and the rate of airflow. The effective solubility (Henry) coefficient of HNO_2 is close to that of sulfur dioxide at physiologic pH (*22*), and increases with increasing pH over the range from 2 to 6 (*23*). Comparisons of the respiratory effects of acidic gases and aerosols of varying compositions indicate that the hydrogen ion content of the substance is one of the important determinants of the effect on airways. A study of the effect of inhaled acid aerosols in asthmatics has suggested that titratable acidity, as well as the specific chemical composition and pH, are important determinants of the potency of acid in producing effects on lung mechanics (*24*).

Asthmatics were selected for the present study because of previously demonstrated susceptibility to airway effects of inhaled acidic aerosol (*9*). Increased sensitivity of asthmatic subjects to acidic aerosols has not been seen in all such studies (*11,12*). The duration of the exposure in this study was three times as long as the exposures reported by Avol et al. (*11*) and Aris et al. (*12*), and may account for the significant effect on lung function seen in the present study. Bronchoconstriction was not seen at this dose and duration, even though forced vital capacity was reduced. Further study will be needed to determine whether asthmatics differ in their susceptibility to the effects of vapor-phase HNO_2 from nonasthmatics and whether airway constriction is seen at dose–duration combinations higher than those used in this study.

In summary, when exposed for 3 hr with intermittent, moderate exercise to 650 ppb HNO_2, mildly asthmatic subjects experienced a small decrease in FVC which was apparent within 25 min of the onset of exposure. They also reported a slightly higher aggregate rate of respiratory and mucous membrane symptoms, although at this dose they were not able to distinguish exposure from control days. These data suggest that the experimental dose of HNO_2 used is slightly above but very close to the threshold for respiratory effects of HNO_2.

References

1. Eatough DJ, Benner CL, Bayona JM, Richards G, Lamb JD, Lee ML, Lewis EA, Hansen LD. Chemical composition of environmental tobacco smoke. 1. Gas-phase acids and bases. Environ Sci Technol 23:679–687 (1989).
2. Harris GW, Carter WPL, Winer AM, Pitts JN, Platt U, Perner D. Observations of nitrous acid in Los Angeles atmosphere and implications for predictions on ozone-precursor relationships. Environ Sci Technol 16:414–419 (1982).
3. Brauer M, Koutrakis P, Keeler GJ, Spengler JD. Indoor and outdoor concentrations of inorganic acidic aerosols and gases. J Air Waste Manage Assoc 41:171–181 (1991).
4. Leaderer BP, Stowe M, Li R, Sullivan J, Koutrakis P, Wolfson JM, Wilson W. Residential levels of particle and vapor phase acids associated with combustion sources. In: Proceedings of the 6th international conference on indoor air quality and climate, Helsinki, Finland, vol 3. Helsinki, Finland:Finnish Society of Indoor Air Quality and Climate, 1993;147–152.
5. Pitts JN, Wallington TJ, Bierman HW, Winer AM. Identification and measurement of nitrous acid in an indoor environment. Atmos Environ 19:763–767 (1985).
6. Brauer M, Rasmussen TR, Kjaergaard SK, Spengler JD. Nitrous acid formation in an experimental exposure chamber. Indoor Air 3:94–105 (1993).
7. Postlethwait EM, Bidani A. Mechanisms of pulmonary NO_2 absorption. Toxicology 89:217–237 (1994).
8. Goldstein E, Peek N, Parks N, Hines H, Steffey E, Tarkington B. Fate and distribution of inhaled nitrogen dioxide in rhesus monkeys. Am Rev Respir Dis 115:403–412 (1977).
9. Bates DV, Utell MJ. Health effects of atmospheric acids and their precursors. Report of an ATS Workshop. Am Rev Respir Dis 144:464–467 (1991).
10. Koenig JQ, Pierson WE, Horike M. The effects of inhaled sulfuric acid on pulmonary function in adolescent asthmatics. Am Rev Respir Dis 128:221–225 (1983).
11. Avol El, Linn WS, Shamoo DA, Anderson KR, Peng R-C, Hackney JD. Respiratory responses of young asthmatic volunteers in controlled exposures to sulfuric acid aerosol. Am Rev Respir Dis 142:343–348 (1993).
12. Aris R, Christian D, Sheppard D, Balmes JR. Lack of bronchoconstrictor response to sulfuric acid aerosols and fogs. Am Rev Respir Dis 143:744–750 (1991).
13. Morris JF, Koski A, Johnson LC. Spirometric standards for non-smoking adults. Am Rev Respir Dis 103:57–67 (1981).
14. American Thoracic Society. ATS Snowbird workshop. Standardization of spirometry. Am Rev Respir Dis 119:831–838 (1979).
15. Taira M, Kanda Y. Continuous generation system for low-concentration gaseous nitrous acid. Anal Chem 62:630–633 (1990).
16. Koutrakis P, Wolfson JM, Slater JL, Brauer M, Spengler JD. Evaluation of an annular denuder/filter pack system to collect acidic aerosols and gases. Environ Sci Technol 22:1463–1468 (1988).
17. Armitage P, Berry G. Statistical Methods in Medical Research. Oxford:Blackwell Scientific Publications, 1987;410–411.
18. Bates DV, Hazucha MJ, Bromberg PA. Mechanism of action of ozone on human lung. J Appl Physiol 67:1535–1541 (1989).
19. Pearson SD, Gan JC. The inactivation of plasma alpha-1 proteinase inhibitor by nitrous acid. Int J Biochem 17:1252–1262 (1985).
20. Pryor WA, Lightsey JW. Mechanisms of nitrogen dioxide reactions: initiation of lipid peroxidation and the production of nitrous acid. Science 214:435–437 (1981).
21. Postlethwait EM, Bidani A. Pulmonary NO_2 uptake proceeds via HNO_2 formation (abstract). FASEB J 2(4):A373 (1985).
22. Park JY, Lee Y-N. Aqueous solubility and reactivity of nitrous acid (abstract no. 46). In: Proceedings: Symposium on acid rain, American Chemical Society, 13–18 April 1986. Upton, NY: Brookhaven National Laboratory, 1973.
23. Larson TV. The influence of chemical and physical forms of ambient air acids on airway doses. Environ Health Perspect 79:7–13 (1989).
24. Fine J, Gordon T, Thompson J, Sheppard D. The role of titratable acidity in acid aerosol-induced bronchoconstriction. Am Rev Respir Dis 135:826–830 (1987).

Toward Less Misleading Comparisons of Uncertain Risks: The Example of Aflatoxin and Alar

Adam M. Finkel

Cecil and Ida Green Center for the Study of Sciences and Society, University of Texas, Richardson, TX 75080 USA

Critics of comparative risk assessment (CRA), the increasingly common practice of juxtaposing disparate risks for the purpose of declaring which one is the "larger" or the "more important," have long focused their concern on the difficulties in accommodating the qualitative differences among risks. To be sure, people may disagree vehemently about whether "larger" necessarily implies "more serious," but the attention to this aspect of CRA presupposes that science can in fact discern which of two risks has the larger statistical magnitude. This assumption, encouraged by the indiscriminate calculation of risk ratios using arbitrary point estimates, is often incorrect: the fact that environmental and health risks differ in unknown quantitative respects is at least as important a caution to CRA as the fact that risks differ in known qualitative ways. To show how misleading CRA can be when uncertainty is ignored, this article revisits the claim that aflatoxin contamination of peanut butter was "18 times worse" than Alar contamination of apple juice. Using Monte Carlo simulation, the number 18 is shown to lie within a distribution of plausible risk ratios that ranges from nearly 400:1 in favor of aflatoxin to nearly 40:1 in the opposite direction. The analysis also shows that the "best estimates" of the relative risk of aflatoxin to Alar are much closer to 1:1 than to 18:1. The implications of these findings for risk communication and individual and societal decision-making are discussed, with an eye toward improving the general practice of CRA while acknowledging that its outputs are uncertain, rather than abandoning it for the wrong reasons. *Key words*: carcinogenic risk, HERP index, natural and synthetic pesticides, risk comparison, uncertainty. *Environ Health Perspect* 103: 376–385 (1995)

To assess risk is to compare risks. Comparisons are hidden or overt virtually any time data and models are used to quantify some environmental or health hazard. This holds true whether the social purpose involves setting a standard (which entails comparing the risk without any intervention to the magnitude, uncertainty, and distribution of risks after intervening), communicating the findings of science (disembodied risk estimates are meaningless to most people without reference to background rates or other numerical indices), or setting priorities (without comparisons, either nothing would be a priority or, equivalently, everything would). And yet, against the countless person-years of effort that have gone into refining and codifying the methodology for quantifying one risk at a time, there has been virtually no progress in developing principles and methods for quantifying risk comparisons.

Comparative risk assessment (CRA) is too important to do poorly. Not only do government agencies use CRA to influence the way people think about different risks, but they are increasingly using it to make irrevocable choices about which risks to control and which to accept. Government must decide, for example, whether to promote, mandate, or restrict alternative fuels such as methyl tert-butyl ether (MTBE) for automobiles; its only choice is whether to use CRA to compare gasoline and MTBE or instead to make the decision on intuitive, political, or other grounds. Either way, choices such as these will be made, but reliance on a misleading analytic tool might be worse than undertaking no analysis at all.

At its current state of development, however, CRA may be sufficiently flawed that on balance it causes more harm than good. Decision-makers cannot use CRA without asking whether merely knowing which of two risks is statistically larger is sufficient to guide regulatory policy or individual choice. Even putting this aside, however, there remains a purely scientific question: With current methods of CRA, would we know a "larger" risk when we saw it?

This article explores a largely unrecognized but fundamental flaw in how CRAs are performed, using a well-known risk comparison—the allegation that exposure to the naturally occurring carcinogen aflatoxin was definitely and substantially riskier than exposure to the pesticide Alar—to demonstrate the implications of analytic overconfidence. From this example, general lessons will be gleaned to offer an improved paradigm for comparing environmental risks.

Background

CRA fell into some disrepute during the last decade, largely because one particular form of it, the quantitative contrasting of markedly dissimilar risks [such as being overweight versus being exposed to benzene (*1*)], was increasingly regarded as unresponsive to important perceptual judgments and hence as needlessly manipulative (*2,3*). Nevertheless, many other brands of CRA have flourished during the same period, while CRA of dissimilar risks seems to be making a comeback of late (*4,5*). In this regard, it is useful to distinguish between what could be termed "small" and "large" versions of CRA. The former involves the quantitative comparison of single risks that are generally less dissimilar than the overweight/benzene sort of comparison. Prominent examples of different types of "small" uses of CRA include the ranking of various hazardous waste sites in the Hazard Ranking System developed by the Environmental Protection Agency (EPA), the analysis of "risk/risk tradeoffs" such as the choice between cancer risks due to the disinfection of drinking water and pathogenic risks due to the failure to disinfect (*6*), and the ranking of various common pollutants (both naturally-occurring and synthetic), either in order of inherent toxicologic potency or of excess risk under specified exposure conditions (*7*).

"Large" CRA involves the comparison of categories of risks and is increasingly being invoked as a means of putting the United States' allegedly haphazard environmental priorities in a "rational" sequence (*8–11*). For example, a recent magazine article cites as strong evidence that "we still haven't figured out what is really worth worrying about" the disparity between the $0.1 billion society spends annually on controlling indoor radon, which EPA estimates may cause as many as 20,000 lung cancer deaths each year, and the $6 billion spent on cleaning up hazardous waste sites, which purportedly cause fewer than 500 annual cancer deaths (*12*).

Misplaced Criticism of Comparative Risk Assessment

By far the most commonly criticized attribute of CRA is its alleged reliance on juxtaposing partially or totally incommensurable situations: the classic "you can't compare apples and oranges" problem [see Covello et al. (*13*) for this criticism applied to small CRA; see Hornstein (*14*) for an application to large CRA]. Although CRA is indeed difficult to do

This article was largely written while the author was at the Center for Risk Management, Resources in the Future, Washington, DC. The following persons generously provided additional data or made helpful suggestions: John Brauman, Leonard Gianessi, Robert Goble, George Gray, Edward Groth, Barbara Petersen, Paul Portney, Barry Ryan, Phil Szuromi, James Wilson, Lauren Zeise, and several anonymous reviewers. Partial support for this research was generously provided by the Office of Policy, Planning, and Evaluation, U.S. EPA.

Received 4 August 1994; accepted 19 December 1994.

and easy to botch, the major obstacle is not the qualitative differences between risks, but a completely different and largely ignored problem: the uncertainty in quantitative risk magnitude. Ironically, critics of CRA thus may well be right, but for the wrong reasons.

The impotence of the accusation of incommensurability is relatively easy to demonstrate. We all routinely compare highly dissimilar states by the simple (at least conceptually) cognitive process that involves: 1) disaggregating each situation or choice into its salient attributes (in the literal apple/orange comparison, these would be price, taste, nutritive value, appearance, etc.); 2) gauging how much of each attribute each situation or choice embodies; 3) assessing how much we value each attribute; and 4) aggregating the individual value judgments into a composite evaluation for comparison.

So apples and oranges are not incommensurable at all, and neither are disparate risks to health. In fact, when researchers tested empirically the most widely accepted predictions about how laypeople were supposed to react to various kinds of risk comparisons, the responses either did not support or else contradicted the thesis that the more dissimilar the comparison, the less acceptable and more aggravating the recipients would find it (*3,15*). For example, those surveyed by Roth et al. (*3*) generally regarded a hypothetical comparison of two different estimates of the same pollutant risk [a type of comparison Slovic (*15*) had put in their "first rank" of very acceptable communication techniques] as less reassuring, informative, and trust-engendering than a comparison of the pollutant risk with the risk of lightning, hurricanes, and insect bites (one of the Slovic et al.'s "fifth rank" or "rarely acceptable" comparisons).

The real problem in comparing risks is not that they differ in (known) qualitative respects, but that they differ in unknown quantitative respects. No amount of careful thought could make a choice between buying apples or buying oranges anything but arbitrary if one could neither discern nor control the price, taste, or appearance of either commodity. A numerical comparison between uncertain health risks, made without taking account of the uncertainty, is like shopping for produce sight unseen when one foodstuff might be expensive and rotten and the other cheap and flawless. And yet this is exactly how environmental risk assessors routinely make risk comparisons.

The further irony in this situation is that the analytic tool for making honest comparisons of uncertain risks—quantitative uncertainty analysis—is already well developed but languishes unused for this important application. For almost as long as risk assessment has existed, researchers have used tools such as expert judgment, Bayesian analysis, and Monte Carlo simulation to estimate the uncertainty surrounding single risks (*16,17*). These uncertainties arise, among other sources, from our inability to measure precisely the quantities that drive the risk assessment models we use (parameter uncertainty) and from our inability to know which of two or more alternative models is in fact correct or most useful (model uncertainty). The most recent report on risk assessment by the National Research Council (*18*) contains numerous recommendations instructing EPA (which has lagged behind the advances in academia) to abandon its reliance on point estimates of risk for standard setting and to instead quantify uncertainty in risk using existing data and methodologies. However, none of the academic literature on uncertainty in risk, nor any of the practical applications conducted by EPA and other stakeholders in risk management policy, has ever applied the methodology to risk comparison.

This omission is particularly glaring because the mathematics of uncertainty dictate that dividing one uncertain risk by another to arrive at a comparative assessment magnifies rather than attenuates or cancels the uncertainty present in each risk (as long as the uncertainties do not arise from identical sources). For example, suppose you can guess the weight of person *A* to within a factor of 1.2 (e.g., your best guess is 180 pounds but you are confident *A* weighs between 150 and 216 pounds), and you can also guess the weight of *B* within a factor of 1.2 (e.g., your best guess is 150 and the range is between 125 and 180). Then, your best estimate of their relative weights would be 1.2 (180/150), but the uncertainty about this comparative estimate would range between 0.83 (150/180) and 1.73 (216/125). The uncertainty about the ratio estimate is now a factor of 1.44 on either side of the central estimate, larger than was present for either risk alone. Notice further that one cannot say with confidence that *A* weighs more than *B*. Thus, it is precisely for those applications where we can be least confident in our results that we devote the least effort to exploring how error-prone our answers might be.

