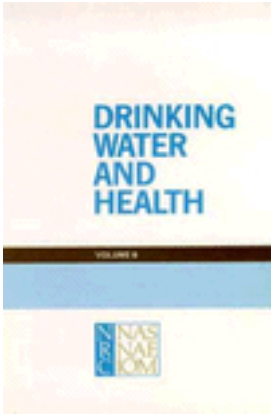

Drinking Water and Health, Volume 6

Safe Drinking Water Committee, Board on Toxicology and Environmental Health Hazards, National Research Council

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Drinking Water and Health

Volume 6

Richard D. Thomas, Study Director and Editor

Safe Drinking Water Committee
Board on Toxicology and Environmental Health Hazards
Commission on Life Sciences
National Research Council

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List of Participants

Safe Drinking Water Committee

DANIEL B. MENZEL (*Chairman*), Duke University Medical Center, Durham, North Carolina
BYRON E. BUTTERWORTH (*Vice Chairman*), Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina
MELVIN E. ANDERSEN, Air Force Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio
JAMES V. BRUCKNER, University of Georgia College of Pharmacy, Athens, Georgia
SEYMOUR L. FRIESS, Drill, Friess, Hays, Loomis & Shaffer, Inc., Arlington, Virginia
MICHAEL A. GALLO, University of Medicine and Dentistry of New Jersey-Rutgers Medical School, Piscataway, New Jersey
JOHN N. HATHCOCK, Iowa State University, Ames, Iowa
CLARK W. HEATH, JR., Emory University School of Medicine, Atlanta, Georgia
MICHAEL E. HOGAN, Princeton University, Princeton, New Jersey
DAVID J. JOLLOW, Medical University of South Carolina, Charleston, South Carolina
JEANNE M. MANSON, Smith Kline & French Laboratories, Philadelphia, Pennsylvania
FREDERICA P. PERERA, Columbia University School of Public Health, New York, New York
MARVIN A. SCHNEIDERMAN, Uniformed Services University of the Health Sciences, Bethesda, Maryland
PETER S. SPENCER, Albert Einstein College of Medicine, Bronx, New York

Advisors, Consultants, and Contributors

EILEEN HAYES, University of Medicine and Dentistry of New Jersey—Rutgers Medical School, Piscataway, New Jersey

DEBRA LASKIN, Rutgers University College of Pharmacy, Piscataway, New Jersey

MATTHEW MILLER, Sterling-Winthrop Research Institute, Rensselaer, New York

BRADLEY SCHWAB, University of Michigan School of Public Health, Ann Arbor, Michigan

GISELA WITZ, University of Medicine and Dentistry of New Jersey—Rutgers Medical School, Piscataway, New Jersey

PETER WORKING, Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina

National Research Council Staff

RICHARD D. THOMAS, *Study Director and Editor*
LESLYE G. WAKEFIELD, *Associate Staff Officer*
EDNA B. SMITH, *Study Secretary*
TRACY L. BRANDT, *Study Secretary*
MIREILLE J. MESIAS, *Administrative Secretary*

Environmental Protection Agency Project Officer

KRISHAN KHANNA, EPA Office of Water Supply, Washington, D.C.

Board On Toxicology and Environmental Health Hazards

GERALD N. WOGAN (*Chairman*), Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts.

DONALD HORNING (*Co-Vice-Chairman*), School of Public Health, Harvard University, Boston, Massachusetts

PHILIP LANDRIGAN (*Co-Vice-Chairman*), Environmental Sciences Laboratory, Department of Community Medicine, Mount Sinai Medical Center, New York, New York

JOHN DOULL, Department of Pharmacology, University of Kansas Medical Center, Kansas City, Kansas

HERMAN N. EISEN, Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts

EMMANUEL FARBER, Department of Pathology, University of Toronto, Toronto, Ontario, Canada

RICHARD MERRILL, School of Law, University of Virginia, Charlottesville, Virginia

EMIL PFITZER, Department of Toxicology and Pathology, Hoffmann-La Roche, Inc., Nutley, New Jersey

LIANE B. RUSSELL, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

ELLEN SILBERGELD, Toxics Program, Environmental Defense Fund, Washington, D.C.

PETER SPENCER, Institute of Neurotoxicology, Albert Einstein College of Medicine, Bronx, New York

Ex Officio Members

ROY E. ALBERT, Institute of Environmental Medicine, New York University Medical Center, New York, New York,

GARY P. CARLSON, Purdue University, School of Pharmacy and Pharmacal Science, Department of Pharmacology and Toxicology, Lafayette, Indiana

THOMAS CHALMERS, Mount Sinai Medical Center, New York, New York

ARTHUR B. DUBOIS, John B. Pierce Foundation Laboratory, New Haven, Connecticut

ALAN M. GOLDBERG, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland

MAUREEN M. HENDERSON, Department of Epidemiology, University of Washington, Seattle, Washington

ROGER O. MCCLELLAN, Lovelace Inhalation Toxicology Research Institute, Albuquerque, New Mexico

DANIEL B. MENZEL, Department of Pharmacology, Duke University Medical Center, Durham, North Carolina

NORTON NELSON, Institute of Environmental Medicine, New York University Medical Center, New York, New York

National Research Council Staff

DEVRA LEE DAVIS, *Executive Director*

JACQUELINE PRINCE, *Staff Assistant*

Preface

The Safe Drinking Water Act of 1974 (PL 93-523) mandated that the U.S. Environmental Protection Agency (EPA) establish federal standards to protect humans from harmful contaminants in drinking water. This law authorized EPA to seek the expertise of a National Research Council committee to identify the health effects associated with specified contaminants, areas of insufficient knowledge, and recommendations for future research. Since 1977, committees of the Research Council have issued five volumes of *Drinking Water and Health*, each of which includes a review of toxicological data and estimates of the risks associated with specific contaminants found in drinking water.

The most recently constituted Safe Drinking Water Committee conducted the study reported in this sixth volume of the series. At the request of EPA, the committee examined current practices in risk assessment and identified likely areas of innovation in the assessment of noncancer toxic responses and in the use of toxicokinetic data to estimate delivered dose and response. To respond to the agency's immediate need for assessments, the study also entailed literature reviews and risk assessments for 14 specific compounds of interest to EPA and the research community.

Many of the principles examined by the committee are not limited to the exposure of humans to toxicants in drinking water. In addition, discussions of the basic principles of dose-response relationships can be found throughout the various chapters. Thus, this volume should be useful as a general reference work in many areas of toxicology and public health. Developmental and reproductive effects are reviewed in Chapters 2 and 3. The temporal relationships between exposure and effect are discussed

in detail to provide a framework for the critical examination of dose-response relationships in laboratory animals. Because of the broad spectrum of possible developmental effects that can occur, the committee reviewed the pathological response mechanisms found in experimental animal studies. Chapter 2 also provides principles for extrapolating from laboratory data to determine potential developmental risks in humans.

General concern has been raised about the effect of chemicals on the reproductive ability of males and nonpregnant females. In Chapter 3, the committee addresses this issue in detail, reviewing three aspects of reproductive toxicity in females: impaired maturation of the female reproductive system, toxicity to oocytes, and alteration of the reproductive endocrine system. For males, the committee examines effects on the maturation of the male reproductive system, spermatogenesis, and fertility. Although these topics are selective, the text provides a comprehensive description of present test systems and their ability to predict human risk for reproductive toxicity. The appendix to Chapter 3 is a summary of test protocols for reproductive toxicity testing.

Many compounds have been identified as neurotoxicants in humans, and there are a number of mechanisms whereby chemicals exert neurotoxic effects. In Chapter 4, the committee examines the data on neurotoxicity in both humans and animals.

Chemical carcinogenesis is discussed extensively in Chapter 5, which describes the most useful methods of short-term assessment that can be integrated into the prediction of carcinogenesis in humans. The committee uses the principles described in Chapter 8 for the assessment of risk for chemically induced cancer in humans.

Some of the chemicals in drinking water are by-products or accidental contaminants of commercial or industrial applications. Many of the dose-response data on these substances, such as small molecular weight solvents, were obtained from inhalation exposures. In Chapter 6, using physiologically and anatomically based mathematical modeling, the committee presents a method for using inhalation exposure data in estimating the kinetics of exposure by ingestion. The approach departs from earlier methods by its dependence on anatomically based compartments.

The collection of human toxicity data through epidemiological studies is discussed in Chapter 7, which focuses on the reliability of these data in estimating human risks. Methods and limitations of such studies are reported.

The overall theme of the volume, relating dose-response relationships to estimates of human health risk, is integrated in Chapter 8, Risk Assessment. The committee presents quantitative risk assessment in concrete rather than abstract terms and delineates those areas in which uncertainty exists.