Exploring Overconfidence in Risk Comparison

To develop and explore the implications of a more technically sound paradigm for CRA, I reexamined one of the most influential examples of small CRA: the conclusion reached by a group led by Ames (*19*) that the aflatoxin B_1 contained in a daily ration of peanut butter posed 18 times greater risk than the growth regulator daminozide (Alar) in a daily ration of apple juice (a risk largely due to Alar's hydrolysis product unsymmetrical dimethylhydrazine, or UDMH, a potent rodent carcinogen). [This point estimate of risk has undergone some minor metamorphoses since it first appeared. Originally, Ames and Gold (*19*) presented the HERP (human exposure/rodent potency) index for aflatoxin (0.03%) as 17.6 times that of UDMH (0.0017%). Some weeks later, Ames cited a ratio of 10:1 (*20*), and later in 1989 then-FDA Commissioner Frank Young attributed to Ames a ratio of 30:1 (*21*). More recently, Uniroyal Chemical Company, the manufacturer of Alar, cited a ratio of 300:1 (*22*). In their most recent update of the HERP table (*23*), Ames and colleagues provided more information on the inputs to these numbers, but the implicit ratio remained essentially the same (0.03%/0.002%, or 15:1).] Whatever the precise number touted, it consists of the ratio of two risk estimates, each of which is composed of at least two uncertain inputs (at the highest level of aggregation, exposure and carcinogenic potency). Thus, any comparison of two HERP values (or other risk estimates) to generate a risk ratio entails calculating the uncertain quotient of two uncertain quotients. The sign and the magnitude of these estimates of the aflatoxin/Alar risk ratio have been cited to support the view that the "artificial" hazard of Alar is (or was) trivial compared to the magnitude of the risk from aflatoxin, a "natural" risk consumers supposedly deem acceptable (*24*).

It is conceivable, of course, that any estimate of this particular risk ratio, even if surrounded by a range of uncertainty, is meaningless because one or both of the substances involved are not carcinogenic in humans. A superficial look at Alar and aflatoxin might suggest that the latter is a "known" human carcinogen while the former is only known to cause tumors in rodents. But that would be a premature judgment. First, although in a few cases, such as saccharin and unleaded gasoline, directed research on chemical-specific mechanisms has cast serious doubt on whether certain animal carcinogens present any risk to humans at low doses, no such evidence or theory currently exists in the case of UDMH that would explain a qualitative interspecies difference. Besides, the lack of epidemiologic data (positive or negative) on UDMH does not necessarily distinguish it from an extensively studied chemical like aflatoxin. In no single case has "negative" epidemiologic data alone been of sufficient power to invalidate positive animal data (*25*); the fact that UDMH is not a "known" human carcinogen says more about what we know than about

what properties the chemical truly does or does not possess. In particular, the human data on these two substances may only differ because one (aflatoxin) is associated with a rare cancer (primary hepatocellular carcinoma) that stands out from the background, while the other may well increase the incidence of some more common tumor type(s) that could not be detected in a typical epidemiologic study. In any event, the method used here to quantify uncertainty in carcinogenic potency explicitly accounts for the additional uncertainty caused by the possibility that UDMH may pose zero or near-zero risks at low doses because we cannot be confident that the rodent tumors are relevant to humans. Finally, recently emerging evidence suggests that aflatoxin may not be a significant contributor to human liver cancer. Campbell et al. (*26*) claim that previous analyses of the epidemiologic data on aflatoxin were confounded by the failure to control for dietary variables and that aflatoxin is "an unnecessary and insufficient cause" as compared to viral and nutritional factors. The CRA presented here, like all previous ones, will not directly account for the model uncertainty contributed by the possibility that one or both contaminants are noncarcinogenic in humans, but will instead concentrate on the substantial amount of uncertainty present even assuming both substances pose non-zero risk.

Methods

The excess cancer risk to an individual consumer (i) of peanut butter or apple juice (j) is a function of three factors: 1) the amount of the foodstuff consumed each day (A_i); 2) the concentration of aflatoxin or UDMH in the foodstuff (C_{ij}); and 3) the carcinogenic potency of each contaminant ($ß_j$). The first two of these quantities can be measured reasonably precisely, but they vary substantially among individuals; the third might be invariant across the population (if each person had equal biological susceptibility to the carcinogenic stimulus), but it clearly cannot be estimated without considerable ambiguity. With the appropriate units specified, risk is simply the product of these three quantities divided by the body weight of the individual (in this example, body weight was assumed to be invariant; the value 20 kg was chosen to represent a 4-year-old-child).

$$R_{ij} = [A_{ij}\,(\text{g/day}) \times C_{ij}\,(\text{ppb}) \times 10^{-6}\,(\text{mg/ng})] \times ß_j\,(\text{excess lifetime risk per mg/kg-day})]/20\ \text{kg}$$

Point estimates such as the 18:1 risk ratio are derived by multiplying single values for consumption, concentration, and potency and reporting the quotient of the two resulting risk estimates as a single number. Since each of the three inputs for each risk estimate can be described more correctly by a probability density function (PDF) than by an arbitrary point estimate, the raw material for a more sound approach to CRA entails first deriving these PDFs and then combining them to yield an estimate of the risk ratio with its associated uncertainty. Combining the PDFs is now computationally simple, with the advent of microcomputers to perform Monte Carlo simulation. In this method, a value from each PDF is chosen at random via an algorithm that ensures that the probability of selecting any value is the same as the underlying probability in the PDF. A single Monte Carlo iteration consists of a random draw from each PDF followed by the appropriate functional combination thereof (in this case, multiplication of three numbers to estimate each risk, followed by division of one risk estimate by the other). With repeated iterations (20,000 in this analysis), a PDF emerges for the output which asymptotically matches the distribution that would be obtained if the individual PDFs could be combined analytically [for this analysis, the Monte Carlo software "@RISK" (version 1.1 for Microsoft Excel, Palisade Corp., Newfield, New York) was used].

Data Sources

Food consumption. Data on the amount of peanut butter and apple juice consumed by children were obtained from a nationwide survey conducted by the U.S. Department of Agriculture (*27*). This survey of almost 38,000 persons, including 1,719 children ages 3–5, provides information on the average quantity of each foodstuff consumed each day, and also gives seven percentile points of the cumulative distribution of consumption across the population. In this analysis, the PDF for peanut butter consumption was well-approximated via a lognormal distribution with a median of 8 g/day and logarithmic standard deviation $\sigma_{ln} = 0.84$. The data on apple juice consumption were also well approximated by a lognormal PDF with a median of 83 g/day and a logarithmic standard deviation of 1.0. For reference, the point estimates of consumption Ames (*19*) apparently used (32 g/day for peanut butter and 120 g/day for apple juice) lie at approximately the 95th and the 64th percentiles of their respective PDFs. Without the distributional information, one would not be aware that these point estimates differ in their degree of "conservatism" (in such a way as to help make aflatoxin seem riskier than Alar), or that neither estimate reasonably approximates the amount of each food eaten either by frequent or by sporadic consumers.

Residue levels. Data on aflatoxin levels in 44,788 samples of peanut butter made from the 1986, 1987, and 1988 peanut crops were provided by the National Peanut Council (*28*). Data from the three crop years were combined to yield a discrete distribution consisting of 13 different possible residue levels and their associated probabilities; the overall mean of this distribution was 2.82 ppb (this distribution was approximately lognormal in shape, but because it had a slightly shorter right-hand "tail" than the continuous distribution would have yielded, the measured discretized values were used instead). The point estimate of concentration used by Ames [2 ppb (*19*)] lies at approximately the 40th percentile of this distribution. In contrast, *Consumer Reports* noted in 1990 (*29*) that 86 samples of peanut butter tested averaged 5.7 ppb of aflatoxin. However, they deliberately oversampled from less well-known brands (*30*).

Residue levels for UDMH in apple juice were provided courtesy of the Uniroyal Chemical Company (*31*). Uniroyal analyzed 71 samples of apple juice for UDMH content; the juice came from the 1985 or 1986 apple crops. The sample mean was 13.8 ppb, and the maximum concentration was 83 ppb. [There is a separate category of "baby apple juice," the small jars that infants (and some toddlers) consume. The mean UDMH content in the 71 samples of baby apple juice was nearly twice that of the adult product, and the maximum single value was 112 ppb (*31*). Thus, using only "adult" apple juice data tends to underestimate both the relative and absolute risk of UDMH exposure.] Due to the small number of samples and the fact that the data clumped into at least four modal groups (35 of the 71 values were clustered either around 1, 8, 13, or 33 ppb), the PDF used in the analysis consisted of the data points themselves; in the Monte Carlo procedure, 1 of these 71 values was chosen at random at each iteration. Ames (*19*) apparently assumed that apple juice always contains about 7.5 ppb UDMH; this value lies at about the 45th percentile of the distribution of measured residue levels.

Carcinogenic potency. The most difficult portion of the analysis was the generation of the PDFs for cancer potency, as no standard methods currently exist for deriving such distributions (*32*). Two different methods were used here, reflecting the distinction between a "known human carcinogen" (aflatoxin) and a substance (UDMH) for which only animal bioassay data are available.

The distribution for the potency of aflatoxin (Table 1) was derived from a risk assessment recently completed by the California Environmental Protection Agency (CalEPA) (*33*), which made use of

new epidemiologic data compiled by Yeh et al. (*34*). This was a cohort study of approximately 8000 persons in Guangxi, China, examining the relationship between aflatoxin exposure and primary hepatocellular carcinoma, controlling for concurrent infection with the hepatitis B virus (HBV). CalEPA tested five mathematical models and recommended the interactive effects form of the excess risk model, based on its fit to the Guangxi data, the stability of the parameter estimates obtained, and its ability to predict liver cancer incidence in the United States given reasonable assumptions about HBV prevalence and aflatoxin exposures. [The interactive excess risk model has the form $y = a + ß_1H + ß_2d + ß_3Hd$, where y is liver cancer incidence, a is the background incidence (in the absence of HBV infection or aflatoxin exposure), d is the daily dose of aflatoxin, H is a dummy variable indexing HBV carrier status (1 = positive, 0 = negative), and the $ß_i$ are fitted coefficients representing the HBV effect, the potency of aflatoxin, and the interactive effect, respectively.] Using the CalEPA regression equations and the standard errors they reported, maximum likelihood estimates (MLEs) and 5th and 95th percentile values for $ß^-$ (the potency of aflatoxin in an HBV-negative person) and $ß^+$ (the potency in an HBV-positive person) were derived (see Table 1). In the @RISK spreadsheet, these normal distributions were truncated at zero so that negative values for potency could not occur. At each iteration in the Monte Carlo simulation, the potency of aflatoxin is determined with reference to f, the assumed prevalence of HBV-positive individuals in the population. According to the CalEPA report (*33*), plausible values for f in the U.S. population range between 0.1% and 1%; a value of 1% for f was chosen here, an assumption that tends to overstate the relative and absolute risk of aflatoxin exposure. The Monte Carlo process then randomly chooses values from either the $ß^+$ or $ß^-$ PDFs, in a 1:99 ratio, thereby preserving the bimodality of the PDF for the potency of aflatoxin to a randomly chosen person in the population.

The PDF for the potency of UDMH is derived by a rather different procedure because no human data exist for this substance. There are various troublesome sources of uncertainty in analyzing an animal bioassay and extrapolating the results to humans, including the choice of dose–response model, interspecies scaling of exposure and susceptibility, and random sampling error affecting the small groups of rodents tested. EPA pays some attention to the last of these three uncertainties by publishing the 95th percentile upper confidence limit (UCL) on the slope of the linearized dose–response function that fits the observed tumorigenicity data acceptably well. In addition, EPA usually includes the caveat that the true slope at low doses "could be as low as zero." There are several problems with this approach: 1) for each case, it provides the risk manager and the public no idea how likely the UCL, zero, and all values in between are to be true, or even whether the value zero is plausible at all; 2) it gives no information on the nature and implications of the 5% of the distribution above the UCL; 3) it does not allow for nonlinear dose–response functions, in effect treating "potency" as a scalar independent of dose; and 4) it assumes, probably incorrectly, that the asymptotic confidence limits (derived by examining changes in the log-likelihood function with reference to the χ^2 distribution) are valid for the case of small samples and constrained (non-negative) optimization of the regression coefficients (*35,36*).

I have adapted work of Guess et al. (*35*), Sielken (*37*), and others to develop a method for quantifying potency uncertainty that addresses these four problems [but that, like EPA's approach, does not deal fully with model uncertainty in dose response (e.g., the possibility that a threshold exists) or in interspecies scaling (*38*)]. The method involves performing a bootstrap analysis of the observed bioassay data. For example, if the original bioassay had a single positive dose group in which 20 animals out of 50 tested developed tumors, the simulated bioassays would have tumor responses ranging from perhaps 15 to 25 animals, depending on the assumption made about the sampling error inherent in the single data point. If 10,000 such simulations were generated, and the resulting (linear) dose–response functions were put in ascending order of steepness, the 500th highest observation of the slope of the line would provide an alternative estimate of the 95th UCL of potency. The method uses the computer program "MSTAGE87" (version 1.1, courtesy E. Crouch, Cambridge, Massachusetts) to calculate the best-fitting polynomial for each simulated data set. By keeping track of all the coefficients, potency can depend on higher-order terms when the linear term is estimated to be near zero (i.e., the distinction between "the potency is zero" and "the dose–response curve is quadratic at low doses" is not muddled).

The bootstrap uncertainty analysis was applied to a new bioassay of UDMH carcinogenicity sponsored by Uniroyal (*39*). Table 2 shows the results of the new UDMH bioassay; because individually coded data for each test animal were not available, only the primary tumor response (hemangiosarcomas plus hemangiomas) was considered, not the total number of animals with tumors at any site (this would include pulmonary neoplasms as well). CalEPA recently completed an analysis of this bioassay (*40*) and calculated a potency value somewhat higher than EPA's. CalEPA used the tumor site that gave the highest UCL for potency, namely, pulmonary carcinomas/adenomas; here, EPA's assumptions about the appropriate data set were used, largely because the blood vessel tumors were so rare in the control animals, in contrast to the pulmonary tumors (35/100 pulmonary tumors among controls, as opposed to only 5/100 vascular tumors among controls).

The bootstrap resampling consisted of 5000 simulated data sets (see Table 3). The fitted values for $ß_1$, the linear term in the multistage polynomial, ranged from 0 (5.2% of all values) to 1.54; the median value for $ß_1$ was 0.508, and the 5th and 95th percentiles were 0 and 0.850, respectively. The PDF is approximately normal, as would be expected when the observed bioassay data are roughly linear; when the observed data can best be fit by a polynomial with no linear term, the PDF for $ß_1$ is approximately exponential in shape (*38*). The 5000 pairs of $ß_1$ and $ß_2$ values were sampled at random in the Monte Carlo process. The risk of

Table 1. Input probability density functions for potency of aflatoxin[a]

	HBV status	
	Negative	Positive
Mean	15.4	202.9
SD	7.3	55.7
5th Percentile	3.4	111.3
95th Percentile	27.4	294.5

[a]Potency is expressed in units of $(mg/kg\text{-}day)^{-1}$; the values in this table have been standardized via the surface area correction to apply to a 20-kg child.

Table 2. Data on carcinogenicity bioassay of unsymmetrical dimethylhydrazine (UMDH) in male mice (*39*)

UDMH concentration in drinking water (ppm)	Dose (mg/kg-day)	Human equivalent dose (mg/kg-day)[a]	Incidence of hemangiosarcomas/ hemangiomas
0	0	0	5/66
40	7.34	0.8797	31/67
80	13.7	1.65	43/68

[a]Rodent doses were converted to the equivalent doses for a 20-kg child by dividing by the factor $(20/0.035)^{1/3}$, where 0.035 kg is the average weight of a male mouse. This procedure assumes that children and mice are equally susceptible to UDMH on a dose per surface-area basis.

UDMH exposure was calculated at each iteration as ($ß_1 d + ß_2 d^2$), where the dose d was defined as intake × residue concentration/body weight). Thus, the computations account for the possible sublinearity of the UDMH dose–response function and permit some probability that the risk to humans at low doses is essentially zero.

Note that the new bioassay data give similar values for the potency of UDMH to those of the controversial Toth study (*41*), although the extent to which the new study should be interpreted as confirming, modifying, or invalidating the earlier one still seems to be a subject of controversy (*42–44*). The maxiumum likelihood estimate and UCL for $ß_1$ in the Toth study (if adjusted to a 20-kg child) were 0.680 and 0.907, respectively.

Table 4 summarizes some key parameters for each of the six input distributions. The sizes of the uncertainties in these parameters are typical of those encountered in previous assessments of the uncertainty in risks assessed singly. Several of the parameters have rather "tight" distributions (i.e., their 95th-percentile values are less than 10 times higher than their 5th percentile values), while one (UDMH residue) varies by nearly 100-fold, and another (UDMH potency) is "infinitely uncertain" in the sense that its lower bound could be zero. For comparison, Finley et al. (*45*) suggested distributions for 12 of the parameters commonly encountered in more complicated multimedia exposure assessments. Some of the distributions they recommend are as tight as some of those in Table 4 (e.g., inhalation rates among adults vary between approximately 8 and 16 m^3/day, to a 90% degree of confidence), while others (e.g., the number of years an individual is likely to live at one residence before moving) vary by more than 100-fold, and still others (e.g., the amount of soil a child ingests each day) resemble the UDMH potency distribution in that there is a nontrivial probability that zero is the true value.

Table 3. Summary statistics for uncertainty in carcinogenic potency ($ß_1$) of unsymmetrical dimethylhydrazine (UMDH) (adjusted to 20-kg child)

	CalEPA data set	U.S. EPA data set
Probability $ß_1 = 0$	3.9%	5.2%
5th Percentile	0.067	0
10th Percentile	0.26	0.13
25th Percentile	0.60	0.35
50th Percentile	0.82	0.51
75th Percentile	1.0	0.62
90th Percentile	1.18	0.73
95th Percentile	1.35	0.85
Asymptotic[a] (5th, MLE, 95th)	0.36,0.92,1.16	0.20,0.58, 0.72

[a]The asymptotic values for $ß_1$ were calculated in the same manner as EPA does, by determining the slope of the linearized dose–reponse function that maximizes the likelihood function given the observed bioassay data (the maximum likelihood estimate; MLE), and then increasing or decreasing the linear coefficient of the dose-response function until it could be rejected as not fitting the data at an upper or lower $p = 0.05$ level of confidence (via reference to the χ^2 distribution). Note that the bootstrap resampling technique described in the main text yields distributions that are somewhat broader than those generated by the EPA method.

Results

Figure 1 shows the cumulative probability distribution functions (CDFs) for the excess lifetime risks of peanut butter and apple juice consumption. Selected summary statistics of these distributions are presented in Table 5. The CDF for UDMH risk has a slightly higher median than the aflatoxin risk CDF, but because the former distribution has a much longer right-hand tail, its mean is nearly twice as high as the latter distribution's mean value.

The effect of this overlapping of the two risk distributions is shown in Figure 2, which depicts the PDF for the common logarithm of the ratio of the UDMH risk to the aflatoxin risk. Several features of this PDF are noteworthy, in light of the deter-

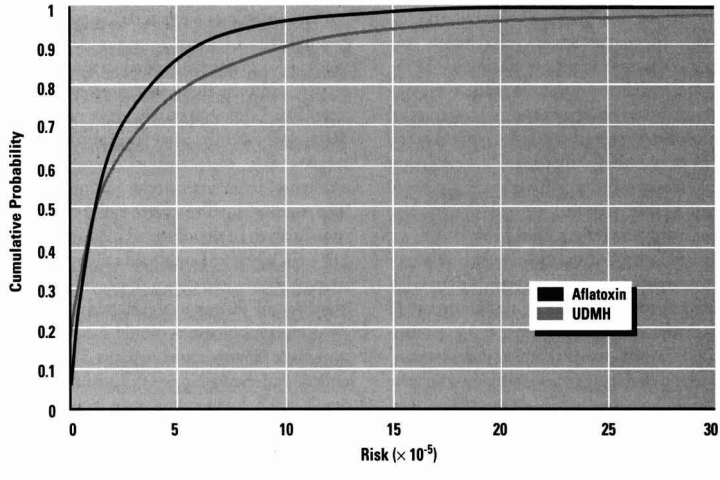


Figure 1. Cumulative distribution functions (CDFs) for the excess lifetime risk of peanut butter (blue curve) and apple juice ingestion (red curve). In either curve, the X-coordinate corresponding to a given value on the Y-axis represents the risk level that with probability y is less than or equal to the true but unknown value of risk. For example, the curves intersect at approximately $y = 0.5$, so there is roughly a 50% chance that either risk is less than about 1.3×10^{-5} (see Table 5 for a tabular representation of this figure). The red curve lies below the blue curve above $y = 0.5$, which means that as one approaches "worst-case" conditions, unsymmetrical dimethylhydrazine (UDMH) is (much) riskier than aflatoxin (e.g., there is a 5% chance the risk of aflatoxin exceeds 1×10^{-4}, whereas continuing horizontally from $y = 0.95$, the UDMH curve is not intersected until the risk level equals 2×10^{-4}).

Table 4. Characteristics of the probability density functions (PDFs) for the input variables

Variable	Units	Median	Mean	5th %ile	95th %ile	Uncertainty factor[a]	Percentile location of mean[b]
Peanut butter consumption	g/day	8.00	11.38	2.00	31.86	15.93	66
Apple juice consumption	g/day	83.00	136.84	16.02	430.02	26.84	69
Aflatoxin residue	ppb	2.50	2.82	1.00	6.50	6.50	61
UDMH residue	ppb	9.00	13.75	0.5	42.00	84.00	67
Aflatoxin potency (population average)	$(mg/kg\text{-}day)^{-1}$	15.40	17.50	4.02	28.23	7.02	61
UDMH potency (linear term only)	$(mg/kg\text{-}day)^{-1}$	0.508	0.490	0	0.850	—	43

UMDH, unsymmetrical dimethylhydrazine.
[a]Ratio of the 95th to the 5th percentiles.
[b]Percentile of the PDF where the arithmetic mean is located; a measure both of skewness and heaviness of tail.

ministic point estimates of Ames and others that this ratio is approximately 1:18.

The central tendency estimates (both the median and the mode) of this ratio are virtually indistinguishable from 1:1. This indicates a comparative risk for apple juice consumption at least an order of magnitude higher than any of the point estimates cited (*19–23*). Contrasting this result with previous risk comparisons reveals another intrinsic flaw in the use of point estimates. Because previous investigations failed to place the point estimates of inputs and results in context (i.e., were they central, lower-bound, or upper-bound numbers?), it is unclear whether the difference between 18:1 and 1:1 is due to a shift in the conservativeness of these estimators, due to changes in the input data (e.g., the newer bioassay of UDMH), or both.

More important than any single estimate of the comparative risk is the large uncertainty revealed here to affect that comparison. It happens that the central estimate of this particular risk ratio is so close to unity that it is clearly reckless to conclude that either risk is definitely greater than the other. The faint signal that aflatoxin may on average be 1.03 times riskier than UDMH is far outweighed by the "noise" in the comparison, which extends over four orders of magnitude at a 90% confidence level (from 376:1 in favor of aflatoxin to 34:1 in favor of Alar, a difference of a factor of 12,700). A nonparametric measure of the amount of overlap in the two risks was also computed to supplement this comparison of the median of the risk ratio to its own variance. By the Wilcoxon rank-sum test (*46*), the two risk PDFs are indistinguishable ($z = 0.525$, $p \approx 0.3$), so the hypothesis that the two PDFs are different must be rejected. Readers who sense that there is a paradox here (how can the two risks be simultaneously "the same" and yet differ by 30 or 300-fold?) may be caught in a semantic trap. There is no inconsistency in believing both parts of that statement. It is the distributions that are statistically indistinguishable; since the true value of either risk could fall anywhere within its own PDF, two independent risks with similar PDFs may, in fact differ wildly.

The major point of this article (and of improving CRA in general) is not to engage in "dueling point estimates," but to progress beyond any single point estimate comparison by changing the currency with which risks are expressed. In other words, this analysis shows that 18:1, 1:1, 1:18, and other answers are all legitimate, but that none of them alone expresses the risk correctly. Assuming this analysis is computationally sound, the only informative way to express the comparative risk of aflatoxin and Alar is to acknowledge the multiplicity of legitimate quantitative conclusions. A statement such as "to a reasonably high degree of confidence, aflatoxin is no more than 376 times riskier than Alar; on the other hand, Alar could be as much as 34 times riskier than aflatoxin" (see Table 5) has the virtue of candor and of revealing the complexity of any decision to control (or be concerned about) one or the other substance preferentially. Its drawback, that it does not lend itself to black-and-white conclusions, is equally prominent, but one must balance the tidiness of a point estimate such as 18:1 against the virtual certainty that other comparative risk estimates (and hence other social or personal decisions) are at least equally valid.

The impact of the uncertainty on the comparative risk assessment is robust to computational differences between this and previous analyses and to assumptions about the human carcinogenicity of UDMH. Again, even though the contrast between 18:1 and 1:1 is subsidiary to the larger difference between point estimates and expressions acknowledging uncertainty, Figure 2 reveals that even if this analysis suffered from a hidden systematic flaw that biased it toward overstating the relative risk of UDMH (which I argue is not a strong possibility), the general point still stands that a facile comparison is vulnerable to serious error. Suppose, for the sake of argument, that such a hidden flaw was found and the entire PDF in Figure 2 was shifted 18-fold to the left (that is, matching the central tendency exactly to Ames's 18:1 estimate). There would still be a roughly 10% chance that apple juice was riskier than peanut butter, and a roughly 1% chance it was more than 10 times riskier. Similarly, even if those convinced that UDMH is not a human carcinogen (see above) could successfully argue (presumably bringing to the table some concrete evidence, either direct or indirect) that there was a 90% probability its risk was zero, there would still be about a 5% chance that UDMH was riskier than aflatoxin. It is entirely a question of policy and values, not of science, whether even an analysis that might have shown such a 90/10 or 95/5 split could legitimately be reduced to the overconfident pronouncement that "peanut butter is riskier."

The PDF is not obviously biased toward overstating or understating the extent of uncertainty. Since the three factors analyzed are only some of the major uncertainties and variabilities affecting these two risk assessments, the results presented here might well understate the true ambiguity in the risk ratio. For example, the analysis assumes that every person is equally susceptible to the carcinogenic effects of aflatoxin or of UDMH; this assumption, though commonly made, ignores evidence that inborn and acquired variations in enzymatic metabolism, DNA repair, immune surveillance, and other factors cause individual susceptibility to cancer for a given exposure to vary widely [with perhaps three to four orders of magnitude separating the most susceptible and least susceptible portions of the "normal" population (*47*)]. This omission tends to bias both of the absolute risk estimates downward (*18*). On the other hand, there are several features of this analysis that

Table 5. Summary statistics for output distributions ($N = 20,000$)

	UDMH risk	Aflatoxin risk	Ratio UDMH/aflatoxin[a]
Minimum	0	3.99×10^{-9}	0
1st percentile	1.97×10^{-11}	4.42×10^{-7}	1.58×10^{-6} (1:632,911)
2.5th Percentile	4.14×10^{-10}	9.26×10^{-7}	3.43×10^{-5} (1:29,155)
5th Percentile	6.38×10^{-8}	1.46×10^{-6}	2.66×10^{-3} (1:376)
10th Percentile	6.15×10^{-7}	2.35×10^{-6}	2.79×10^{-2} (1:36)
25th Percentile	3.22×10^{-6}	5.31×10^{-6}	0.193 (1:5)
50th Percentile	1.33×10^{-5}	1.26×10^{-5}	0.972 (1:1.03)
75th Percentile	4.29×10^{-5}	2.95×10^{-5}	4.33
90th Percentile	1.09×10^{-4}	6.18×10^{-5}	15.96
95th Percentile	1.83×10^{-4}	9.57×10^{-5}	33.88
97.5th Percentile	2.97×10^{-4}	1.39×10^{-4}	64.52
99th Percentile	4.81×10^{-4}	2.15×10^{-4}	139.82
Maximum	5.97×10^{-3}	1.44×10^{-3}	17,545.6
Mean[b]	4.60×10^{-5}	2.72×10^{-5}	

UMDH, unsymmetrical dimethylhydrazine

[a]Note that the values in the third column of numbers are not simply the quotients of the numbers in the first and second columns; the third column contains the summary statistics of a separate probability density function (PDF) derived from the Monte Carlo simulation that takes into account the possibility that one risk truly lies in the left-hand tail of its own PDF while the other true value lies in its own right-hand tail (and, with equivalent probability, vice versa).

[b]Note that the arithmetic mean of the distribution of ratios is a nonsensical statistic, and hence is not reported here. Since ratios are inherently geometric (as opposed to arithmetic) quantities, their arithmetic mean gives disproportionate weight to cases where the numerator exceeds the denominator, and is thus entirely an artifact of which risk is placed in the numerator (i.e., the means of A/B and of B/A might both be greater than 1).

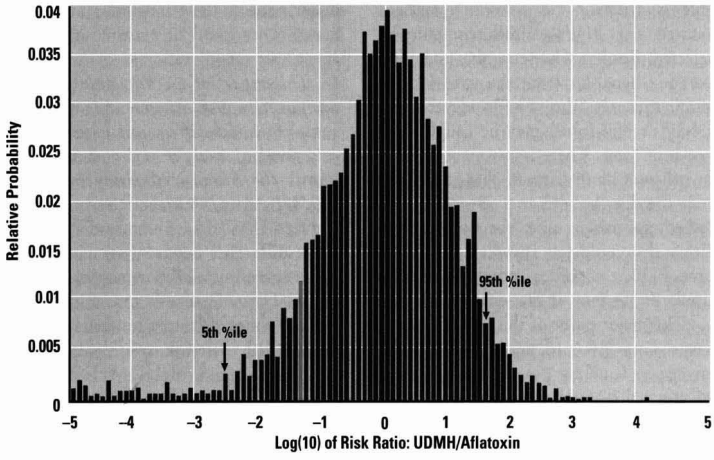


Figure 2. Probability density function (PDF) for the ratio of the risk of unsymmetrical dimethylhydrazine (UDMH) to the risk of aflatoxin, generated via 20,000 realizations from the Monte Carlo simulation described in the text. The X-axis denotes the common logarithm of the risk ratio (hence, $x = 3$ represents a risk of UDMH 1,000 times that of aflatoxin; $x = -2$ represents a risk ratio of 100:1 in the opposite direction). The height of the histogram at any point denotes the relative probability of that value compared to other possible values (the area under the smooth curve approximated by this histogram equals unity). The deterministic point estimate (1:18) of Ames et al. (*19*) lies at $x = -1.25$ (pink bar); the 5th and 95th percentiles (as shown by the two arrows) lie at $x = -2.57$ and $x = 1.53$, respectively.

might contribute to an overstatement of the total uncertainty in the comparison. Notably, the use of the entire distribution of measured residue data implicitly assumes that some consumers ingest products with high (or low) contaminant levels day after day, rather than being exposed at random to the whole spectrum of contaminant concentrations over long periods. Because residue levels are correlated to some degree with brand name and with geographic market, this assumption may not be far off the mark. Similarly, the ingestion rates for peanut butter and apple juice may not be statistically independent. If avid consumers of one product tend to be high consumers of the other (the more peanut butter ingested, the more liquid needed to wash it down?), this analysis would overstate the variability in the ratio of the two exposures.