Chapter 9 is intended by the committee to serve as an example of how the principles of toxicology can be applied to quantitative analysis of risks to humans. Those principles suggested in the preceding chapters are applied whenever possible in assessing the risk from 14 chemical contaminants in drinking water. Some of the chemicals were examined in earlier volumes of *Drinking Water and Health*. Thus, only recent data are presented herein. Chemicals not previously reviewed are discussed in greater detail.

This volume could not have been completed without the generous, dedicated service of many colleagues. Richard Thomas served as study director of this volume. Leslye Giese Wakefield was professional staff associate. Marvin Schneiderman provided valuable assistance to the committee in analyzing the data used in risk assessments and providing guidance on risk assessment methodology as assessments were developed. Linda Starke provided editorial assistance to the staff and committee as the report was being written. Barbara Ream served as bibliographer. In addition, Gail Charnley, Kulbir Bakshi, Victor Miller, Edna Paulson, Andrew Pope, Mary Ellen Scheckenbach, Diane Wagener, and Chris Wendel, staff of the Board on Toxicology and Environmental Health Hazards, provided able assistance in conducting literature reviews, calculating the risks, and verifying the risk assessments. Eugenie Pascual assisted in typing the manuscript.

Special thanks are extended to Kenneth Crump and Ralph Kodell of K. S. Crump and Company, Charles Brown of the National Cancer Institute, and Murray Cohn of the Consumer Product Safety Commission, who provided the committee with both carcinogenicity risk estimates and helpful discussions of problems related to these estimates. Eileen Hayes, Debra Laskin, and Gisela Witz of Rutgers University; Matthew Miller, Sterling-Winthrop Research Institute; Bradley Schwab, University of Michigan; Robert Wolpert and Douglas Keller, Duke University; Peter Working, Paul Miller, and Thomas Starr, Chemical Industry Institute of Toxicology; and Michael Symons, University of North Carolina, served as advisers to the committee. Devra Davis, Executive Director of the Board on Toxicology and Environmental Health Hazards, provided valuable assistance in developing the program and in final completion of the study. We are grateful for the helpful advice and guidance of Frederick Robbins and Alvin Lazen, Chairman and Executive Director, respectively, of the Commission on Life Sciences. We also acknowledge the generous assistance of members of the EPA's Office of Drinking Water staff, especially Krishan Khanna, Technical Officer, Penelope Fenner-Crisp, Toxicologist, and Joseph Cotruvo, Director.

Volume 6 represents many hours of effort by my dedicated colleagues on the Safe Drinking Water Committee. Their hard work and learned

discussions are only partly recorded herein. The committee particularly wishes to recognize the contribution of Jeanne Manson to Chapters 2 and 3, Peter Spencer to Chapter 4, and Melvin Andersen to Chapter 6. My thanks to all members of the committee, consultants, collaborators, and especially to the staff and officers of the National Research Council cannot be adequately expressed.

The committee notes with regret the death of Sergio Fabro, who provided expert advice on risk assessment in the areas of reproductive and developmental toxicology for this volume. Others who assisted in this field through a workshop were Carole Kimmel, Gary Kimmel, Ellen Silbergeld, and Peter Voytek. Participating in a neurotoxicology risk assessment workshop to advise the committee were Mohamed Abou-Donia, Elizabeth Anderson, Kent Anger, Barbara Bass, Richard Butcher, Roger Cortesi, Donald Fox, John O'Donoghue, Charles Rebert, Patricia Rodier, Joseph Rodricks, and William Sette.

DANIEL B. MENZEL, CHAIRMAN
SAFE DRINKING WATER COMMITTEE

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Drinking Water and Health

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Executive Summary

Risk assessment of chemicals constitutes the linchpin for many regulatory decisions. In this sixth volume of *Drinking Water and Health*, the Safe Drinking Water Committee reviews the state of the art in the developing field of risk assessment and provides some risk assessments for drinking water exposure to 14 compounds of interest to the U.S. Environmental Protection Agency (EPA) as the agency develops its regulatory program. Reflecting the tendency of past toxicological studies to focus on cancer as the main health effect of concern, most of the risk assessments in this report were estimated for carcinogenicity. Wherever data were adequate, however, the committee also developed risk assessments for noncancer end points.

In 1977, the first volume of this series contained the advice that both carcinogenicity and other health effects should be evaluated when studying exposures to contaminants in drinking water and elsewhere. Until quite recently, however, the assessment of these noncancer diseases has remained fairly primitive, characterized by relatively little work on modeling, methods, and materials. For some common and widespread effects of exposure, such as reproductive and developmental impairments and neurotoxicity, risk assessments have been conducted only infrequently. A major portion of this report is devoted to an overview of these noncancer diseases and general advice on methods for estimating risks associated with chemical pollutants. There is also extensive discussion of the many factors that must be considered in the risk estimation process. The committee has thus laid the groundwork for innovative approaches to assessing cancer and noncancer risks, including the assessment of risks

to development, reproduction, and neurologic functioning. The committee also assessed methods for using epidemiological data in conducting risk assessments and methods for extrapolating results of laboratory animal inhalation studies to estimate human risk from ingestion. In addition to the use of safety factors, the committee has described mathematical modeling approaches currently available or under development for the assessment of noncancer risks.

DEVELOPMENTAL TOXICITY

Developmental effects encompass embryo and fetal death, growth retardation, and malformations, all of which can be highly sensitive to chemical exposures. Up to 25% of all pregnancies end in spontaneous abortion, but relatively little is known about the causes of these terminated pregnancies. When defects that become apparent only later in life are included, the frequency of major and minor malformations in the U.S. population increases to about 16%, but here again, few causes have been firmly established.

Applying the results of developmental toxicity studies to risk assessment remains problematic, because the underlying events leading to impaired development are very complex. Some of these events may be reversible, whereas others clearly are not. Moreover, the most appropriate animal models for extrapolating developmental toxicity data to humans have not yet been identified. Major factors that influence developmental toxicity include timing of exposure and different patterns of dose response for growth retardation, embryo death, and teratogenicity in laboratory animals. These must all be considered when examining data from mammalian, nonmammalian, and *in vitro* studies to estimate human risks from developmental toxicants. Risk assessors must also determine whether to select a no-observed-effect level (NOEL) or the lowest-observed-effect level (LOEL) and must choose a model for estimating the risks of developmental toxicity. These decisions depend on the extent of the data, whether data on humans are available, and the potential associated hazard. The committee recognized that several different models (e.g., the one-hit model) could be used for estimating developmental toxicity but that much additional research needs to be done to establish their relative biological reliability.

REPRODUCTIVE TOXICITY

Reproductive toxicity may affect anything within a continuum of events, ranging from germ cell formation and sexual functioning in the parents through sexual maturation of the offspring. The relationship between exposure and reproductive dysfunction is highly complex, because exposure

of the mother, father, or both may influence reproductive outcome. In addition, critical exposures may include maternal exposures long before or immediately prior to conception or exposure of the mother and fetus during gestation.

Sexual development and maturation of the male and female reproductive systems are highly susceptible to general environmental exposures, but they involve vastly different durations and stages of vulnerability. The committee noted that a variety of xenobiotic substances, including polycyclic aromatic hydrocarbons and certain antineoplastic agents (e.g., adriamycin, 5-fluorouracil, bleomycin, vincristine), have been associated with reproductive toxicity in animals, but the applicability of these findings to humans is not known, since only limited relevant studies have been conducted in humans. Risk assessment of reproductive toxicity necessarily relies on the results of *in vivo* and *in vitro* models of animal studies during the distinct stages of reproduction when important developments occur.

Mutagenic activity in the germ cells of test animals must be regarded as evidence of potential germ cell toxicity in people. Demonstrated ability to cause somatic cell mutation, combined with evidence that a substance can reach and interact with the germ cells, is evidence of potential mutagenicity in humans. Thus, the committee recommends that a battery of short-term tests, including the dominant lethal assay and the heritable translocation test, be conducted to confirm activity in germ cells. Among the factors that should be studied are DNA damage and repair in spermatocytes, sperm morphology, and distribution and binding sites. Unfortunately, there have been few attempts to develop assays for measuring chemically induced mutations in the germ cells of females.

Accurate information concerning the potential mutagenic hazards of chemicals in humans has been difficult to obtain for several reasons, but primarily because genetic alterations such as recessive mutation are not easily observed in humans. Chromosome damage in the embryo may be the undocumented and unrecognized cause of an unknown proportion of spontaneous abortions.

Two factors complicate the assessment of mutagenic risk for humans: there has been little systematic thinking about the factors that influence the determination of safety factors to be applied to experimental data, and mathematical models of the mutagenic process often cannot be applied because of insufficient experimental data. Consequently, the committee did not estimate risks of germ cell mutagenesis for the compounds reviewed in this report.

NEUROTOXICITY

The nervous system is especially vulnerable to chemical insult. This recent finding has led to an increased interest in the toxicology of that

system. More than 850 chemicals have been identified as known neurotoxicants in humans or animals; many of them are pesticides. Of the 350,000 cases of pesticide intoxication that occur worldwide annually, most have involved some form of neurotoxicity. Neurotoxicity data have served as the bases for threshold limit values (TLVs) recommended by the American Conference of Governmental Industrial Hygienists for 30% of all frequently encountered industrial chemicals.

Neurotoxicity in humans encompasses a vast variety of effects, ranging from cognitive, sensory, and motor impairments to immune system deficits. Because of this complexity, classification of chemical neurotoxic action is constantly evolving. Although tentative, broad, and subject to change as knowledge increases, a classification by cellular target and period of peak vulnerability has been proposed by the committee.

As with developmental and reproductive toxicity, major considerations affecting risk assessment for neurotoxicants include the permanence of effects, the amount and timing of exposure, the mechanisms underlying the toxicity, and heightened sensitivity of various subpopulations. Also, as for other chronic noncancer diseases, neurological impairment has rarely been studied in humans (with the important exception of lead-poisoning studies and the adverse effects of a few clinical agents). Nevertheless, there are valid *in vivo* and *in vitro* models, and others are being developed. Human neurological disorders can be modeled in many animals given doses of chemical substances for a test period simulating human exposure. In the absence of data on humans, therefore, convincing demonstration that a substance is neurotoxic in a test animal can be regarded as evidence that the agent may be neurotoxic in humans.

The application of data from studies in animals to estimate human risks of neurologic disease is complicated by several factors: major differences in the degree to which neurotoxic responses occur in animals and in humans as a result of exposures to comparable levels of a given chemical; the existence of thresholds for some end points in some species that may not be clearly expressed in other species; incompleteness of information on dose-response relationships for most environmental chemicals (unlike that for therapeutic agents); and the influence of timing and exposure patterns on outcomes. For these reasons, the committee advised caution in assessing risk for neurotoxicants and suggested that such assessments might best be applied to distinct neurologic processes and segments of the nervous system.

CARCINOGENESIS

Current theories and experimental research in animals indicate that there are at least three distinct stages in the development of carcinogenesis: initiation, promotion, and progression. Each of these stages apppears to

be influenced by such factors as age, heredity, diet, metabolic activity, and previous and current exposures to xenobiotic compounds, which may interact and potentiate, antagonize, or synergistically influence the development of carcinogenesis. The committee examined the mouse skin as a model for chemical carcinogenesis and the model's additional utility for studying the mechanisms of action of initiators and promoters. Rat and mouse liver models have also led to useful discoveries about stages of tumor development, and promotion has been found in other tissues, such as the lung and breast. Some promoters, such as asbestos, estrogens, and some constituents of cigarette smoke, appear to be tissue-specific, whereas others, such as 2-AAF (2-acetylaminofluorene), can act as promoters in the mouse bladder but as initiators in the liver of the same animal. The effect of a promoter can vary, depending on the sequence in which it is administered with the initiator. Therefore, it may be desirable to test potential promoters and inhibitors of promotion *in vivo* in several species and strains, to examine several organs for the response, and to test different sequences of administration.

Recent investigations into the role of oncogenes seem to support the multistage theory of carcinogenesis. Of the approximately 20 oncogenes thus far discovered, most have been either of two types, immortalizing genes or transforming genes, which may be regarded as the biological counterparts of initiation and promotion in tumor induction. A third type of oncogene recently identified may cause a cancer cell to metastasize. Ideally, then, the risk modeler should consider at least three stages of tumor induction to be consistent with human and experimental evidence. Risk models must also take into account the diversity of promoting agents and their multiplicity of action at the cellular level.

The immune system is particularly sensitive to the toxic effects of xenobiotic agents and can be suppressed or overstimulated by both noncarcinogens and carcinogens. Either effect may lead to increased cancer incidence. Suppression of the immune response typically produces decreased resistance to viruses, parasites, bacteria, and tumor cell grafts, whereas enhancement results in the development of autoimmune diseases and hypersensitivity reactions.

The most widely used tests for carcinogen evaluation and regulation are long-term animal bioassays, which are considered to be qualitative predictors of response in humans. However, animal bioassays are not only time consuming but also are difficult to conduct, are extremely expensive, do not necessarily predict the target of action in humans, and cannot duplicate the wide range of susceptibilities, simultaneous exposures, and genetic backgrounds of the human experience.

Short-term cell culture *in vitro* assays, requiring only a fraction of the time, can test for toxic end points manifested during the initiation phase of carcinogenesis in the widely accepted multistage theories of carcino

genesis. Other tests, such as cell transformation assays, examine toxic end points for later events in carcinogenesis.

There is a need to develop short-term tests for promotion. The committee concluded that a battery of tests is necessary for screening chemicals for genetic toxicity. It cautions that false-negative and false-positive results may occur because the tests do not reflect the complexity of interactions in the whole animal and that although knowledge of the chemical's principal mechanism of action is crucial to the selection of short-term tests with meaningful predictive value, this knowledge is rarely available. Despite the fact that *in vitro* tests are technically difficult to design and interpret, the committee believes that they have considerable potential and should be developed further.

DOSE-ROUTE EXTRAPOLATION

It is difficult to estimate the risks of exposure to volatile compounds in drinking water because of the nature of their chemical properties. The principal route of exposure is ingestion, although the dermal and inhalation routes may account for fairly large exposures during bathing, showering, cooking, and other activities involving the use of water. Volatile compounds cannot easily be added to water for testing purposes because their solubility in water is limited. In addition, the volume of water consumed by a rodent each day is insufficient to provide an adequate simulation of the water-conveyed exposure of humans for many volatile compounds. Consequently, a substantial amount of data on many volatile substances derives from subchronic or chronic toxicological studies of inhalation exposures.

When results of inhalation toxicity studies in animals are used to predict the risks associated with human consumption of contaminated drinking water, both a dose-route and interspecies extrapolation of the toxicity data must be performed. The committee found that inhalation studies, in which chemicals are absorbed at fairly uniform rates over a specified exposure period, are not very different from ideally designed drinking water studies, in which chemicals are absorbed at a variable but moderate rate over the course of a day. In fact, well-designed inhalation toxicity studies may serve as excellent experimental models for deriving drinking water standards for a variety of volatile chemicals.

The committee developed an innovative model for extrapolating from inhalation to ingestion exposures, taking into account the metabolic and pharmacokinetic differences between these different primary routes of exposure. The model also incorporates time-dependent physiological and metabolic changes, scaling data from the studies on the rat up to a standard, healthy, young adult human male. The committee did not address problems

of extrapolating from healthy rodents to very young or old humans or to other susceptible human subpopulations.

THE USE OF EPIDEMIOLOGICAL STUDIES IN RISK ASSESSMENTS

The strength of epidemiology lies in its use of direct human observations. Its major limitation is that epidemiological observations of humans are rarely made under adequately controlled conditions. Accordingly, the design, analysis, and interpretation of epidemiological studies require special care to ensure that observations are valid and that populations under study are comparable. Epidemiological studies are best suited to confirming past risks, but they can also be used for estimating future risks when the substance of concern resembles another substance for which adequate data exist. Epidemiological studies are also valuable for generating hypotheses about possible etiologic agents, but they can rarely pinpoint precise causes. Negative epidemiological studies require careful interpretation, especially where low-level exposures or risks are involved. The committee noted that for many chemicals to which people are exposed, few or no epidemiological data are likely to become available because of these limitations.

Epidemiological studies are of value principally for conducting qualitative risk assessments of specific chemicals or industrial processes. Quantitative risk assessments are possible only when the data have been generated in well-conducted studies of well-defined cohorts whose exposures to a specific substance have been carefully measured or estimated and when sufficient time has elapsed from first exposure to the expression of disease. Such conditions are rarely encountered, however, since much human exposure involves complex mixtures. There are even problems in developing risk assessments for the general population from data on well-studied industrial hazards. For example, one cannot directly extrapolate from studies of healthy workers to the general population, which includes hypersusceptible people, the very young and the elderly, and others of less than robust health. Moreover, industrial exposures occur over an 8- to 10-hour workday, ceasing on weekends, whereas environmental exposures can be lower but continuous. The committee also noted that epidemiological studies of small populations are seldom able to detect risks from low exposures, which may nevertheless have significant long-term public health effects.

A FRAMEWORK FOR RISK ASSESSMENT

The theoretical basis for risk assessments discussed in the first *Drinking Water and Health* report has been extended considerably by the present

committee's detailed consideration of cancer and noncancer diseases that should be included in risk assessments. The first volume distinguished between injuries produced by chemicals likely to have a threshold and those for which no threshold can safely be presumed to exist: "It is more prudent to treat some kinds of toxic effects that may be self-propagating or strictly cumulative, or both, as if there were no threshold and to estimate the upper limits of risk for any given exposure." Included among self-propagating effects were alteration in cellular genetics transmitted by cell propagation and injuries such as the destruction of neurons.