Using alternative assumptions or data sets could also shift the entire PDF upwards or downwards (without affecting its variance). For example, the central estimate of the UDMH/aflatoxin ratio would increase by approximately a factor of two if the tumor site chosen by CalEPA (*40*) was used to analyze the UDMH bioassay data, and it would have increased further if more recent data reflecting increased apple juice consumption in the United States during the 1980s had been available or if residue levels in "apple juice for infants" had been included (*31*). Similarly, it is plausible that the estimate of UDMH's potency, like all current estimates based on rodent studies, is approximately seven times lower than the true value because the animals were only exposed for less than 2 years out of their natural life span (*48*). However, the fact that other researchers could legitimately generate other PDFs for the risk ratio, differing in mean and/or variance from the one in Figure 2, is not a weakness of this analysis. Rather, it illustrates a fundamental point: arguments about the precise extent of uncertainty reveal the bankruptcy of the practice of expressing risks and risk ratios via point estimates that admit no possible imprecision. The type of analysis undertaken here differs in kind as well as result from previous analyses: disputes about which point estimates are "correct" are resolved by the improved type of analysis. Remaining disagreements about exactly how to compute the uncertainty are not trivial, but they are second-order questions that should not obscure the fact that *a priori,* any CRA that results in a distribution of values is superior to any one that yields only a single value. To argue otherwise is to claim that scientists, decision-makers, and the public are better off with a guess that hides the fact it is a guess (i.e., the point estimate of relative or absolute risk) than with an analysis that attempts, but may conceivably fail, to precisely quantify the magnitude of the guesswork.

Discussion

These results have some important implications for risk management and risk communication, both specific to the case analyzed and for the general goal of improving decisions based on CRA.

Both Alar-contaminated apple juice and aflatoxin-contaminated peanut butter posed small, but not insignificant, individual and population risks. A central-tendency estimate of either risk falls at approximately 10^{-5}, an order of magnitude higher than the one-in-one-million benchmark generally regarded as the dividing line between minimal and significant risk when the exposed population is large. However, while these central tendency estimates are only mildly troubling, the UCLs lie in the range of 1 in 10,000 to 1 in 5,000. Thus, for the deliberately added contaminant (Alar/UDMH), a substantial number of preventable deaths may have been occurring each year in the population of children consuming apple juice, and a substantial number of children may have been subjected to risk levels several hundred times higher than those generally deemed to be acceptable. [For a rough calculation, consider only the subset of the approximately 2.5 million children born each year in the United States who faced risks from UDMH of at least 2×10^{-4}/lifetime. If this group made up 5% of each cohort, as Table 5 suggests, then each year there would be at least 25 excess deaths attributable to UDMH exposure in this subgroup. This figure is roughly numerically equivalent to the annual number of children murdered in public schools (*4*), a problem most people view as quite serious.]

Ironically, despite the various differences in the underlying data and despite the fact that this is a quantitative uncertainty analysis rather than a point-estimation exercise, the UCL in Table 5 is very close to the "plausible upper bound" of 1 in 4,000 that the Natural Resources Defense Council computed for UDMH in its much-maligned "Intolerable Risk" report (*49*). Actually, neither a Monte Carlo simulation nor a complicated point-estimation exercise is necessary to derive the approximate 1 in 4,000 lifetime risk estimate. Simply multiplying estimates for consumption (two 8-oz glasses per day) and for residue level (20 ppb) that each lie between the mean and the reasonable upper bound of their distributions yields a dose estimate about 2,000 times smaller than the surface-area-adjusted dose (approximately 1 mg/kg/day) that produced about a 50% tumor incidence in mice (in two different studies). As long as the assumption of proportionality is reasonable, 1/2,000 of this TD_{50} represents a risk of (0.5) (1/2000), or approximately 1 in 4,000 (*44*).

Overconfident pronouncements are not a good way to rank risks for decision-makers and the public. A social/political controversy continues to ferment as to whether numerical comparisons of risk should be used in isolation to inform peo-

ple what to worry about (*11*), given that people may legitimately regard a "smaller" risk as more worthy of attention or control than a "larger" one, depending on factors outside the purview of such quantitative rankings (e.g., issues involving dread, feasibility of control, locus of responsibility, and distributional equity). Even if analysts could somehow be sure that their numerical results would be used to supplement rather than monopolize this much larger priority-setting arena, however, they remain responsible for at least reporting in a thorough and honest fashion the narrower comparisons they purport to make. In this case, even if all other relevant factors had no effect on the risk comparison, it would be misleading to declare peanut butter the larger risk, when there is a 50% chance (if this analysis is exactly correct) or a 10% chance (even if this analysis is off by 18-fold in a particular direction) that such a statement is not true, even in a limited numerical sense. Recently, Ames' colleague Gold has claimed that their body of work on risk comparison was not designed to make or to encourage quantitative risk comparisons (*50*). Gold states that because of their well-known belief that there is little or no scientific basis for extrapolating from animal bioassays to human environmental risks, readers of their papers understand that they are not actually presenting risk estimates, but "merely ranking possible hazards." If these rankings are so uncertain as to be meaningless, however, then why express all the HERP indices to two significant figures, and why write that "the public might be better served if EPA were to present its risk assessments as comparisons to its estimates of risks from cups of coffee, beers, and so forth" (*51*)? A number cannot simultaneously be both extremely precise and infinitely uncertain; I maintain that quantitative uncertainty analysis is far superior to point estimation, no matter how many retrospective caveats are later placed on the point estimates.

The problems created by overconfident point estimates only increase with large CRA, because the kind of risks EPA, the U.S. Office of Management and Budget, Congress, and others wish to rank are much less straightforward to compare than even this rather uncertain comparison of two carcinogenic food contaminants. Returning to the radon/hazardous waste example cited at the beginning of this article, the CRA presented here should cast doubt on the definitive statements from EPA and the media that radon is exactly 40 times "worse" (or 2,400 times less efficient, if the additional and uncertain dimension of cost is also included) than the Superfund problem.

Uncertain risk comparisons, despite their complexity, are much preferable to avoiding quantification altogether. Although it is always simpler to criticize a misleading practice than to thoroughly describe a practical alternative, there are three cornerstones of decision-making under uncertainty that should help improve the way we calculate and communicate environmental and health risk comparisons. The message of this article is certainly not that we should eschew priority-setting—that would itself be contradictory, as priorities set by default or inertia are no less real than ones set consciously. Rather, the goal is to understand how formal analysis can inform priority-setting and where it must leave off and allow for creativity and subjectivity.

First, individual and social decision-makers must use the depiction of uncertainty to evaluate the probabilities and the consequences of making errors in their decisions, not just as another tool to answer an intellectual question about the magnitude of two disembodied problems. The decision determines how confident one needs to be that the larger risk is indeed larger. If the stakes are not high and large errors are not extremely more dangerous than smaller errors, then the central tendency of the risk ratio may be enough to go on. For example, if you are most concerned about picking the fruit with fewer calories, it may be sufficient to know that the average apple has, say, 80 calories to the average orange's 90, even if both values can range 30 calories above or below their averages. In this hypothetical case, you might be content to be only reasonably sure that apples were less caloric than oranges, given that even the worst portion of the rest of the distribution (the apple really has 110 calories to the orange's 60) does not lead to a decision costly enough to outweigh the benefits of being right on average. On the other hand, high stakes and/or asymmetries in the decision problem make it more important for the thoughtful decision-maker or risk communicator to consider the full range of possibilities and carefully evaluate which decision is best, rather than simply which risk is larger.

For the practical rather than the intellectual exercise, risk management thus involves, among other goals, trying to minimize the regret associated with the chosen option (where "regret" is a personal judgment related to the various costs incurred if the option chosen turns out to be inferior to another available one) (*52*). In the Alar/aflatoxin example, the question of which risk is worse is only a proxy for the real question of what to do about either or both substances. In the latter context, and given the results in Table 5, the individual or the regulator must balance, say, the 5% chance that ignoring or delaying action on Alar would erroneously leave unaddressed a problem 34 times greater than aflatoxin, against an equal chance that the opposite decision would focus attention on a problem 376 times smaller than aflatoxin (or, assuming that nothing more can be done about the natural carcinogen, the choice becomes one between some probability of spending resources on a problem manyfold smaller than a background risk already accepted by society, versus ignoring a problem erroneously deemed smaller than the tolerated risk). Again, needlessly definitive statements that one of these risks is exactly x times worse than the other robs the listener of the knowledge that the simplistic choice might be wrong by any criterion he might use to value the risks.

Second, decision-makers and analysts also need to understand that there is nothing wrong with using point estimates to inform and simplify their tasks. After all, the quantitative aspect of environmental decisions hinges on numbers, not on abstract curves that subsume an infinite set of discrete estimates. But different kinds of point estimates are appropriate for different decision-making goals, and the unwitting choice of an estimate can confound the decision. For example, if the decision-maker's goal here was simply to maximize the probability of addressing the larger risk, then the median of the risk-ratio PDF would be the appropriate anchor, and either of the possible decisions would have a virtually identical error rate. If the goal instead was to minimize the expected cost of the decision (assuming cost was proportional to the true absolute difference between the two risks, so that incorrectly ignoring a much larger risk would be costlier than ignoring a slightly larger risk), then a comparison of the means of each PDF would be appropriate, and Alar would emerge as the higher priority. And if the goal was to minimize the chance of an extremely bad decision, the appropriate choice of a summary point estimate would depend on whether the decision-maker was more averse to gross errors of overspending or underprotecting (or to errors that favor ignoring a deliberately added contaminant versus those that favor ignoring a naturally occurring toxin). Because the UDMH risk distribution has both a longer right-hand tail and a longer left-hand tail than the aflatoxin PDF, either risk could be the priority depending on which percentile (near the 5th or near the 95th) corresponded to the eventuality the decision-maker particularly wished to avoid.

Finally, optimal decision-making requires careful attention to the twin influences of uncertainty and interindividual

variability. The latter is a property of the system being studied which causes different estimates to be valid for different individuals (and which is generally irreducible through further study); the former is a property of the investigator (and his limited knowledge of the system) which generally can be reduced through further study (*18*). The results presented to this point deliberately intermingle uncertainty and variability. For societal decision-making, the two phenomena can be usefully combined. The PDFs in Table 5 essentially represent the uncertainty in risk to a person selected at random from the exposed population. Thus, the fact that the 95th percentile risk estimate for UDMH is 1.83×10^{-4} does not necessarily mean that 5% of the population faced risks at this level or higher, nor does it necessarily mean there was a 5% chance everyone's risk was this large; rather, it means that knowing nothing about the consumption habits or exposure history of an individual, there is a 5% chance his or her individual risk was above this value. Similarly, the mean of 4.6×10^{-5} can be interpreted as $1/N$ times the expected number of excess deaths in a random population of N persons exposed to UDMH.

The PDFs summarized in Table 5 are really made up of a family of uncertainty distributions, which average out to the composite statistics presented; each distribution is applicable to a person at a particular fractile of the underlying variability distribution. For example, one could replace two of the three input PDFs in the spreadsheet (for consumption and residue levels) with deterministic values and arrive at statements of the following type: for an individual whose exposure to UDMH puts him at the 95th percentile of the population, there is an 80% chance (here due entirely to uncertainty in carcinogenic potency) that his risk is between 1.1×10^{-4} and 6.5×10^{-4}, with a median value of 4.4×10^{-4}. Therefore, even though both variability and uncertainty are irreducible (if a decision must be made today) from the government's vantage point, the individual can reduce uncertainty by considering where he or she falls in the population with respect to the characteristics that are variable. Of course, some of the components of variability in this example are easier to resolve than others. Although it would not be apparent from the definitive nature of the 18:1 pronouncements, a frequent peanut butter consumer might realize that in relative terms, Alar was even less of a problem than this assessment suggests, and conversely for the frequent apple juice consumer (whose absolute risk might closely approximate the narrower PDF referenced earlier in this paragraph). Even an individual's relative risk due to residue levels might to some extent be clarified, as government or private organizations could analyze and publish the variation in residue levels by region, brand, or type of product (e.g., store-bought peanut butter versus the more highly contaminated "grind-your-own") (*29*). And, at least in the case of aflatoxin risk, motivated citizens could learn more about their own biologic susceptibility (to the extent that tests for hepatitis B virus antibodies accurately indicate higher risk).

Social decision-makers can also profit from attempts to decouple uncertainty and variability, as they can then intelligently rephrase the questions at hand. The questions of whether aflatoxin is riskier than Alar or whether radon is a bigger problem than Superfund sites are needlessly overaggregated; both for thorough risk communication and for more creative control strategies, more useful questions would be, for whom is risk A worse than risk B? Thus, rather than declaring that radon abatement should increase at the expense of waste-site cleanups, EPA might try to identify particular situations where marginal decreases in risk from the latter problem might be foregone to target efforts at "hot spots" of radon risk. Similarly, in situations where societal decision-makers wished to compare risks solely based on their expected population consequences (i.e., without regard to individual risk levels or their distribution), substituting deterministic average values for consumption and residue levels would yield a narrower distribution measuring only the uncertainty in the expected number of excess fatalities. In this case, of course, the aflatoxin and UDMH distributions would still substantially overlap.

Conclusions

CRA will never be both technically valid and acceptable to citizens and government unless it tells people both what they want to know and how well they can know it. Deciding what to compare is inherently difficult because any two risks differ in many ways. Risk assessors will naturally gravitate toward presenting statistical measures of harm rather than comparing other dimensions of risk that may have more influence on individual and public judgment (e.g., citizens may rather save fewer lives by spending more on Superfund sites than on radon abatement because they perceive the former as also redressing an injustice committed in the past). This focus on risk estimates need not be counterproductive, as long as analysts and regulators understand that risk statistics are like the proverbial lamp post: if the lost keys are underneath it one need not look further, but one should not be surprised not to find them there.

In considering how to compare risk statistics, on the other hand, it is only slightly more difficult to do it well than to do it badly. At a minimum, analysts should estimate and communicate some measure of the lower and upper bounds of each risk ratio, rather than just a measure of central tendency or a qualitative pronouncement about which risk is definitely "worse." In cases where one risk is almost certainly larger than another, this mode of communication should reinforce the distinction and increase confidence and trust (e.g., risk A is at least 10 times larger than risk B, and may be as much as 500 times larger). In other cases such as the Alar/aflatoxin example, where the lower and upper bounds reveal an ambiguous rank order, this fact should not be hidden, but turned from an adversary into an ally by one simple step: admitting that any rank ordering or any decision that flows from it will not be iron-clad, but will be informed by what the numbers say and what they don't (or cannot yet) say. Point estimates of uncertain risk comparisons offer a simplicity that makes decisions easier but makes wrong decisions well-nigh inevitable. Rather than either blinding ourselves to the numbers or letting the numbers usurp all our power to discern and choose, we should start fresh with Schopenhauer's apt advice: "the value of what one knows is doubled if one confesses to not knowing what one does not know."