For effects other than carcinogenicity and mutagenicity, earlier committees and the present committee have relied on widely accepted safety factors that reflect the level of uncertainty associated with a particular set of toxicological data: an uncertainty factor of 10 is applied when there are valid results obtained from humans, a factor of 100 is applied when there are data from valid animal studies but human data are not available or are scanty, and a factor of 1,000 is applied when human data are not available and where the animal data are very limited. As in the past, the committee endorsed basing the assessments on a 70-kg human and an average daily consumption of 2 liters of drinking water. The committee agreed that noningestion exposure (such as exposures that occur during bathing) may be important, but it did not directly estimate such exposures. Although risks were not assessed for the 10-kg child who drinks proportionately more water (1 liter/day) than the adult, the committee noted that risks posed by carcinogens in drinking water would be higher for children than in adults.

The committee relied on conversion factors based on body surface area rather than on body weight for extrapolating animal data to humans, since effects have been more directly correlated to that factor in cancer chemotherapy studies. For the noncancer end points considered by the committee, much additional work must be done, both in the identification of suitable animal models for estimating human risk and in the development of theoretical models for using animal data, once obtained. The committee is encouraged by recent activity in these areas.

The committee concluded that data from animal studies are useful in *qualitative* risk assessment and for setting priorities. When such data are used for *quantitative* estimates of risk, there is less consensus about the most appropriate models, data sets, and conversion factors. Despite these uncertainties, however, there are compelling arguments favoring the use of animal data for quantitative risk assessments.

In assessing risk of exposure to carcinogens, the committee noted the importance of interspecies variations, interindividual variability in the human population, and a variety of other factors identified by the Office of Science and Technology Policy in 1984 and discussed by the State of

California and the EPA in their development of guidelines on cancer risk assessment at the end of the same year. The committee also examined models for extrapolating from high animal doses to estimate the responses of humans at more frequently encountered low exposures to carcinogenic materials. These models include the tolerance distribution models (logistic, log-normal, probit), the so-called hitness models, time-to-tumor occurrence (or to death from cancer) models, and models derived from the multistage hypothesis of cancer causation (e.g, the Armitage-Doll model). A recently developed mathematical model (i.e., the Moolgavkar-Knudson model) extends the multistage concepts by adding the concept of a differential birth-death process for cells in a preneoplastic stage. The committee believes that this model has the potential for improving quantitative risk assessments for cancer-causing materials.

Most experts agree that current techniques for assessing cancer risk cannot generate a single precise estimate of human risk and that risks may best be expressed in terms of ranges or confidence intervals. One way of ensuring that such estimates are not overinterpreted is to state the assumptions underlying specific assessments and to discuss the uncertainties surrounding the numerical estimates, including both point estimates and upper confidence limits for all extrapolation models. The committee noted that these models diverge chiefly on the matter of low-dose extrapolation. For carcinogens assessed in this study, the committee generally relied on the multistage model, believing that this model most nearly reflects biological mechanisms of cancer, despite the existence of substantial individual differences in sensitivity to carcinogens and potential for detoxification.

TOXICOLOGICAL ASSESSMENT OF 14 COMPOUNDS

Quantitative risk assessment includes four distinct components: hazard identification, exposure assessment, dose-response assessment, and characterization of human risk at projected levels and patterns of exposure. Such assessments have been done for approximately 205 drinking water contaminants in the previous five volumes of this series. In this volume, compounds that demonstrate important toxicological issues within the research community were selected from those on the regulatory agenda of the EPA.

In Chapters 2 through 8, the committee has suggested several methods for risk assessment. Examples of the use of most of these methods are provided in the evaluations of individual contaminants. Pharmacokinetic principles have not been applied to risk assessment, however, due to a general lack of data to support this exercise for the compounds reviewed in this volume. The committee hopes that potential gains from using

pharmacokinetic information to extrapolate response from high to low doses can be explored in the future to improve risk estimation techniques.

The 14 compounds reviewed by the committee were, in order of discussion, acrylamide, aldicarb, diallate, sulfallate, dibromochloropropane, 1,2-dichloropropane, 1,2,3-trichloropropane, 1,3-dichloropropene, di(2-ethylhexyl) phthalate, mono(2-ethylhexyl) phthalate, ethylene dibromide, nitrofen, pentachlorophenol, and trichlorfon. For each compound, the committee reviewed all available data on metabolism, health effects in humans and laboratory animals, mutagenicity, carcinogenicity, and teratogenicity.

Cancer risk was estimated for several substances, and when data were adequate, the committee also developed risk assessments for noncancer health effects. When adequate evidence suggested that a chemical was not likely to be a carcinogen or a mutagen, the committee identified levels of exposure that would not be expected to cause adverse health effects in humans, i.e., suggested no-adverse-response levels, or SNARLs, using the uncertainty factors described above. The committee realized the experimental difficulty of determining NOELs. In addition, a different NOEL or LOEL may be determined for each end point examined; the more sensitive end points will have lower values. As discussed in Chapters 3 and 8, for example, data on reproductive toxicity produced lower estimates of LOELs than did data from subchronic toxicity studies for 35% of compounds tested, the same estimates for another 35%, and higher estimates for 30%.

For some compounds reviewed in Chapter 9, the data base was sufficient for estimating the magnitude of inter-or intraspecies variability and, thus, for selecting a safety factor on the basis of that estimation. For carcinogens, the committee used the multistage model to estimate both the maximal likelihood estimate and the upper 95% confidence limit of risk associated with a daily exposure to 2 liters of drinking water containing 1-µg/liter concentrations of the carcinogen.

The committee recognizes that the assessment and the management of risk are two distinct responsibilities; the former is chiefly scientific, whereas the latter requires policy judgments beyond the purview of any scientific committee. Thus, in the development of standards, the risk estimates provided in this report are offered as general guidance to policymakers for evaluating the risks to the public posed by exposures to contaminants in drinking water.

2

Developmental Effects of Chemical Contaminants

The discussion in this chapter is limited to embryo (fetal) death, growth retardation, and malformations—the only end points measured in the Food and Drug Administration's (FDA) guidelines for Segment II developmental toxicity studies of drugs (Collins, 1978) and, therefore, the only end points for which there are sufficient data bases for analysis. Postnatal functional impairment is not covered, despite its relevance, since there is no well-established data base with which to make cross-species comparisons. The much broader spectrum of end points included under the heading of reproductive toxicology, e.g., germ cell toxicity, infertility, and dysfunction of the adult reproductive system, are covered in Chapter 3.

Embryo lethality is defined and reported in the literature as the ratio of resorptions or dead fetuses in a litter at term to the number of implantation sites. Growth retardation is measured by weighing and taking crown-to-rump measurements of live fetuses at term. The frequency and type of structural anomalies are determined by gross inspection of the fetuses and by detailed skeletal and soft tissue analysis. The occurrence of embryo death precludes measurements of growth retardation or identification of malformations, because these two end points are noted only on live fetuses.

Developmental toxicity includes any detrimental effect produced by exposures during embryonic stages of development. Such lesions can be either irreversible or reversible. Embryolethal lesions result in resorption, spontaneous abortion, or stillbirth. Persistent lesions that cause overall growth retardation or delayed growth of specific organ systems are generally referred to as embryotoxic. For a chemical to be labeled a teratogen, it must significantly increase the occurrence of irreversible structural or

functional abnormalities in live offspring after it is administered to either parent before conception, to the female during pregnancy, or directly to the developing organism.

Many teratologists believe that any chemical administered in appropriate dosages at certain developmental stages can cause some disturbances in embryonic development in some laboratory species (Fabro et al., 1982; Karnofsky, 1965; Staples, 1975). For an agent to be classified as a developmental toxicant, it must produce adverse effects on the conceptus at exposure levels that do not induce severe toxicity in the mother (e.g., substantial reduction in maternal weight gain, persistent emesis, hypo-or hyperactivity, or convulsions), so that the effects are not secondary to the stress on the maternal system. The main reason for conducting developmental toxicity studies is to ascertain whether an agent causes specific or unique toxic effects on the conceptus. If these studies are conducted under extreme conditions of maternal toxicity, then identification of exposures uniquely toxic to the conceptus or pregnant animal is not possible. (This is discussed in more detail later in this chapter.) In some cases, however, chemical agents are deliberately administered at maternally toxic doses to determine the threshold level for adverse effects on the offspring. As a result, conclusions can be qualified to indicate that adverse effects on the conceptus were obtained at maternally toxic exposure levels and may not be indicative of selective or unique developmental toxicity.

INFLUENCE OF TIME OF EXPOSURE

Compared to adults, developing organisms undergo rapid and complex changes within a relatively short period. Consequently, the susceptibility of the conceptus to chemical insult varies dramatically within each of the major developmental stages, i.e., the preimplantation, embryonic, fetal, and neonatal stages. As shown in Table 2-1, the time between ovulation and preimplantation development is similar among several mammalian species, regardless of gestation length (Brinster, 1975). Alterations in the hormonal milieu as well as direct secretion of chemicals into uterine fluids during this period can interfere with implantation and result in embryo death. The preimplantation embryo appears to be more susceptible to death than to teratogenicity following chemical insult. In studies with preimplantation embryo cultures, severe toxicity was manifested by rapid death of the embryo, and the less severe effects included decreased cleavage rates and arrested development (Brinster, 1975). There have been few studies on the effects of sublethal exposures to preimplantation embryos, and the possibilities of persistent biochemical or morphological alterations have not been adequately explored.

TABLE 2-1 Timing of Early Development in Some Mammalian Species[a]

	Times of Early Development (days from ovulation)			
Mammal	Blastocyst Formation	Implantation	Organogenesis	Length of Gestation (days)
Mouse	3-4	4-5	6-15	21
Rat	3-4	5-6	6-15	22
Rabbit	3-4	7-8	6-18	30
Sheep	6-7	17-18	14-36	150
Monkey (rhesus)	5-7	9-11	20-45	164
Human	5-8	8-13	21-56	270

[a] Adapted from Brinster, 1975.

Following implantation, organogenesis takes place. During that period, there are highly specific periods of vulnerability for different organ systems, thus making the embryo extremely susceptible to the induction of structural birth defects. The periods when the major embryonic organ systems of the rat are most sensitive to teratogenic insult are shown in Figure 2-1. Administration of a teratogen on day 10 of rat gestation is likely to result in a high level of brain and eye defects, intermediate levels of heart and skeletal defects, and a low level of urogenital defects. If the same agent were administered on day 11, a different spectrum of malformations would be anticipated, predominantly effects on the brain and palate. Figure 2-1 also illustrates that exposure to teratogens usually results in a spectrum of malformations involving a number of organ systems, reflecting the overlap of critical periods for individual organ systems. This is most evident in species such as rodents, which have short gestation periods, but can also be observed in humans. Most teratogens have been found to influence the development of several organ systems in humans and to cause clusters of malformations rather than single anomalies.

The critical period of inducing anomalies in individual organ systems may be as short as 1 day or may extend throughout organogenesis. In the rat, for example, urogenital effects can be produced by drug treatment from the 9th to 18th day of gestation. This implies that development of the urogenital system is multiphasic and that individual stages may have different sensitivities to chemical insult. Depending on the mechanism of action of the agent and the time of administration, it is possible that only one or a few of these steps will be affected but that succeeding stages will be disrupted as a result of the original alteration. The persistence of the agent also influences the malformation pattern, as discussed later in

this chapter. Processes governing embryonic differentiation are not well understood, yet they are most likely to determine the intrinsic susceptibility of individual organ systems to teratogenic insult.

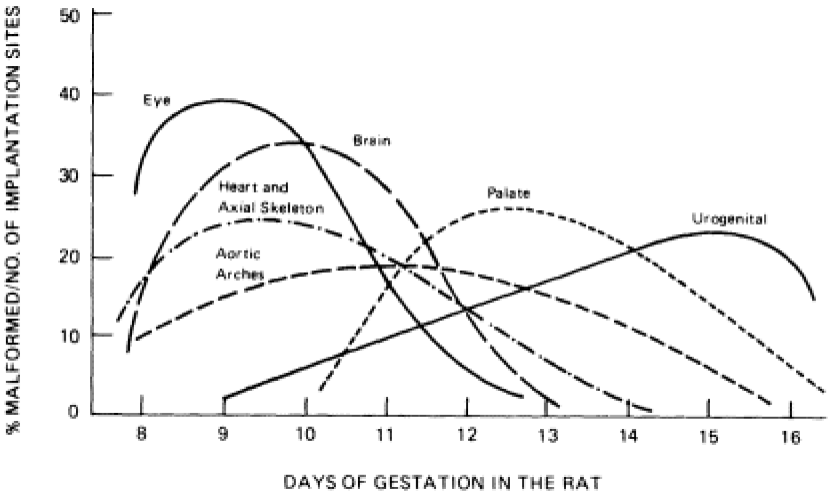

Figure 2-1
Hypothetical pattern of the susceptibility of rudimentary embryonic organs to teratogenic insult. Adapted from Wilson, 1965.

Histogenesis, functional maturation, and growth are the major processes occurring during the fetal and neonatal (i.e., perinatal) periods. Insult at these later developmental stages leads to a broad spectrum of effects that can generally be manifested as growth retardation, functional disorders, or transplacental carcinogenesis. The fetus is more resistant to lethal effects than is the embryo, but the incidence of stillbirths is measurable. The perinatal period is a time of high susceptibility to carcinogenesis. At least three factors contribute to this enhanced susceptibility: high cellular replication rates, ontogeny of xenobiotic-metabolizing enzymes, and low immunocompetence. Several childhood tumors occur so early in life that prenatal origin is considered likely. Among these are acute lymphocytic (but not myelogenous) leukemia, Wilms' tumor, neuroblastoma, carcinoma of the liver, and presacral teratoma (Miller, 1973). In 1976 cancer was the chief cause of death from disease among children under the age of 15 in the United States, accounting for 11.3% of all childhood deaths. Leukemias and lymphoma accounted for approximately half of these deaths, followed by cancers of the central nervous system, soft tissues, kidney, and bone (ACS, 1980).

Studies of direct-acting transplacental carcinogens, such as ethylnitrosourea (ENU), indicate that susceptibility to carcinogens in rodents begins

after completion of organogenesis. In one study, tumors in offspring occurred primarily when ENU was given during the fetal period, whereas birth defects and embryo deaths predominated when exposures were administered earlier in organogenesis (Ivankovic, 1979). This is not to imply that teratogenesis and carcinogenesis are mutually exclusive processes, however. Birth defects and neoplasms occur together in the same offspring with unusually high frequency, but not necessarily at the same site. Teratogenesis and carcinogenesis can be considered graded responses of the embryo to injury, teratogenesis representing the grosser response involving major necrosis. Bolande (1977) has postulated that certain agents cause teratogenesis in early, relatively undifferentiated embryos; combined carcinogenesis and teratogenesis when older embryos are exposed; and carcinogenesis alone when exposure occurs during the perinatal period. Prenatal insult may also predispose the offspring to tumor induction in later life.

PATTERNS OF DOSE RESPONSE IN LABORATORY ANIMAL STUDIES

Functional deficits and perinatally induced cancers, such as those caused by diethylstilbestrol (DES) (Herbst et al., 1977), are often not manifested until adolescence or later. They are usually examined as end points in themselves without correlation to outcomes observable at the time of birth. Observations made at the time of birth indicate that the major effects from prenatal exposure are embryo death, malformations, and growth retardation. The relationship between these outcomes is complex, and varies with the agent, the time of exposure, and the dose.

Some developmental toxicants can cause malformations in the entire litter at exposure levels not causing embryo death. The dose-response pattern for such agents is shown in Figure 2-2A. If the dose is increased beyond that causing malformations of the entire litter, embryo death can occur, but often in conjunction with maternal toxicity. Fetal malformations are usually accompanied by growth retardation. Note that the curves for these two end points are parallel and slightly displaced from one another in Figure 2-2A. This pattern of response is rare, indicating that the agents have high teratogenic potency. Both natural and synthetic glucocorticoids cause this kind of dose-response pattern. The target-organ specificity of glucocorticoids is related to the concentration of glucocorticoid receptor protein, which is higher in the craniofacial region than in other parts of the embryo (Pratt and Salomon, 1981). Thus, pharmacological doses administered to laboratory animals at midgestation induce malformations of the palate. Glucocorticoid induction of cleft palate in the absence of other major malformations, embryo death, extensive necrosis, or growth retar

dations is an example of developmental toxicity with selective teratogenic potency.