References

1. Cohen BL, Lee IS. A catalog of risks. Health Phys 36:707–722 (1979).
2. Rothschild's numerate arrogance. Nature 276:429 (1978).
3. Roth E, Morgan MG, Fischhoff B, Lave L, and Bostrom A. What do we know about making risk comparisons? Risk Anal 10:375–392 (1990).
4. Office of Technology Assessment, U.S. Congress. Risks to children in school. Environment Program, OTA (in press).
5. Abelson PH. Reflections on the environment. Science 263:591 (1994).
6. Graham JD, Weiner J. Risk versus risk: resolving tradeoffs in health and environmental protection. Cambridge, MA:Harvard University Press (in press).
7. Wilson R, Crouch EAC. Risk assessment and comparisons: an introduction. Science 236:267–270 (1987).
8. Roberts L. Counting on science at EPA. Science 249:616–618 (1990).
9. Finkel AM. Taking aim at environmental risks: questions of feasibility and desirability. The Geneva Papers on Risk and Insurance 17:343–354 (1992).
10. U.S. EPA. Reducing risk: setting priorities and strategies for environmental protection, SAB-EC-90-021. Washington, DC:Environmental Protection Agency, 1990.
11. Finkel AM, Golding D, eds. Worst things first?

The debate over risk-based national environmental priorities. Washington, DC:Resources for the future, 1994.
12. Main J. The big cleanup gets it wrong. Fortune 123:95–101 (1991).
13. Covello VT, Sandman PM, Slovic P. Risk communication, risk statistics, and risk comparisons: a manual for plant managers. Washington, DC:Chemical Manufacturers Association, 1988.
14. Hornstein D. Reclaiming environmental law: a normative critique of comparative risk analysis. Columbia Law Review 92:501–571 (1992).
15. Slovic P, Kraus N, Covello V. Comment: What *should* we know about making risk comparisons? Risk Anal 10:389–392 (1990).
16. Morgan MG, Henrion M. Uncertainty: a guide to dealing with uncertainty in quantitative risk and policy analysis. New York:Cambridge University Press, 1990.
17. McKone TE, Bogen KT. Predicting the uncertainties in risk assessment. Environ Sci Technol 25:1674–1681 (1991).
18. National Research Council. Science and judgment in risk assessment. Washington, DC: National Academy Press, 1994.
19. Ames BN, Gold LS. Pesticides, risk, and applesauce. Science 244:755–757 (1989).
20. Ames BN. Letter to Hewitt D (CBS Inc., New York, NY), 29 June 1989.
21. Young FE. Weighing food safety risks. FDA Consumer September:8–13 (1989).
22. Hageman FJ. Letter to Finkel AM, 5 August 1991.
23. Gold LS, Stone TH, Stern BR, Manley NB, Ames BN. Rodent carcinogens: setting priorities. Science 258:261–265 (1992).
24. Smith K. Alar three years later: science unmasks a hypothetical health scare. New York:American Council on Science and Health, 1992.
25. Goodman G, Wilson R. Quantitative prediction of human cancer risk from rodent carcinogenic potencies: a closer look at the epidemiological evidence for some chemicals not definitively carcinogenic in humans. Regulatory Toxicology Pharmacology 14:118–146 (1991).
26. Campbell TC, Chen JS, Liu CB, Li JY, Parpia B. Nonassociation of aflatoxin with primary liver cancer in a cross-sectional ecological survey in the People's Republic of China. Cancer Res 50:6882–6893 (1990).
27. Pao EM, Fleming KH, Guenther PM, Mickle SJ. Foods commonly eaten by individuals: amount per day and per eating occasion. Home Economics Research Report no. 44, Washington, DC:U.S. Deptartment of Agriculture.
28. Cutchins KJ. Letter to Finkel AM, 28 June 1991.
29. Anon. The nuttiest peanut butter. Consumer Reports 588–591 (Sept 1990).
30. Groth N. Letter to Finkel AM, 25 June 1991.
31. Ball JO. Letter to Berteau PE (California Department of Health Services, Berkeley, CA), 3 April 1989.
32. Crouch E, Wilson R. Regulation of carcinogens. Risk Anal 1:47–57 (1981).
33. Cal EPA. Risk-specific intake levels for the proposition 65 carcinogen aflatoxin. Berkeley, CA:California Environmental Protection Agency, 1991.
34. Yeh FS, Yu MC, Mo CC, Luo S, Tong MJ, Henderson BE. Hepatitis B virus, aflatoxins, and hepatocellular carcinoma in Southern Guangxi, China. Cancer Res 49:2506–2509 (1989).
35. Guess H, Crump K, Peto R. Uncertainty estimates for low-dose-rate extrapolations of animal carcinogenicity data. Cancer Research 37:3475–3483 (1977).
36. Portier C, Hoel D. Low-dose-rate extrapolations using the multistage model. Biometrics 39:897–906 (1983).
37. Sielken RL. GEN.T: a general tool for incorporating cancer dose-response extrapolation techniques into quantitative risk assessments, User's manual, version 1. Bryan, TX:Sielken, Inc., 1988.
38. Finkel AM. Computing uncertainty in carcinogenic potency: a bootstrap approach incorporating bayesian prior information. Report to the Office of Policy, Planning, and Evaluation, U.S. EPA. Washington, DC:Resources for the Future, 1988.
39. Goldenthal EI. Two-year oncogenicity study in [CD-1] mice with UDMH. Unpublished report prepared by International Research and Development Corp., submitted to U.S. EPA by Uniroyal Chemical Co., report #IRDC 399-065, MRID # 413780–01, 1990.
40. Cal EPA. Risk-specific intake level for the proposition 65 carcinogen 1,1-dimethylhydrazine (UDMH). Berkeley, CA:California Environmental Protection Agency, 1992.
41. Toth B. 1,1-Dimethylhydrazine (unsymmetrical) carcinogenesis in mice: light microscopic and ultrastructural studies on neoplastic blood vessels. J Nat Cancer Inst 50:181–187 (1973).
42. Marshall E. A is for apple, Alar, and . . . alarmist? Science 254:20–22 (1991).
43 Kimm VJ. Alar's risks. Science 254:1276 (1991).
44. Finkel AM. Alar: the aftermath. Science 255:664–665 (1992).
45. Finley B, Proctor D, Scott P, Price P, Harrington N, Paustenbach D. Recommended distributions for exposure factors frequently used in health risk assessment. Risk Anal 14:533–552 (1994).
46. Freund JE. Mathematical statistics. NJ:Prentice Hall, 1971.
47. Finkel AM. A quantitative estimate of the extent of human susceptibility to cancer and its implications for risk management. In: Low-dose extrapolation of cancer risks: issues and perspectives (Farland W, Olin S, Park C, Rhomberg L, Scheuplein R, Starr T, Wilson J, eds). Washington, DC:International Life Sciences Institute Press (in press).
48. Peto R, Gray R, Brantom P, Grasso P. Dose and time relationships for tumor induction in the liver and esophagus of 4,080 inbred rats by chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine. Cancer Res 51:6452–6469 (1991).
49. NRDC. Intolerable risk: pesticides in our children's food, Washington, DC:Natural Resources Defense Council,1989.
50. Gold LS. Letter to Finkel AM, 31 July 1994.
51. Ames BN. Gold LS. Carcinogens and human health. Science 251:607–608 (1991).
52. Bell D. Regret in decision-making under uncertainty. Operations Res 30:961–981 (1982).

Molecular and Cellular Approaches to Extrapolation for Risk Assessment

Thomas R. Sutter

Department of Environmental Health Sciences, Johns Hopkins School of Hygiene and Public Health, Baltimore, MD 21205 USA

A workshop, "Molecular and Cellular Approaches to Extrapolation for Risk Assessment," was held in Baltimore, Maryland, USA, 5–6 May 1993. The workshop was hosted by the Johns Hopkins Center for Alternatives to Animal Testing and was sponsored by the American Forest and Paper Association, Washington, DC. Forty representatives of government, industry, and academia met to discuss the opportunity to use *in vitro* data to improve evaluations of human risk, citing information gaps between animal and human data sets, and the relatively limited use of *in vitro* data in risk assessments.

The scientific methods of toxicology are most often used to identify potential hazards or to evaluate the safety of specific substances under certain experimental conditions. Although current scientific risk assessments are quantitative in nature, they are based upon assumptions inherent in methods of safety evaluation and hazard identification.

To date, the predominant method is to test animals and then to extrapolate the results to humans. Interspecies extrapolations include route of exposure, dose, and response. Due to the lack of information about relevant human responses to chemical exposures, such extrapolations lead to uncertainties; these uncertainties result in decreased confidence in risk estimates. Even in tier test systems that are significantly based on alternative tests, confirmation is obtained through selective testing in animal species. *In vitro* data are often viewed as an additional level of extrapolation. In such schemes, *in vitro* data may be used to bolster knowledge about specific issues of extrapolation, but the data themselves represent an additional source of uncertainty (Fig. 1A) (*1–6*).

In this workshop we considered an alternate scheme, based on the parallelogram approach to extrapolation to man, that was proposed in the late 1970s by Sobels (*7–9*). Although this approach was originally described for its application to chemical mutagenesis, its underlying principal, "to obtain information on damage that is hard to measure directly" (*8*), is relevant to most, if not all, biological endpoints of toxicity. In Figure 1B, the parallelogram has been modified to emphasize two important issues that were considered in this workshop: interspecies and *in vitro–in vivo* extrapolation. This parallelogram provides a framework for the discussion of molecular and cellular approaches to extrapolation for risk assessment and provides a process for systematic, comparative biology. *In vitro* data are used to support investigations of mechanism of action and, more specifically, to evaluate the assumption of conserved mechanism of action among different species. By superimposing the parallelogram onto the components of toxicity identified in Figure 2, a rationale is established for systematic stepwise comparisons of specific mechanism of action. Through such comparisons, it should be possible to establish whether specific mechanistic steps are conserved among species. Furthermore, once conservation of mechanism is established, subsequent studies can be used to determine and to compare quantitative aspects of dose–response relationships between species. Thus, as previously noted by Sobels, the parallelogram approach can be used to provide both qualitative and quantitative information that is directly relevant to estimates of human risk.

Workshop sessions were organized to emphasize each corner of the parallelogram or to highlight related issues. Before the meeting, each speaker provided a statement of his or her beliefs concerning the three most relevant issues and opportunities associated with molecular and cellular approaches to extrapolation for risk assessment. Compilation of these statements identified issues related to four major topics: 1) predictions, 2) humans, 3) mechanisms, and 4) regulatory agencies and risk assessment.

Predictions

In vivo responses are often the result of complex pathological processes, i.e., the long-term result of multiple factor, multicellular interactions. Even when using sensitive molecular and cellular approaches, can *in vitro* data be used to predict likely outcomes of such processes? For example, can early markers predict chronic toxicity? A related issue of prediction concerns the equivalency of sensitive biological responses. If different concentration–response curves are determined for different markers, which one(s) predicts *in vivo* toxicity?

These issues related to *in vitro–in vivo* extrapolations are significant. When viewed within the context of a complex process, e.g., carcinogenesis, it is difficult to conceptualize an approach for addressing these difficulties. However, if complex biological processes are broken down into a biologically based dose–response paradigm, then the specific *in vitro–in vivo* comparisons become more focused. As shown in Figure 2, complex processes can be subdivided into discrete components that provide a context for investigations of specific mechanistic steps. While each individual component is truly a connected series of mechanistic steps, the broader picture depicted in Figure 2 emphasizes that, for the current status of risk assessments, it may be more useful to obtain increased knowledge of the entire process, albeit at a less comprehensive level, than it is to have complete knowledge about one or more steps in the process, with little knowledge of others. In general, the overall assessment will only be as good as the least understood component in the mechanism of the endpoint of interest.

Several speakers raised an interesting question concerning prediction: are the responses observed in rodents valid predictors of human toxicity? As discussed above, the parallelogram provides a framework in which to test the hypothesis of conserved mechanism of action among different species.

Molecular and cellular approaches, combined with comparative *in vitro* systems, provide a method to explore early biological responses to chemical or physical agents and the role of these early effects in altered cellular structure and function. Such studies may lead to an improved understanding of mechanism of action and biological determinants of specificity. Also,

Address correspondence to T.R. Sutter, Division of Toxicological Sciences, Johns Hopkins Medical Institutions, Hygiene, Room 7032, 615 N. Wolfe Street, Baltimore, MD 21205 USA.

I thank the members of Center for Alternatives to Animal Testing that contibuted to this workshop and report: A. Goldberg, J. Zurlo, J. Frazier, D. Rudacille, M. Principe, A. Kerr, and R. Lewis. I thank R. Hill for his discussions of the parallelogram approach and the members of my laboratory that assisted with this report: C. Hayes, J. Gastel, and N. Walker.

To obtain a complete technical report of this workshop, contact Richelle Lewis, Johns Hopkins Center for Alternatives to Animal Testing, 111 Market Place, Suite 840, Baltimore, MD 21202-6709 USA.

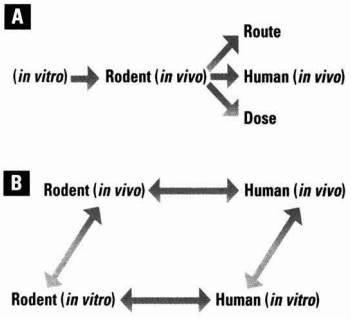


Figure 1. The role of *in vitro* data in extrapolations for risk assessment. (A) The traditional approach. *In vitro* data provides specific knowledge about important issues of extrapolation: route, species and dose. (B) The parallelogram approach. Modified from Sobels (*8*) to emphasize the issues of interspecies and *in vitro–in vivo* extrapolation. In this four-cornered experimental approach to knowledge of mechanism, *in vitro* data is used to test the hypothesis that a specific mechanism of action is conserved among rodent and human species. Note that the alternate hypothesis will still provide information about the action of the test compound in humans.

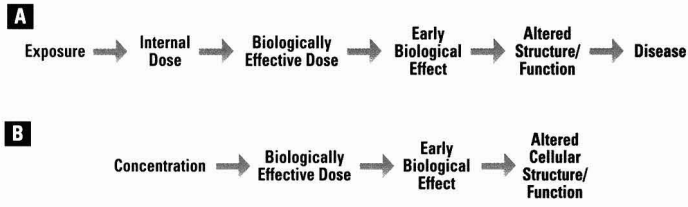


Figure 2. The principle of dose–response in risk and safety assessments. In the exposure–dose–response paradigm [adapted from the National Research Council Committee on Biological Markers (*20*)], complex biological processes are divided into discrete components that provide a context for investigations of specific mechanistic steps. (A) Components of *in vivo* toxicity. (B) Components of *in vitro* toxicity. Note that most of the components of *in vivo* toxicity can be studied *in vitro*.

studies of the relationship between concentration and biologically effective dose may provide insights into the shape of the dose–response curve in humans, including even lower levels of exposure. The potential for this latter opportunity (high to low dose) comes from the sensitivity of biological endpoints that are based on specific molecular and cellular targets.

In terms of linking exposure to dose–response relationships, several significant advancements have been made in the area of physiologically based pharmacokinetic (PBPK) and pharmacodynamic modeling. The concept of surrogate dose, or dose at the site of molecular action, provides a bridge between *in vivo* exposure and specific biological responses measured either *in vivo* or *in vitro*. As such, these modeling techniques may provide a continuum in investigations of mechanism, as experimental systems move between animals and cells in culture (*10*).

Human Cells

At present, the mechanism of action of many chemicals in humans is not fully understood. This includes knowledge of distribution, metabolism, specific cellular targets, sensitivity of specific cell populations, and repair capacity. This general lack of information concerning toxicity in humans is further complicated by the exposure of people to multiple chemicals over a lifetime that is considerably longer than that of rodents. A second issue relates to the limited availability of human specimens.