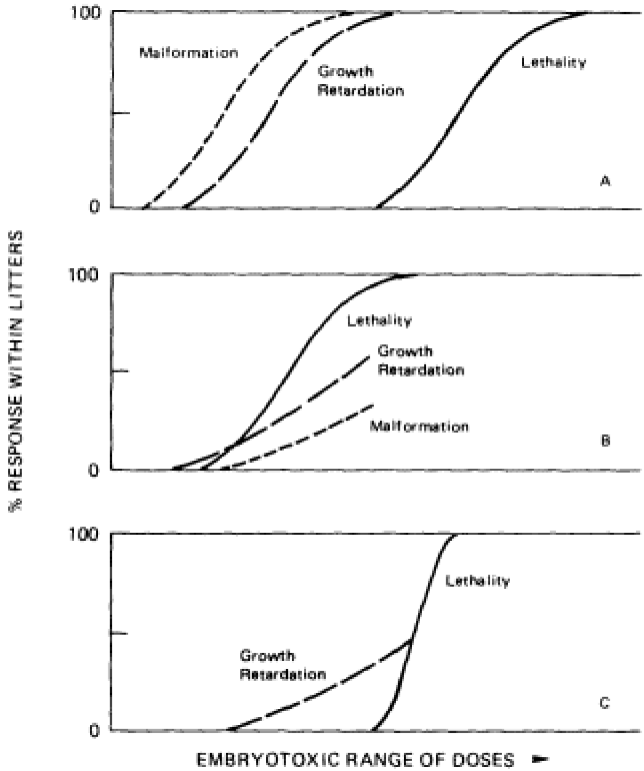

Figure 2-2
Theoretical dose-response patterns for different types of embryotoxic agents. Adapted from Neubert et al., 1980.

A more common dose-response pattern involves a combination of resorptions, malformations, growth retardations, and unaffected fetuses after exposure to a developmentally toxic range of doses of an agent (Figure 2-2B). Lower doses may cause predominantly resorptions or malformations, depending on the teratogenic potency of the agent. As the dosage increases, however, embryo death predominates until the entire litter is resorbed. Growth retardation can precede both outcomes or parallel the malformation curve. This response pattern is typical of agents that are cytotoxic to replicating cells by alterating replication, transcription, translation, or cell division. These agents include alkylating, antineoplastic, and many mutagenic substances. The susceptibility of the embryo to these agents derives from the high rate of cell division during organogenesis.

Low doses of cytotoxic agents administered relatively early in the critical period may kill cells at rates low enough that the cells can be replaced through compensatory hyperplasia, resulting in growth-retarded but morphologically normal fetuses at term. Higher doses administered later during the critical period may substantially deplete cell number, leaving insufficient time for replacement before critical morphogenetic events occur. The resulting hypoplasia of the rudimentary organs and retarded proliferation of surviving cells are the initial events leading to the induction of malformations. High levels of exposure may damage too many cells and organ systems to be compatible with survival, thus resulting in embryo death (Ritter, 1977). Exposure to cytotoxic agents during organogenesis can produce all three outcomes both within and among litters. Some litters may be totally resorbed, others may include only growth-retarded fetuses at term, and still others may include a mixture of malformed or growth-retarded fetuses and resorption sites.

A third dose-response pattern consists of growth retardation and embryo death without malformations (Figure 2-2C). The dose-response curve for embryo death in this case is usually steep, which may imply a dose threshold for survival of the embryo. Growth retardation of surviving fetuses usually precedes a significant increase in lethality. Agents producing this pattern of response would be considered embryotoxic or embryolethal substances but not teratogenic. When such a pattern is observed, it is necessary to conduct additional studies with doses within the range causing growth retardation and embryo death. Results obtained at these intermediate doses can indicate whether teratogenicity has been masked by the deaths of the embryos. Agents in this class include the mitochondrial protein synthesis inhibitors chloramphenicol and thiamphenicol (Neubert et al., 1980). On days 10 and 11 of treatment with thiamphenicol, the dose-response curve for embryo death is steep, increasing from baseline to 100% mortality at doses between 100 and 125 mg/kg body weight per day (Bass et al., 1978). In the same study, dose-dependent inhibition of mitochondrial respiration, adenosine triphosphate (ATP) content, and cytochrome oxidase activity in embryonic tissue was correlated with growth retardation and death of the embryos. There is no basis for target-organ susceptibility to perturbation of such fundamental cellular processes in the early embryo. Consequently, all tissues appear to be equally affected. An early sign of perturbation is overall growth retardation, which progresses to death of the entire litter once a threshold for cellular energy requirements is exceeded. These conditions are incompatible with teratogenicity, which can induce irreversible lesions in some tissues while sparing others, thus permitting survival of abnormal embryos to term.

For some agents, i.e., those cytotoxic to replicating cells (Figure 2-2B), growth retardation, embryo death, and teratogenicity are viewed as

different degrees of manifestation of the same primary insult, cytotoxicity. For others, there is a qualitative difference in response, and the primary insult leads to embryo death alone (Figure 2-2C) or to teratogenicity alone (Figure 2-2A). Separate evaluations of growth retardation, teratogenicity, and embryo death for increasingly higher doses are necessary to determine the agent's primary mode of action.

In safety studies, the usual sequence of testing begins with dose range-finding studies in relatively small numbers of pregnant rodents. On days 6 through 15 of gestation, animals are exposed to the test agent at doses up to and including those causing limited maternal toxicity or developmental toxicity (e.g., death or severe growth retardation). The purpose of this type of study is to obtain a qualitative yes-or-no signal about the potential developmental toxicity of the agent. At the next level of testing, larger numbers of animals are exposed on days 6 to 15 of gestation to obtain quantitative information on dose-response relationships. The highest dose should cause measurable maternal toxicity (e.g., significant depression of weight gain) or developmental toxicity (e.g., significant depression of fetal body weight or increased embryo death), and the low dose should cause no observable effects. If evidence of selective developmental toxicity is obtained from this study, it may be necessary to conduct a third study, exposing dams on single days during organogenesis at doses that are not maternally toxic, to obtain a clear definition of the dose-response pattern of developmental toxicity.

EXTRAPOLATION OF ANIMAL DATA TO HUMANS

Spectrum of End Points

The timing of exposure and the patterns of dose response obtained in animal studies have important implications for extrapolating the resultant data to humans. The major implication is that a spectrum of end points can be produced, under the controlled conditions of timing and exposure that can be achieved in animal studies. In some cases, the spectrum comprises a continuum of response: depressed birth weight or functional impairment at low doses, birth defects at intermediate doses, and death at high doses. Less commonly, birth defects alone or deaths alone are produced. Consequently, in estimating risks to humans, all exposure-specific adverse outcomes must be taken into consideration—not just birth defects. Most often neglected in extrapolation of animal data to humans is fetal growth retardation, despite the strong evidence concerning the adverse consequences of low birth weight in human infants (Hull et al., 1978). Fetal growth retardation in the absence of a significant reduction

in maternal weight gain is an important event to be considered in cross-species extrapolation.

TABLE 2-2 Frequency of Selected Adverse Pregnancy Outcomes in Humans[a]

Event	Frequency per 100 Pregnancies
Spontaneous abortions, 8 to 28 weeks	10-20
Chromosome anomalies in spontaneous abortions, 8 to 28 weeks	30-40
Chromosome anomalies detected by amniocentesis	2
Stillbirths	2-4
Low birth weight (<2,500 g) among live births	7
Major malformations among live births	2-3
Chromosome anomalies among live births	0.2
Severe mental retardation among children <15 years old	0.4

[a] Adapted from Edmonds et al., 1981.

A similar spectrum of response has been observed in humans after prenatal exposure to developmental toxicants. The specific effects in that spectrum are determined by the time and duration of exposure, magnitude of exposure, interindividual differences in sensitivity, interactions with other types of exposure, and interactions among all these factors (Fraser, 1977). Consequently, manifestations of developmental toxicity cannot be presumed to be constant or specific across species; i.e., an animal model cannot be expected to forecast exactly the human response to a given exposure. For instance, an agent that induces cleft palate in the mouse may elevate the frequency of spontaneous abortion or intrauterine growth retardation in humans. *Any* manifestation of exposure-related developmental toxicity in animal studies can be indicative of a variety of responses in humans (Kimmel et al., 1984).

Table 2-2 illustrates another factor to be considered in cross-species extrapolation. The most common adverse pregnancy outcome in humans is spontaneous abortion or early fetal loss (before the 28th week of pregnancy), occurring in at least 10% to 20% of all recognized pregnancies. Estimates from prospective studies range even higher: between 20% and 25% of all conceptions spontaneously abort (Edmonds et al., 1981). The incidence of spontaneous abortions is high during early pregnancy, especially during the first 12 weeks, and gradually decreases to the 20th

week, after which fetal loss is uncommon. Approximately one-third of the specimens obtained from spontaneous abortions occurring between 8 and 28 weeks of gestation contain chromosome aberrations. The frequency of such aberrations is at least 60-fold higher among spontaneous abortions than among term births. Among the spontaneous abortions without chromosome aberrations, approximately half have structural malformations (Edmonds et al., 1981). The frequency of such malformations is not as well documented as that of chromosome aberrations, because they are difficult to observe in specimens that are often macerated or incomplete. In the remaining one-third of the specimens, the incidence of placental inflammations suggests that uterine infections can be high (Ornoy et al., 1981).

These observations suggest that the majority of human embryos bearing chromosome aberrations or morphological abnormalities are lost through early miscarriage. Epidemiological approaches to monitoring the frequency of early fetal loss and detecting such fetal abnormalities have only been used to a limited extent. Most studies of humans focus on the examination of adverse effects, such as major malformations, stillbirths, low birth weight, and neonatal deaths, at the time of birth or later. Underestimation of adverse pregnancy outcome, and thus true risk and pattern of response, is unavoidable in human studies whenever measurements are made only from the time of birth onward. Moreover, it is difficult to design studies to work within the limitations of statistical power and to document exposure of humans, even when observations are confined to the time of birth onward (Edmonds et al., 1981; IRLG Epidemiology Work Group, 1981).

Developmental toxicants with dose-response patterns resembling those in Figure 2-2A could be detected by monitoring malformations at the time of birth, especially if the malformations were rare (such as those resulting from thalidomide), or if the exposed populations were large (such as those with rubella infections). The possibility of concordance in the pattern of malformation across species would be greatest for potent teratogens operating in the pattern shown in part A of Figure 2-2, because they tend to demonstrate target-organ specificity. Agents with patterns shown in parts B and C would probably be missed, because early fetal loss is not routinely monitored in human populations, even though it has been done successfully in isolated groups (Kline et al., 1977).

Concordance of Results from Animal and Human Studies

For several well-studied developmental toxicants, there is good evidence for dose-response correspondence between humans and animals. The correspondence is nearly 100% when data on animals have been expected to

provide a qualitative yes-or-no signal, i.e., when *any* exposure-related adverse effect from an animal study is taken into consideration and not just those specific outcomes that are also seen in humans. Thalidomide is the only toxicant known to produce developmental abnormalities in humans but not to produce such effects consistently in conventional laboratory animal species (see reviews by Brent, 1972; FDA, 1980; Frankos, 1985; Fraser, 1977; Nisbet and Karch, 1983; Nishimura and Tanimura, 1976; Schardein, 1976; Shepard, 1980; Strobino et al., 1978; and Wilson, 1973). An FDA review of the literature (FDA, 1980; Frankos, 1985) indicates that of 38 compounds having demonstrated or suspected teratogenic activity in humans, all except one tested positive in at least one animal species. Furthermore, more than 80% were positive in more than one species. Eighty-five percent of the 38 compounds were teratogens in mice, 80% in rats, 60% in rabbits, 45% in hamsters, and a low of 30% in primates. Other species (i.e., the cat, ferret, and guinea pig) have been used to test only a few of these substances.

Nisbet and Karch (1983) reported that humans appear to be as sensitive to thalidomide as the most sensitive species tested (the cat), and 5 to 10 times more sensitive than those species with comparable target-organ specificity for limb defects (the rabbit and various primates). These authors also compared the minimally effective doses in animals and humans of eight known teratogens where there was cross-species concordance in target organs. When dose was converted to mg/kg body weight per day for these teratogens [i.e., thalidomide, polychlorinated biphenyls (PCBs), alcohol, aminopterin, methotrexate, methylmercury, DES, and diphenylhydantoin], humans were shown to be more sensitive than laboratory animals by factors ranging from 1.8 (for PCBs) to 50 (for methylmercury). If exposure was expressed in units of dose per unit body surface area per day, the ratios of human to animal sensitivity ranged from 0.3 to 8.0 (Nisbet and Karch, 1983).

These findings of qualitative (yes-or-no signals) and quantitative (dose-response) concordance support the use of animal studies for predicting risk to humans. However, there are qualifications that must be placed on their direct application to risk estimation. These comparisons of response have necessarily been limited to agents for which there have been established effects in humans and substantial data bases from animal studies. Only the eight agents tested could be found to meet these criteria (Nisbet and Karch, 1983). In the *Catalog of Teratogenic Agents*, Shepard (1980) has listed more than 600 agents that cause congenital anomalies at any dose in laboratory animals. Only 20 of these are confirmed or suspected developmental toxicants in humans. Consequently, the major concern in risk assessment today is that far more agents have been shown to be positive in animal studies than have been identified in human studies for

one of three reasons: no studies have been conducted in humans with the animal teratogen; the true risk for adverse pregnancy outcome in humans has been underestimated in epidemiological studies; or the animal studies have yielded false-positive results because of test conditions and interpretation.

The aforementioned FDA literature review indicated that of 165 compounds with no evidence of teratogenesis in humans, 29% were negative in all animal species tested and 50% were negative in more than one species. However, 41% of these 165 compounds were positive in more than one animal species. A "nonpositive" response to agents with no evidence of human teratogenesis was observed for 80% of the primates tested, for 70% of the rabbits, for 50% of the rats, for 35% of the mice, and for 35% of the hamsters (Frankos, 1985).

Estimates of risk from exposure to individual chemicals are usually based on animal data alone. An understanding of the potential for underestimating human reproductive risk in epidemiological studies is important in the selection of the safety factor. The appropriateness of using any safety factor in interspecies extrapolation is open to debate; however, factors ranging from 1 to 50 have been found to be adequate for those few agents for which data on minimally effective doses, or lowest-observed-effect levels (LOELs), in humans and animals are comparable (Nisbet and Karch, 1983). That range is based on outcomes measurable at birth and is likely to be inadequate for taking into account early fetal loss in humans. Selection of the no-observed-effect level (NOEL) or the LOEL for use with these safety factors has received more experimental attention. This is discussed in the following section.

INTERPRETATION OF ANIMAL DATA

When interpreting and extrapolating developmental toxicity data obtained from studies in animals, three factors should be considered: the quality and quantity of data available, the relationship between maternal and developmental toxicity, and the selection of the LOEL or NOEL.

Quality and Quantity of Data

The quality and quantity of the data are the primary factors in determining whether cross-species extrapolation can be performed and the degree of confidence to place in the extrapolation. The quality of data is influenced primarily by the protocol or guidelines under which a study is conducted. An indication of data quality can be obtained by comparing a study protocol with accepted guidelines, such as those developed by the IRLG Testing Standards and Guidelines Work Group (1981) or by the

FDA (Collins, 1978; FDA, 1970). In Segment II developmental toxicity studies, pregnant females are exposed during organogenesis and killed one day before estimated delivery, and observations are made on maternal body weight, embryo death, intrauterine growth retardation, and the presence of external, visceral, and skeletal anomalies. Two species are tested, preferably one rodent and one nonrodent. The minimum requirement is three treatment groups and a concurrent control group, each containing 20 rodents or 10 nonrodents (e.g., rabbits). Extrapolation to humans should be made with data on the most sensitive animal species tested, unless there is evidence that this species is not appropriate because of major differences from humans in pharmacokinetics or pharmacodynamics.

The quantity of data required is determined largely by statistical power. If an experimental result is found to be statistically significant, i.e., there is a statistically significant association ($p < 0.05$) at a certain alpha level between exposure and end point, then that study had enough power to detect that difference at the chosen level of significance. If no statistically significant association is found, the study may have had insufficient power to detect a smaller but real effect, or there may have been no effect.

Power is related to the number of subjects in a group, to the rarity of the end point, and to the variability in the frequency of the end point's occurrence. In general, the rarer the end point, the fewer the excess occurrences above background needed for detecting an effect. Thalidomide and DES, for example, induced lesions (e.g., phocomelia and vaginal adenocarcinoma) that rarely occur in control populations (e.g., one in 10,000 to 100,000). Thus, it only took several cases to elevate the incidence of these lesions far above the background.

Most developmental toxicants tested in animal and human studies cause more common effects, such as intrauterine growth retardation and increases in the occurrence of minor anomalies and variants rather than major malformations. In evaluating these end points, it is necessary to consider their background incidence as well as their variance in estimating the sample sizes needed for their detection.

Historical control data on the frequency of major and minor fetal malformations in conventional laboratory animals are listed in Table 2-3. In developmental toxicity studies, major malformations are considered to be an insensitive indicator of embryotoxicity. Major malformations tend to occur within a relatively narrow range of doses, and unless the compound is a potent teratogen (pattern A in Figure 2-2), large numbers of animals are required to detect a statistically significant increase in their occurrence. With 10 to 20 litters per treatment group, differences of 40% to 50% between treatment and control means are required before statistical significance is achieved (Palmer, 1981). When more common outcomes, such as embryonic death, intrauterine growth retardation, and elevated

incidence of common variants and minor anomalies, are taken into consideration, a more sensitive appraisal of developmental toxicity can be obtained.

TABLE 2-3 Historical Control Data on Major and Minor Fetal Malformations[a]

			Minor Malformations (%)	
Laboratory Animal	No. of Fetuses Examined	Major Malformations (%)	Visceral	Skeletal
New Zealand white rabbits	36,508	0.74	2.53	8.60
CD rats	51,349	0.41	2.02	2.35
CD1 mice	22,389	0.84	3.68	5.32

[a] Adapted from Palmer, 1978.