Human specimens, including tissues, slices, organ cultures, cocultures, or primary cells in culture, provide tremendous opportunity to investigate human biological response(s) to a variety of chemical and physical agents (*11*). When combined with modern methods of molecular biology and biochemistry to provide human recombinant DNA probes and expressed and purified human proteins, such studies can be used to identify primary biological endpoints relevant to human exposures (*12*) and to determine if the same critical cellular target (*13*) and mechanism (*14*) responsible for toxicity in animals exist in people. Corollary to this approach is the understanding that the methods developed using human *in vitro* systems can be easily imported as biomarkers into human epidemiology studies. Thus human *in vitro* studies support both human corners of the parallelogram and provide an opportunity for improved understanding of human *in vivo* responses (*15,16*). Without such improved sensitivity of the methods of human epidemiology or the incorporation of human *in vitro* data into the risk characterization process, biologically based risk assessments will simply represent improved models for the interpretation of data generated by animal experimentation.

Mechanisms

Should all tests be relevant mechanistically? Is correlation sufficient, especially as it relates to screens, or is it necessary to demonstrate a mechanistic link to biological response? Considerable discussion centered on the importance of knowledge of mechanism in decision-making processes. Both lectures on strategies for implementation made it clear that correlation is sufficient as a criteria for the application of screens (rapid tests to determine general or specific toxicity). In these cases knowledge of the mechanism resulting in the endpoint of toxicity is not required. For screens, current and future uses of *in vitro* data offer great potential to reduce, or eventually eliminate, the use of animals (*17,18*). The importance of these advancements should not be understated. However, it should be noted that while correlative studies may provide useful in-house information for decision-making, they advance neither the specific understanding of the endpoint of toxicity nor the methods to detect and quantitate such toxicity. For example, the Draize test, an *in vivo* screen for ocular and dermal irritancy, was widely used as a correlative screen for human-use product safety assessments. If more emphasis had been placed on obtaining a mechanistic understanding of this test, its replacement by cell or organ culture methods would have been greatly facilitated. Correlative studies do not provide a foundation for scientific advancement and, as such, should be used judiciously to immediately reduce the use of animals, while mechanistically based screen replacements with inherent potential for continued improvement are developed.

Mechanism-based approaches to risk assessment tend toward identification of true risk. Risk assessments that are based on such information will be based on the best available science. In turn, this should motivate good research and promote a self-advancing field that provides an improved understanding of human risk. Computer-based chemical databases facilitate the collection, storage, and retrieval of large amounts of information. Inherent in these chemical structures are features that determine biological activity (*19*). Studies of structure activity relationships provide the opportunity to advance from chemical specific risk assessments to chemical class-based risk assessments. Both the concepts of structure activity and surrogate dose imply the presence of a critical cellular target. Mechanism-based approaches implore the identification of such targets and raise the question of their conservation among species.

Regulatory Agencies and Risk Assessment

Several issues were identified that relate to certainty and uncertainty in risk estimates. Currently, both the regulatory and legal systems attempt to classify everything as safe or hazardous. Is it possible to move away from this toward a weight-of-evi-

MOLECULAR AND CELLULAR APPROACHES TO EXTRAPOLATION FOR RISK ASSESSMENT

Program Committee: A.M. Goldberg, J. Zurlo, and T.R. Sutter

Program Sessions

Plenary Lecture

J. M. Frazier	New perspectives on *in vitro/in vivo* extrapolation for risk assessment

***In Vivo* Responses: From Other Animals to Humans**

G.A. Boorman	Animal toxicity/carcinogenicity studies
J.D. Groopman	Human epidemiology/biomarkers: aflatoxin and liver cancer as a model

Research Supporting Extrapolations

M.E. Andersen	How will we know whether *in vitro* and molecular approaches really tell us about what goes on in the living animal?
B.J. Smith	Ovarian toxicity of 4-vinylcyclohexene and related compounds
L.D. Lehman-McKeeman	Male rat specific $\alpha 2_u$-globulin nephropathy: *in vivo* and *in vitro* assessment
R.B. Conolly	Pharmacodynamic modeling: quantitative descriptions of the linkage between tissue dose and toxic response
H.S. Rosenkranz	Application of SAR to extrapolation from *in vitro* to *in vivo* assays

Case Study: Dioxin

W.H. Farland	Dioxin: current and future uses of *in vitro* data
C.A. Bradfield	Molecular modeling of dioxin action
B.D. Abbott	Palatal organ culture in the study of dioxin-induced cleft palate
W.F. Greenlee	Human responses to dioxin: identification of interspecies determinants of specificity

Strategies for Implementation

K.A. Stitzel	Current and future uses of *in vitro* data
S. Green	Current and future uses of *in vitro* data

dence approach? Is the most sensitive response observed in animals necessarily the most relevant for human risk assessment? To what extent must we define a toxic mechanism *in vivo* as a prerequisite to gaining regulatory acceptance?

Current use of *in vitro* data in the risk assessment process is limited. Advancements in our abilities to grow and maintain human specimens, coupled with improvements in the ability to detect and quantitate specific human molecular targets, suggest that an important opportunity exists to improve the understanding of the human component of information available for risk assessment. To understand how to incorporate *in vitro* data into the risk assessment process will be difficult, but achievable.

For most of the topics discussed during this workshop we are currently knowledge-limited, that is, we lack sufficient information to move to a totally *in vitro* based approach. This information gap encompasses fundamental understanding of both knowledge of mechanism of action and the availability of reliable data sets of sufficient size to facilitate the recognition of underlying general principles. Because of these limitations, the prerequisite for a preliminary understanding of mechanism is, in general, currently obtainable only through *in vivo* studies. Such information includes knowledge of 1) distribution, including route of administration, dose, and duration, 2) metabolism and identification of the proximate toxicant, 3) target tissue and target cell, including critical cellular concentration and relative cell and tissue sensitivities, 4) injury progression and cell–cell interactions, 5) the capacity for repair, compensatory responses, and adaptation, and 6) the potential for chemical interactions, including exposures to mixtures, and interactions with endogenous chemicals. In general, for the complex biological responses depicted in Figure 2, we have little understanding of the events that link altered structure and function to disease.

The availability of human specimens is limited. The quality of such samples varies, and this further complicates the issues of intersample and interindividual variability. In addition, little information is available about the influence of cell culture conditions and specific medium constituents on measurements of biological responses determined *in vitro*.

In general, mechanism-based approaches are expensive and time consuming to develop. In addition to being technically demanding, the results tend to be chemical specific and indicative of selective toxicity, as opposed to more general or universal mechanisms.

Current and Future Uses of *In Vitro* Data

Several current uses of *in vitro* data exist: 1) to select the most appropriate animal model of humans; 2) to provide mechanistic information about *in vivo* responses; 3) to screen series of toxicants rapidly; 4) to screen for ocular, dermal, neurological, and developmental toxicity; 5) to establish potential mutagenicity and carcinogenicity; and 6) to further document the hazardous nature of a carcinogen.

Future uses of *in vitro* data include: 1) expanded use as screens; 2) reduction or elimination of the use of animals for assessments of dermal irritation; 3) determination of specific parameters for PBPK models; and 4) expanded use in investigations of mechanism of action, specifically as such information relates to risk assessment.

Conclusions

This workshop explored many aspects of the complex issues related to interspecies extrapolation. The parallelogram approach provides a rationale for systematic step-wise comparisons, including *in vitro–in vivo* comparisons of rodent and human biology that provide knowledge of response and sensitivity to chemical action. Applications of modeling provide important methods to link *in vivo* exposures to other endpoints of *in vivo* and *in vitro* biological response. In reviewing the available methods and experimental systems, a major informational gap was identified concerning the events that mechanistically link altered structure and function to toxicity or disease. Future studies need to focus on this important area of limited knowledge, as it appears to be rate limiting in the overall process to determine accurate risk estimates.

Given the understanding that chemical-specific risk assessments are both time consuming and expensive, considerable concern remains about the issue of selective versus universal mechanisms of toxicity. For now, no simple solution is evident. Minimally, advancements in structure activity relationships should permit us to move from chemical-specific risk assessments to those based on chemical class. Moreover, from the history of mutagenesis, it is clear that complete knowledge of specific mechanisms is not required for effective determinations of risk estimates. As in the case of chemical mutagenesis, unifying concepts of general mechanisms may make it possible to develop systems to

detect and quantify specific chemical activity. It remains possible that such unifying concepts are inherent in other complex biological process such as dermal irritancy or even cancer, and that such concepts will supersede the need for complete and specific knowledge of mechanism of action and permit the development of effective, general screens based on common mechanism.

References

1. Huff J. Issues and controversies surrounding qualitative strategies for identifying and forecasting cancer causing agents in the human environment. Pharmacol Toxicol 72(suppl 1):12–27 (1993).
2. Tennant RW, Margolin BH, Shelby MD, Zeiger E, Haseman JK, Spalding J, Caspary W, Resnick M, Stasiewicz S, Anderson B, Minor R. Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays. Science 236:933–941 (1987).
3. Davidson IWF, Parker JD, Beliles RP. Biological basis for extrapolation across mammalian species. Regul Toxicol Pharmacol 6:211–237 (1986).
4. Cohen SM, Ellwein LB. Risk assessment on high-dose animal exposure experiments. Chem Res Toxicol 5:742–748 (1992).
5. Boorman GA, Eustis SL, Elwell MR, Griesemer RA. Rodent carcinogenesis studies: their value and limitations. In: Assessment of inhalation hazards (Mohr U, Bates DV, Dungworth DL, Lee PN, McCellelan RO, eds). Heidelberg:Springer-Verlag, 1989; 61–68.
6. Frazier JM. Scientific perspectives on the role of *in vitro* toxicity testing in chemical safety evaluation. In: *In vitro* methods in toxicology (Jolles G, Cardier A, eds). New York:Academic Press, 1992;521–529.
7. Sobels FH. Some problems associated with the testing for environmental mutagens and a perspective for studies in comparative mutagenesis. Mutat Res 46:245–260 (1977).
8. Sobels FH. Evaluating the mutagenic potential of chemicals: the minimal battery and extrapolation problems. Arch Toxicol 46:21–30 (1980).
9. Sobels FH. Environmental mutagenesis in retrospect. Mutat Res 181:299–310 (1987).
10. Conolly RB, Andersen ME. Biologically-based pharmacodynamic models: tools for toxicological research and risk assessment. Ann Rev Pharmacol Toxicol 31:503–523 (1991).
11. Haris CC. Human tissues and cells in carcinogenesis research. Cancer Res. 47:1–10 (1987).
12. Greenlee WF, Sutter TR, Marcus C. Molecular basis of dioxin actions on rodent and human target tissues. In: Receptor-mediated biological processes: implications for evaluating carcinogenesis, vol 387 (Spitzer HL, Slaga TJ, Greenlee WF, McClain M, eds). NewYork: Wiley-Liss, 1994;47–57.
13. Lehman-McKeeman LD. Male rat-specific light hydrocarbon nephropathy in toxicology of the kidney. In: Toxicology of the kidney (Goldstein RS, Hook, JB, eds). New York:Raven Press, 1993;477–494.
14. Smith BJ, Sipes IG, Stevens JC, Halpert JR. The biochemical basis for the species difference in hepatic microsomal 4-vinylcyclohexene epoxidation between female mice and rats. Carcinogenesis 11:1951–1957 (1990).
15. Wogan GN. Molecular epidemiology in cancer risk assessment and prevention: recent progress and avenues for future research. Environ Health Perspect 98:167–178 (1992).
16. Groopman JD, Kensler TW. Molecular biomarkers for human chemical carcinogen exposures. Chem Res Toxicol 6:764–770 (1993).
17. Bruner LH. Alternatives to the use of animals in household products and cosmetic testing. J Am Vet Med Assoc 200:669–673 (1992).
18. Green S, Bradlaw J. Regulatory law and the use of *in vitro* methods for the assessment of various toxicities. In: *In vitro* toxicity testing. (Frazier JM, ed). New York:Marcel Dekker, 1992;281–293.
19. Klopman G, Rosenkranz HS. Approaches to SAR in carcinogenesis and mutagenesis-prediction of carcinogenicity/mutagenicity using MULTI-CASE. Mutat Res 305:33–46 (1994).
20. National Research Council Committee on Biological Markers. Biological markers in environmental health research. Environ Health Perspect 74:3–9 (1987).

New Books

Analytical Chemistry for Technicians, 2nd ed.
John Kenkel
Boca Raton, FL: Lewis Publishers 1994, 592 pp. ISBN: 0873719662, $69.95.

Big Green Circus; Inside the Environmental Movement
Charles Galvin Murphy, Jr.
Gulf Breeze, FL: Paw Mark Publications, 1994, 480 pp. ISBN: 096404840X, $19.95.

Biological Effects of Electric and Magnetic Fields, vol. I
David O. Carpenter, Sinerik Ayrapetyan
San Diego, CA: Academic Press, 1994. ISBN: 0121602613, $99.

Bioremediation
Paul E. Flathman, Douglas E. Jerger, Jurgen H. Exner
Boca Raton, FL: Lewis Publishers 1994, 560 pp. ISBN: 0873717406, $79.95.

Butadiene and Styrene; Assessment of Health Hazards
M. Sorsa, et al.
Lyon: International Agency for Research on Cancer, 1994, 440 pp. ISBN: 9283221273, $95.

Contaminants in the Environment: A Multidisciplinary Assessment of Risks to Man and Other Organisms
Aristeo Ranzoni, et al., eds.
Boca Raton, FL: Lewis Publishers, 1994, 286 pp. ISBN: 0873718534, $89.95.

Defending the Land of the Jaguar; A History of Conservation in Mexico
Lane Simonian
Austin, TX: University of Texas Press, 1995. ISBN: 029277690X, no price available.

Elements of Environmental Engineering; Thermodynamics and Kinetics
K. T. Valsaraj
Boca Raton, FL: Lewis Publishers 1995, ISBN: 1566700892, no price available.

Environment and History; The Taming of Nature in the USA and South Africa
William Beinart, Peter Coates
New York: Routledge, 1995. ISBN: 0415114683, $12.95.

Environmental Medical Emergencies
David J. Steedman
New York: Oxford University Press, 1994, 180 pp. ISBN: 0192623931, (cloth acid-free), $59.95. 0192623923 (paper acid-free), $25.00.

Field Sampling Methods for Remedial Investigations
Mark E. Byrnes
Boca Raton, FL: Lewis Publishers 1994, 272 pp. ISBN: 0873716981, $69.95.

Global Ecosystems; Creating Options through Anthropological Perspectives
Pamela J. Puntenney, ed.
Arlington, VA: American Anthropological Association, 1995. ISBN: 0913167703, no price available.

Handbook of Chemistry and Physics, 75th ed.
David R. Lide
Boca Raton, FL: Lewis Publishers 1994, 2624 pp. ISBN: 084930475X, $99.50.

Look to the Mountain; An Ecology of Indigenous Education
Gregory Cajete
Durango, CO: Kivaki Press, 1994, 288 pp. ISBN: 1882308654, $16.95.

Male-Mediated Developmental Toxicity
Andrew F. Olshan, Donald R. Mattison, eds.
New York: Plenum Press, 1994, 298 pp. ISBN: 0306448157, $115.

Managing the Environment; The Role of Economic Instruments
Organisation for Economic Co-operation and Development
Washington, DC: OECD Publications and Information Centre, 1994, 192 pp. ISBN: 9264141367, $34.

Methods of Pesticide Movement into Ground Water; Occurence, Behavior, and Regulation
Richard C. Honeycutt, Daniel J. Schabacker
Boca Raton, FL: Lewis Publishers 1994, 208 pp. ISBN: 0873719263, $69.95

The Natural Contract
Michel Serres
Ann Arbor: University of Michigan Press, 1995, 128 pp. ISBN: 0472095498, (cloth, alk. paper), $39.50. 0472065491 (paper, alk. paper), $14.95.

Neurobehavioral Methods and Effects in Occupational and Environmental Health
Shunichi Araki, ed.
San Diego, CA: Academic Press, 1994, 869 pp. ISBN: 0120597853, $49.

Textbook of Clinical Occupational and Environmental Medicine
Linda Rosenstock, Mark R. Cullen, eds.
Philadelphia, PA: Saunders, 1994, 909 pp. ISBN: 0721634826, $145.

The Vulnerable Brain and Environmental Risks, vol. I
Robert L. Isaacson, Karl F. Jensen
New York: Plenum Press, 1994, 290 pp. ISBN: 0306441489, $65.

Wetland Economics
Jay A. Leitch, Herbert R. Ludwig Jr.
Westport, CT: Greenwood Publishing Group Inc., 1995, 152 pp. ISBN: 0313292868, $65.

When Is Life Too Costly to Save? The Evidence from U.S. Environmental Regulations
George L. Van Houtven, Maureen L. Cropper
Washington, DC: World Bank, 1994. Free.

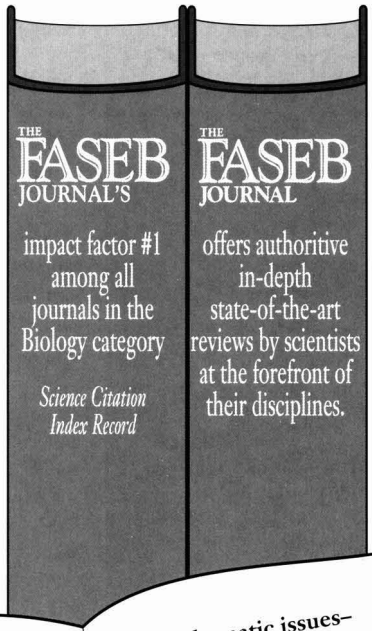
Xenobiotics and Inflammation
Lawrence B. Schook, Debra L. Laskin, eds.
San Diego, CA: Academic Press, 1994, 361 pp. ISBN: 0126289301, $99.

YOUR LIBRARY IS NOT COMPLETE...

without a subscription to

THE
FASEB JOURNAL

A library in itself and the #1 review and communication biology journal.



key biological areas...

- ANATOMY
- BIOCHEMISTRY
- BIOPHYSICS
- CELL BIOLOGY
- DEVELOPMENTAL BIOLOGY
- GENETICS
- IMMUNOBIOLOGY
- MOLECULAR BIOLOGY
- NEUROBIOLOGY
- NUTRITION
- PATHOLOGY
- PHARMACOLOGY
- PHYSIOLOGY

FEATURES

- ✦ Invited reviews
- ✦ Innovations in methodology
- ✦ Original communications
- ✦ Articles covering public affairs
- ✦ News items about research funding people, and institutions
- ✦ Calendar of Scientific Events
- ✦ New Products
- ✦ Employment Opportunities
- ✦ Hypotheses Section

PLUS 2 thematic issues–
Structural Immunology
Vascular Biology

PLUS 3 abstract issues from–
- ✦ Experimental Biology 95 APS, ASPET, ASIP, AAI, AIN, AAA
- ✦ ASBMB/DBC-ACS 1995 Annual Meeting

PLUS the Journal is 100% guaranteed!
We are so certain of your satisfaction, that should you become dissatisfied, you may cancel your subscription at any time and receive a refund for the unused portion.

SUBSCRIPTION RATES:		ADD FOREIGN POSTAGE:	
FASEB Society Members	$ 47	*Expedited delivery except*	
Students	$ 47	*U.S., Canada & Mexico.*	
Nonmembers	$110	Canada & Mexico	$ 20
Institutions	$295	Elsewhere	$ 48

Maryland residents add 5% sales tax
D.C. residents add 6% sales tax
Canadian subscribers add GST number + 7%

For Rates in Japan, contact USACO, Corp., 13-12 Shimbashi 1-Chome, Minato-Ku, Tokyo, 105 Japan; Tel: 011-81-33-502-6473 Fax: 011-81-33-593-2709

☐ VISA ☐ Mastercard

Card Number: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Expiration Date _____

Cardholder's Name _____

Cardholder's Signature _____

Cardholder's Phone Number _____

THE FASEB JOURNAL

SUBSCRIBE

TO EXPEDITE YOUR ORDER: Charge by FAX: 301-530-7001
Questions? Call 800-43-FASEB ext. 7027

☐ ***YES!* Please enter my 1995 subscription to *The FASEB Journal***

(Please print clearly)

NAME _____ TITLE _____

ORGANIZATION _____ DEPARTMENT _____

ADDRESS _____ BLDG/ROOM _____

CITY _____ STATE _____ ZIP _____

POSTAL CODE _____ COUNTRY _____

☐ Check enclosed. *U.S. currency drawn on U.S. bank only.*

Make checks payable to: ***The FASEB Journal.***
Charge & fax your order OR mail your check to: ***The FASEB Journal***
9650 Rockville Pike, Bethesda, MD 20814-3998 U.S.A.

Calendar

If you have a Calendar, Fellowships, Grants, & Awards, or Position Announcements item you would like included, follow the instructions below.

For a Calendar Event

Please provide the name of the event, the dates, location, and who to contact for further information including FAX number and BITNET/Internet address if possible. The entries in this section are brief. If you would like us to advertise additional information about your event, such as an overview of the contents and speakers, we have limited space available for public service advertising.

Fellowships, Grants, & Awards or Position Announcements

Please provide a concise description of the fellowship, grant, or award requirements or the position announcement including the application address and deadline. Send an electronic version via electronic mail or disk if possible.

Public Service Advertising

On a space-available basis, we will accept your public service announcements in a variety of formats. We can accept full, half, and one- or two-column camera-ready ads. If you do not have a formal ad but can provide an electronic version of your text via electronic mail or on a computer disk, we will include it for a limited time in the journal.

How to Submit Material to EHP

For camera-ready copy, computer disk, or text submissions, send your material to:

EHP Announcements
NIEHS/EHP
MD WC-01
PO Box 12233
Research Triangle Park, NC 27709

FAX (919) 541-0273
BITNET/Internet address: niehs.nih.gov.ehp_annc

May

Gene Therapy
May 1–3, Mon–Wed
Crystal Gateway Marriott Hotel, Arlington, Virginia
Information: Ben Keddy, Cambridge Healthtech Institute, Bay Colony Corporate Center, 1000 Winter Street, Suite 3700, Waltham, MA 02154
(617) 487-7989
FAX (617) 487-7937

Pharmacogenetics Optimizing Drug Discovery and Development
May 4–5, Thu–Fri
Hyatt Regency Bethesda, Bethesda, Maryland
Information: IBC USA Conferences Inc., 225 Turnpike Road, Southborough, MA 01772-1749
(508) 481-6400
FAX (508) 481-7911

The Clinical Research Meeting
May 5–8, Fri–Mon
San Diego Convention Center, San Diego, California
Information: Registration Manager
6900 Grove Road
Thorofare, NJ 08086
(609) 848-1000

Lead in the Americas: Strategies for Disease Prevention Symposium
May 8–10, Mon–Wed
Cuernavaca, Mexico
Information: Mauricion Hernandez-Avila Centro de Investigaciones en Salud Publica, Instituto Nacional de Salud Publica, Av. Universidad 655, Col. Sta. Maria Ahuacatitlan, Cuernavaca, Morelos, C.P. 62508 Mexico
FAX 52 73 11 11 48

New England Environmental Expo
May 9–11, Tue–Thu
Boston's Harborside World Trade Center, Belmont, Massachusetts
Information: New England Environmental EXPO, 352 Trapelo Road, Belmont, MA 02178-1891
(617) 489-2302
FAX (617) 489-5534

Conference on Environmental Bone-Seeking Agents
May 12–15, Fri–Mon
Park City, Utah
Information: H. Clarke Anderson, Department of Pathology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160-7410
(913) 588-7070
FAX (913) 588-7073

Toxicology Mechanisms
May 15–16, Mon–Tue
San Francisco, California
Information: Ben Keddy, Cambridge Healthtech Institute, Bay Colony Corporate Center, 1000 Winter Street, Suite 3700, Waltham, MA 02154
(617) 487-7989
FAX (617) 487-7937

American Thoracic Society International Conference
May 20–24, Sat–Wed
Seattle, Washington
Information: 1995 International Conference American Thoracic Society, 1740 Broadway New York, NY 10019-4374

International Conference on Ribosomes
May 20–25, Sat–Thu
Victoria, BC, Canada
Information: A. Matheson, Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, V8W 2Y2 Canada

ASMB Joint Annual Meeting with the Division of Biological Chemistry of the American Chemical Society
May 21–25 Sun–Thu
San Francisco, California
Information: FASEB Office of Scientific Meetings and Conferences, 9650 Rockville Pike, Bethesda, MD 20814-3998

III Ural Atomic Symposium: Human Health and Ecology of Radioactive Contamination in the Ural Mountains of Russia
May 29–June 2 Mon–Fri
Zarechny/Koltsovo, Russia
Information: Victor Chuckanov, Institute of Industrial Ecology, UB RAS 91, Pervomayskaya St. 620219, Ekaterinburg, Russia
(7) 3432-44-59-62
FAX (7) 3432-44-07-71
e mail alpha@ecko. rcupi.e-burg.su

June

Cancer Prognosis Markers
June 7–8, Wed–Thu
Crystal City Marriott Hotel, Arlington, Virginia
Information: Ben Keddy, Cambridge Healthtech Institute, Bay Colony Corporate Center, 1000 Winter Street, Suite 3700, Waltham, MA 02154
(617) 487-7989
FAX (617) 487-7937

14th Annual International Symposium of The Society of Toxicologic Pathologists
June 12–14, Mon–Wed
San Diego Marriott Hotel & Marina, San Diego, California
Information: Society of Toxicologic Pathologists, 875 Kings Highway, Suite 200,
Woodbury, NJ 08096-3172
(609) 845-1720

Second International Conference on Arsenic Exposure and Health Effects
June 12–14, Mon–Wed
Holiday Inn on The Bay at Embarcadero, San Diego, California
Information: Willard R. Chappell, CB 136,University of Colorado at Denver, PO Box 173364
Denver, CO 80217-3364
(303) 556-4520
FAX (303) 556-4292

International Benzene Conference
June 17–20, Sat–Tue
Rutgers University Campus, New Brunswick, New Jersey
Information: Jill Braun, Environmental and Occupational Health Sciences Institute, 681 Frelinghuysen Road, PO Box 1179,
Piscataway, NJ 08855-1179
(908) 932-9271
FAX (908) 932-8726

Joint International Congress on Minimally Invasive Techniques in Neurosurgery and Otolaryngology
June 17–20, Sat–Tue
David L. Lawrence Convention Center, Pittsburgh, Pennsylvania
Information: Allegheny General Hospital Continuing Medical Education, 320 East North Avenue, Pittsburgh, PA 15212
(412) 359-4952
FAX (412) 359-8218

First Interdisciplinary Conference on the Environment
June 21–25, Wed–Sun
Park Plaza Hotel and Towers, Boston, Massachusetts
Information: Demetri Kantarelis, IEA, Economics/Foreign Affairs Department, Assumption College, 500 Salisbury Street,
Worcester, MA 01615-0005
(508) 752-5615 ext 557
FAX (508) 799-4502

Cytokines and Adhesion Molecules in Lung Inflammation
June 22–23, Thu–Fri
Institut Pasteur, Paris, France
Information: Unité de Pharmacologie Cellulaire, Unité Associée IP/INSERM no. 285, Institut Pasteur, 25 Rue du Dr. Roux
75015 Paris, France
(33-1) 45.68.86.82
(33-1) 45.68.87.03

Indoor Air Quality, Immunity and Health
June 22–23, Thu–Fri
McKimmon Center, North Carolina State University, Raleigh, North Carolina
Information: Rodney Dietert
213 Rice Hall,
Cornell University,
Ithaca, NY 14853-5601
(607) 255-7789

SETAC-Europe 1995 Congress: Environmental Science and Vulnerable Ecosystems
June 25–28, Sun–Wed
Copenhagen, Denmark
Information: DIS Congress Service
DK-2730 Herlev, Denmark
45-4492-4492
FAX 45-4492-5050

Risk Assessment of Polycyclic Aromatic Hydrocarbons in the Environment
June 26–28, Mon–Wed
Hyatt Regency Airport Hotel, San Francisco, California
Information: Alex Taylor, JACA Corporation
550 Pinetown Road
Fort Washington, PA 19034
(215) 643-5466
FAX (215) 643-2772

International Symposium on Peroxisomes: Biology and Role in Toxicology and Disease
June 28–July 2, Wed–Sun
The Aspen Institute, Aspen, Colorado
Information: Nancy Starks, Department of Pathology, W127, Northwestern University Medical School, Ward Building, Room 6-204,
303 East Chicago Avenue,
Chicago, IL 606011-3008
(312) 503-8144
FAX (312) 503-8240

July

VII International Congress of Toxicology—Horizons in Toxicology: Preparing for the 21st Century
July 2–6, Sun–Thu
Seattle, Washington
Information: ICT–VII Management Support Staff, The Sterling Group, 9393 W. 110th Street, Suite 253, Overland Park, KS 66210
(913) 345-2228
FAX (913) 345-0893

Modulators of Immune Response; Hiking up the Evolutionary Trail
July 8–15, Sat–Sat
Beaver Run Resort and Conference Center, Breckenridge, Colorado
Information: Joanne Stolen, SOS Publications,
43 DeNormandie Avenue,
Fair Haven, NJ 07704-3303
(908) 530-3199
FAX (908) 530-5896

Eighth International Conference of the International Federation of Science Editors
July 9–12, Sun–Wed
Barcelona, Spain
Information: IFSE-8 Secretariat, Apartado 16009, E-08080 Barcelona, Spain

---

Mid-Atlantic Industrial and Hazardous Waste Conference
July 9–12, Sun–Wed
Lehigh University, Bethlehem, Pennsylvania
Information: Arup K. Sengupta, Office of National Media Relations, 436 Brodhead Avenue, Bethlehem, PA 18015
(610) 758-3171
FAX (610) 758-4522

Vth COMTOX Symposium on Toxicology and Clinical Chemistry of Metals
July 10–13, Mon–Thu
Vancouver, BC, Canada
Information: Secretariat, F. William Sunderman Jr., Departments of Laboratory Medicine and Pharmacology, University of Connecticut Medical School, PO Box 1292, Farmington, CT 06034-1292
(203) 679-2328
FAX (203) 679-2154

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

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

October

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

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 00202-700931

December

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

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

Request for pre-proposals

The Washington State Department of Labor & Industries invites investigators from the United States and Canada to submit pre-proposals for research on chemically related illness (CRI). The department anticipates receiving a legislative appropriation of $1.3 million to fund CRI research in the biennium beginning July 1, 1995 and ending June 30, 1997.

The department has particular interest in supporting studies that clarify the etiology, diagnosis, natural history, and treatment of chemically related illness including but not limited to multiple chemical sensitivity syndrome. Priority will be given to:

1. **Proposals that focus on issues particularly relevant to the people of Washington.** In addition to common chemical exposures, investigators should consider workplace exposures from industries important to Washington, for example: Aerospace, Agriculture, Construction, Health Care Services, High Technology, Logging/Wood Products, Manufacturing, Paper Processing, Public Employees, Retail Trades, Transportation.

2. **Studies involving human subjects.** Animal studies and research limited to the laboratory will be considered only if expected results are clearly relevant to CRI in humans.

3. **Studies with a completion date prior to June 30, 1997.** Investigators should expect to present their findings at a conference in Washington in the spring of 1997.

It is anticipated that up to ten research studies will be funded. In addition to larger scale projects, applicants with pilot projects costing less than $50,000 are encouraged to apply. Indirect costs cannot exceed 27.3%.

Interested investigators are invited to submit a three-page pre-proposal including a preliminary budget and a curriculum vitae by May 22, 1995. Forms for the preparation of pre-proposals and additional information on requirements for applicants can be obtained by written, fax, or E-MAIL request from:

Dr. Gary Franklin, Medical Director, Department of Labor & Industries,
PO Box 44321, Olympia, WA 98504-4321
(FAX 360-902-4249 or E-Mail: MCG5235@ LNI.WA.GOV)

Research pre-proposals must be received by 5 p.m. May 22, 1995. After review by the CRI Scientific Advisory Committee in May, selected investigators will be asked to submit full proposals for final review. Funding for successful proposals is anticipated to occur by September/October 1995.

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

Great Lakes Protection Fund Call for Preproposals

In order to assist potential applicants in planning and coordinating grant requests, the Great Lakes Protection Fund announces adoption of two fixed dates for submission of preproposals–January 2 and July 1. These annual deadlines for general calls for preproposals will apply in 1995 and future years. The fund may also issue a limited call for preproposals to target a specific topic or topics within one of the fund's four goals.

Eligibility: The Fund's priority applicants are nonprofit agencies; however, individuals and proprietary entities may apply if a clear public benefit can be demonstrated and if financial benefits stemming from the proposed work accrue to the public good. Successful applicants must maintain open access to project data, records and financial information. Results must be disseminated so that they are readily accessible to others.

Preproposal Application and Evaluation Process: The two-page preproposal is the first of two steps in the fund's proposal review process. The second step is an invitation to submit a full proposal based upon favorable evaluation of the preproposal.

Preproposals are evaluated strictly against the fund's mission and must address one of the fund's four goals. Proposed projects must be appropriately collaborative among the private, public and independent sectors. The fund seeks to support projects which are supplemental and non-duplicative of other efforts. For multi-year projects, the fund may issue challenge grants to encourage supplemental contributions.

Staff reviews the preproposals and makes recommendations to the fund's grant making committee of the Board of Directors. Preproposals are not sent to outside technical reviewers. Full proposals, however, are sent to at least three independent technical reviewers.

Preproposal Deadline: Preproposals must be received in the office by 5:00 pm Central Time, January 2, 1995. Preproposals received after that date will be considered with preproposals submitted for the July 1, 1995 deadline. *There are no exceptions to these deadlines.*

The fund also supports efforts to promote collaboration, coordination and regional action through planning and discretionary travel grants. For more information on these grants, please contact the fund:

Preproposal Application
Great Lakes Protection Fund
35 East Wacker Drive, Suite 1880
Chicago, IL 60601

Research Announcement for Experimental and Theoretical Studies as Part of the Global Troposhperic Experiment's Pacific Exploratory Mission in the Tropics (PEM-Tropics)

This NASA Research Announcement (NRA) solicits research proposals for the experimental investigations and theoretical studies that will compose the Pacific Exploratory Mission in the central and eastern regions of the tropical Pacific Ocean basin (PEM-Tropics). PEM-Tropics will be conducted as part of NASA's Global Tropospheric Experiment (GTE). The GTE is an ongoing element of the Troposhperic Chemistry Program, a Research and Analysis (R&A) program within the Science Division of NASA's Office for Mission to Planet Earth (OMTPE). The long range goal of the GTE is to contribute substantially to scientific understanding of human impacts on the chemistry of the global troposphere.

The GTE PEM-Tropics mission will utilize both the NASA DC-8 and P3-B aircraft in a coordinated project to study the chemistry of the troposphere over the central and eastern Pacific Ocean with a focus on the tropics. This relatively unexplored region of the troposhere is an outstanding "laboratory" for studying the role of nitrogen oxides in troposheric ozone formation and loss, a problem that has important climate implications. It is expected to yield important new information on chemical changes that are affecting the oxidizing power of global troposhere and, therefore, the rate at which the global atmosphere can cleanse itself of pollutants emitted into it by human activities.

The purpose of this NRA is to solicit proposals for 1) the experimental investigations that will compose the payload to be carried by the two aircraft and 2) for analytical and process-oriented theoretical studies based on the data to be acquired. Because the meteorological context is quite critical to understanding the chemical data, proposals are also solicited to provide analyses of meteorological

information expected to be available from the worldwide meteorological community and instruments aboard the aircraft. The meteorological analyses to be proposed should be directed toward the characterization of the air masses in which the aircraft operate with respect to their origin and transport.

Experimental investigations to be proposed should be based largely on existing capabilities and should be capable of operation aboard the aircraft. They should have detection limits low enough to operate in the clean air environment of the tropical Pacific Ocean basin. Experimental investigators may propose for participation aboard one or both aircraft.

The selected experimental and theoretical investigators will form a Science Team that will do final planning for the mission and lead its field implementation. The Science Team will be chaired by one or more Mission Scientists. A Mission Meteorologist will be selected for each of the two airplanes who will be responsible for meteorological forecasting for the flight operations and for providing input on the origins and destinations of the air masses encountered during the flights. Proposals for Mission Scientist(s) and Mission Meteorologist(s) are solicited, but such proposals should be a part of a broader experimental, theoretical, or data analysis proposal to participate in the PEM-Tropics mission. Theoretical investigations may be proposed for process oriented studies based on the data to be obtained.

Proposals for extensive instrument development are not solicited by this NRA, nor are proposals that would require extensive model development or the acquisition of significant computer hardware and software in order to complete that proposed investigations. Proposals for ground-based or ship-based experiments that would provide important supporting data for the aircraft investigations are invited, but NASA does not expect to provide research ship support, nor is a long-term ground-based monitoring project a part of the GTE PEM-Tropics mission. Proposals for extensive instrument development, model development, and long term ground based measurements will be nonresponsive to this NRA and will not be considered.

Participation in this program is open to all categories of domestic and foreign organizations, including educational institutions, industry, nonprofit institutions, NASA research centers, and other government agencies. Applications for participation in this program can be made through submission of a proposal to the Science Division of OMTPE, National Aeronautics and Space Administration Headquarters, Washington, DC. After a review and evaluation of the proposals received, a selection of the experimental and theoretical investigations to be supported will be made by the Director of the Science Division. Financial support of the selected investigations will be provided by NASA. The initial proposal review will be conducted from mid-May through July, 1995, with selection anticipated in August, 1995. To allow adequate time for evaluation and selection, proposals must be submitted by May 16, 1995. Participation by non-U.S. investigators is encouraged.

Important guidelines specific to this Research Announcement can be obtained from Selecting Official: Director Science Division, OMTPE.

Robert J. McNeal
Manager Tropospheric Chemistry
Program Code YSM
NASA Headquarters
Washington, DC 20546
(202) 358-0239
FAX (202) 358-2771
e-mail jmcneal@mtpe.hq.nasa.gov
or
James M. Hoell
GTE Project Manager
Mail Stop 483
NASA Langley Research Center
Hampton, VA 23681-0001
(804) 864-5826
FAX (804) 864-5841
e-mail j.m. hoell@larc.nasa.gov

Identifier NRA-95-MTPE-01

Proposals should be submitted by mail to:
Tropospheric Chemistry Program
Code YSP-44 (REF: GTE/PEM-TROPICS)
National Aeronautics and Space Administration
300 E Street SW
Washington, DC 20546

Proposals sent by express or commercial deliver should substitute 20024 for the above ZIP code.

NASA welcomes proposals from entities located outside the U.S. in response to this NRA. Foreign Participants should contact:
NASA Headquarters
Office of External Relations,
Mission to Planet Earth Division,
Code IY
300 E Street SW
Washington, DC 20546.

The National Toxicology Program (NTP)
Announces a Public Meeting of the
NTP Board of Scientific Counselors Ad Hoc Working Group
to review criteria for listing substances in the *Biennial Report on Carcinogens*
and to receive public comments on the criteria

April 24–25, 1995
Washington Hilton, 1919 Connecticut Avenue, NW
Washington, DC

To register to attend, make oral comments, submit written comments, or receive the background document provided to the working group, contact:
C.W. Jameson, NIEHS, MD WC-04, PO Box 12233, Research Triangle Park, NC 27709
Phone (919) 541-4096 • Fax (919) 541-2242

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.

Effects of Melatonin on Cell Biology (HNV90)
Gloria Jahnke
(919) 541-3376
Laboratory of Molecular Carcinogenesis,
Maildrop C3-03
The effect of melatonin on the regulation of growth and survival of normal and neoplastic breast epithelial cells is being investigated. Emphasis is being placed on signal transduction pathways that may be affected by melatonin. Applicants should have training in toxicology, cell biology and biochemistry.

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.

National Institutes of Health
Public Health Service
National Institute of Environmental Health Sciences

Applications wanted for Research on the Biological Effects of EMFs

In section 2118 of the 1992 Energy Policy Act, the National Institute of Environmental Health Sciences (NIEHS), an institute of the National Institutes of Health (NIH), has been designated as the lead agency for coordinating and conducting the health effects studies on the possible adverse health effects, if any, of exposure to 60 Hz electric and magnetic fields (EMFs) from the generation, transport and use of electricity. As part of the national program, the NIEHS is required to competitively solicit and select applications to conduct research and communication activities. With the funds provided by this act, the NIEHS established a Research and Public Information Dissemination (RAPID) Program to meet the obligations of this law. In fiscal year 1995 (FY95), the NIEHS is again soliciting grant applications for the NIH Program Announcement 91-53, Research on the Effects of Power Frequency Electric and Magnetic Fields.

PRIORITY AREAS FOR THE FY 95 RAPID PROGRAM

In the FY95, the NEIHS RAPID Program is encouraging investigators to pursue research areas which may be important for understanding the biological actions and possible health effects of exposure to 60Hz EMFs. The following have been identified as priority research areas:

1. Identification and characterization of any biological mechanisms that might lead to adverse health effects in animal models (*in vivo* studies).
2. Identification of any significant changes in normal biological function caused by EMFs that may be plausibly involved in the processes leading to leukemia, breast and brain cancer, and reproductive and neurologic dysfunctions.
3. Validation (and to the extent possible, replication) of bioeffects reported in the peer-reviewed literature related to the mechanisms underlying the diseases listed in item 2.
4. Use of laboratory-based approaches to identify and characterize the specific physical characteristics of EMFs that might be biologically active.
5. Development of scientifically sound risk assessment models and criteria for determining the human health hazards of EMFs.

EMF EXPOSURE SYSTEMS

Many of the problems with exposure systems that have hindered the participation of investigators wishing to pursue research on this problem have been minimized with the recent development of commercial exposure commercial exposure units for *in vitro* and *in vivo* studies. NIEHS can provide investigators a list of commercial vendors and engineers who have expertise and information about EMF exposure units. In addition, NIEHS, through the Department of Energy (DOE), is willing to assist successful investigators by providing advice, helping to identify potential problem areas, and visit laboratories to monitor the functioning of exposure units. NIEHS and DOE have also established regional exposure facilities staffed with knowledgeable investigators who may also be able to provide advice on exposure systems to investigators interested in working in this field.

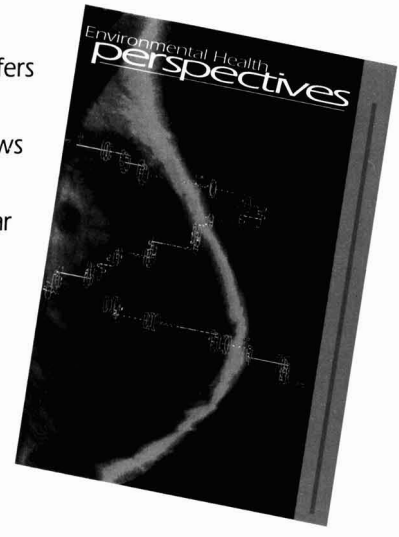
Application receipt date for FY95:
June 1, 1995

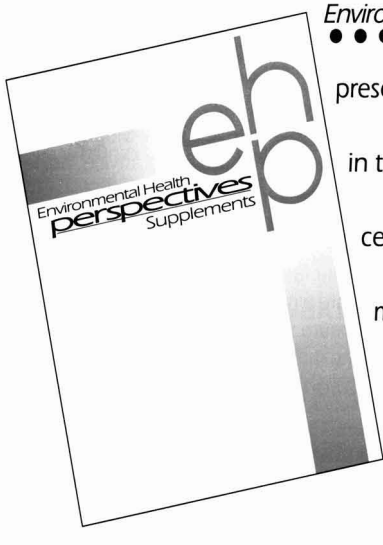
A copy of the program announcement can be obtained by either writing or faxing your mailing address to:

Dr. Michael J. Galvin, Program Administrator
Division of Extramural Research and Training
National Institute of Environmental Health Sciences
Research Triangle Park, NC 27709
Fax (919) 541-2843 Phone (919) 541-3319

Subscription Information

Environmental Health Perspectives offers cutting-edge research articles and news of the environment. To receive one year of *EHP*, fill out the upper form on the facing page.





Environmental Health Perspectives Supplements presents state-of-the-art information in the form of monographs, conference proceedings, and an annual review of environmental science. To receive *EHP Supplements*, fill out the lower form.

Order Processing Code:
*5391

Superintendent of Documents Subscription Order Form

Charge your order. It's easy! MasterCard VISA

To fax your orders (202) 512-2250

To phone your orders (202) 512-1800

☐ **YES,** please send me _____ subscriptions to **Environmental Health Perspectives** (EHPM) at $36 ($45 foreign) per year so I can get cutting-edge research articles and news of the environment.

The total cost of my order is $_____. Price includes regular shipping and handling and is subject to change.

_____
Company or personal name (Please type or print)

_____
Additional address/attention line

_____
Street address

_____
City, state, ZIP code

_____
Daytime phone including area code

_____
Purchase order number (optional)

For privacy, check box below:

☐ Do not make my name available to other mailers.

Check method of payment:

☐ Check payable to the Superintendent of Documents

☐ GPO Deposit Account ☐☐☐☐☐☐–☐

☐ VISA ☐ MasterCard

☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

☐☐☐☐ (expiration date) *Thank you for your order!*

_____
(Authorizing signature) (4/95)

Mail To: Superintendent of Documents
P.O. Box 371954, Pittsburgh, PA 15250-7954

Order Processing Code:
*5572

Superintendent of Documents Subscription Order Form

Charge your order. It's easy! MasterCard VISA

To fax your orders (202) 512-2250

To phone your orders (202) 512-1800

☐ **YES,** please send me _____ subscription(s) to the *Monthly Edition* of **Environmental Health Perspectives Supplements** (EHP) at $79 ($98.75 foreign) per year so I get state-of-the-art information in the form of monographs, conference proceedings, and an annual review of environmental health.

The total cost of my order is $_____. Price includes regular shipping and handling and is subject to change.

_____
Company or personal name (Please type or print)

_____
Additional address/attention line

_____
Street address

_____
City, state, ZIP code

_____
Daytime phone including area code

_____
Purchase order number (optional)

For privacy, check box below:

☐ Do not make my name available to other mailers.

Check method of payment:

☐ Check payable to the Superintendent of Documents

☐ GPO Deposit Account ☐☐☐☐☐☐–☐

☐ VISA ☐ MasterCard

☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

☐☐☐☐ (expiration date) *Thank you for your order!*

_____
(Authorizing signature) (4/95)

Mail To: Superintendent of Documents
P.O. Box 371954, Pittsburgh, PA 15250-7954

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 100 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.

In This Issue

U. S. Department of Health & Human Services
Public Health Service
National Institutes of Health
NIH Publication 95-218

Official Business
Penalty for Private Use $300

Bulk Rate
U.S. Postage
PAID
Permit 2058
St. Louis, Mo.