The influence on power attributable to the end point's historical variability is illustrated in Table 2-4, which shows the number of litters of different strains of rats and mice that would be required to detect 5% and 10% changes in fetal weight or embryo death. From 22 to 50 litters of mice are required to detect a 10% depression in fetal weight, whereas only 12 to 16 rat litters would be required to detect the same magnitude of weight depression. For embryo death, from 235 to 324 litters of mice are necessary to detect a 10% increase in resorptions, compared to 216 to 248 rat litters. Fewer litters are needed to detect a change in fetal weight, probably because this is a continuously distributed end point with relatively low variability. In contrast, embryo death is a highly variable, binomially distributed parameter, and more than 200 litters are required to detect even a 10% change in this response (Nelson and Holson, 1978).

TABLE 2-4 Number of Litters Per Group Required to Detect Designated Changes in Fetal Weight and Embryo Lethality in Rats and Mice[a]

	Change in Fetal Weight		Change in Embryo Lethality	
Test Animal	5%	10%	5%	10%
Mice				
A/J	84	22	1,176	324
C57BL/6	198	50	992	288
CD1	84	22	805	235
Rats				
CD	62	16	858	248
Osborne-Mendel	44	12	723	216

[a] From Nelson and Holson, 1978.

Given the current testing requirements for 20 rats or mice per group, the most sensitive end point in developmental toxicology studies is fetal body weight, as judged by the statistical power of the study. Within the range of normal variability for this response, a 10% change in fetal body weight would be statistically significant ($p < 0.05$) if that change had been observed in 20 rodent litters in a group.

For an accurate *biological* interpretation of depressed fetal weight and embryo death, however, the occurrence of maternal toxicity must be taken into consideration. Most experimental studies of developmental toxicity have been designed to provide information on the basic mechanisms that result in birth defects. Agents are administered under conditions that cause a high incidence of abnormal fetuses, without concern for whether these were secondary to toxic effects in the maternal system. In safety studies, however, failure to recognize that developmental toxicity will inevitably occur at exposure levels causing severe maternal toxicity can lead to false-positive identification of many agents.

Relationship between Maternal and Developmental Toxicity

Regulatory guidelines for determining the developmental toxicity of chemicals call for dose-response studies in pregnant animals, the highest dose being of sufficient magnitude to induce maternal toxicity (slight but statistically significant weight loss and not more than 10% maternal deaths). The rationale for using a maternally toxic dose is to maximize the potential to detect lesions in the fetus (Palmer, 1981). Effects observed in offspring at maternally toxic doses are used as a landmark to focus attention on outcomes at lower doses. If a statistically significant incidence of a particular lesion is found in the high-dose group, the biological significance of a lower and perhaps nonsignificant incidence at lower dose levels is magnified. It can be difficult, however, to interpret some effects observed only at maternally toxic dose levels. Are they indicative of unique and selective developmental toxicity, or are they a function of nonspecific alterations in maternal homeostasis?

It is generally accepted that developmental toxicity in the form of increased resorptions and decreased fetal body weight can occur at maternally toxic dose levels. The role of maternal toxicity in the induction of congenital malformations is not clear, however. Recently, Khera (1984) reviewed more than 85 published studies in mice to examine the relationship of birth defects to maternal toxicity and embryo toxicity. He noted that doses of test agents that caused maternal toxicity, as indicated by reduced maternal body weight, clinical signs of toxicity, or deaths, commonly caused reduction in fetal body weight, increased resorptions, and, rarely, fetal deaths. He identified three patterns of association between

maternal toxicity and malformations: (1) for some compounds, maternal toxicity was not associated with malformations; (2) for others, maternal toxicity was associated with a diverse pattern of malformations, which often included cleft palate; and (3) the maternal toxicity of still others was associated with a characteristic and unique pattern of malformations.

Compounds in the second category are the most difficult to classify in terms of teratogenic potential. Cleft palate has been reported as the principle malformation resulting from food and water deprivation during pregnancy in mice (Szabo and Brent, 1975); however, cleft palate is also a malformation specifically induced in mice by a number of teratogens, most notably the glucocorticoids, without any apparent maternal toxicity. Complete ascertainment of food and water consumption, maternal body weights, and, occasionally, alterations in maternal homeostasis (i.e., organ histopathology, kidney or liver dysfunction, hematological alterations, pharmacologic reactions, and other possible toxic effects) are necessary to distinguish between cleft palate caused by a teratogenic effect of a chemical on the embryo and a nonspecific toxic effect on the dam that secondarily influences embryonic development.

Compounds in the third category were structurally unrelated to test agents administered at maternally toxic doses that caused increased resorptions and decreased fetal body weight. The characteristic pattern of defects induced by these agents was exencephaly; open eyes; fused, missing, or supernumerary ribs; and fused or scrambled sternebrae. The severity and incidence of these defects could be directly related to the degree of maternal toxicity. They were absent or rare at doses that were nontoxic to the dam. Khera (1984) concluded that these defects resulted from maternal toxicity and did not reflect the teratogenic potential of the compounds.

Kavlock et al. (1985) also examined the association between maternal toxicity and malformations by administering 10 chemicals to mice at doses causing low (LD_{10}) to moderate (LD_{40}) maternal mortality rates. Three compounds caused a dose-related increase in the incidence of resorptions, decreased fetal body weight, and malformations that appeared to be indicative of developmental toxicity and not the result of indirect maternal action. For seven compounds, an increased incidence of supernumerary ribs was observed. There was a significant ($p < 0.001$) inverse linear relationship between maternal weight gain during pregnancy and the incidence of extra ribs in groups treated with these chemicals, compared to the respective controls. Under the conditions of this study, there appeared to be a quantitative relationship between supernumerary ribs and nonspecific maternal toxicity. This quantitative relationship needs to be established for the defects attributed to maternal toxicity in Khera's study

(1984), especially for defects classified as major malformations (e.g., exencephaly and open eyes).

As implied from this discussion, not all effects observed in animal studies may be appropriate for use in risk assessment. Although there is little doubt that major effects, such as irreversible and life-threatening malformations and severe embryotoxicity, are deleterious to animals and humans, other effects are of considerable less importance. Common skeletal variants, such as retarded ossification of the sternum or vertebrae, are considered to be reversible and indicative of slight developmental delay and not of teratogenesis. In humans, the incidence of congenital anomalies of the ribs and vertebrae is low (between 0.02% and 0.03%), and these are considered minor variants with little functional consequence (Heinonen et al., 1977). A low level of concern should be attached to common variations observed in animals, especially if they are the only effects observed and if they only occur in conjunction with maternal toxicity. Greater importance should be given to variations that are dose related or that occur at doses not maternally toxic.

Selection of the NOEL/LOEL

If the data are of sufficient quality and quantity, it should be possible to identify the NOEL or the LOEL, the maternally toxic dose levels, and the specific types and incidences of adverse effects in the fetus. When the data are insufficient to identify these parameters, the agent should not be subjected to quantitative risk assessment. In the absence of sufficient data, a qualitative assessment should be conducted to rank agents on the basis of high, moderate, or low potential for developmental toxicity in humans. Agents that selectively induce irreversible developmental toxicity in animals, at low doses that are not maternally toxic, have the highest potential for causing developmental toxicity in humans. If maternally toxic exposure causes irreversible developmental toxicity, or if nontoxic maternal exposure causes reversible variants or minor malformations in animals, the agent should be considered a moderate hazard to humans. A low hazard to humans can be projected if prolonged exposure of animals to high levels of the compound does not result in developmental toxicity.

If the data are sufficient for a quantitative risk assessment, the next decision to be made is whether to establish a NOEL or a LOEL. This decision depends largely on which dose level can most accurately be identified from the data base. Greater experimental confidence can be placed on the LOEL, insofar as this value is empirically derived, whereas the NOEL can be orders of magnitude below the exposure level that would induce developmental toxicity.

Selection of the LOEL need not be restricted to responses that are statistically significant. Trends in the data indicating biologically relevant elevations in the incidence of adverse effects at low doses can be used if there are statistically significant increases in the occurrence of these effects at higher doses. LOELs are most accurately selected when the response is minimal (i.e., it is slightly elevated above background and involves reversible developmental toxicity), indicating that the NOEL is being approached. When statistical significance cannot be used as a guide to select the LOEL, which will often be the case, minimal responses can be regarded as those causing a doubling of the background rate (from concurrent or historical controls) for the particular response. To protect against the possibility that humans may have double the background rate of the response (which for major malformations would represent an unacceptable increase from approximately 60,000 malformed infants per year to 120,000), a large safety factor can be used for LOELs selected under these conditions.

RISK ASSESSMENT

Quantitative Assessment of Developmental Toxicity

Investigators concerned with the regulatory aspects of risk assessment have focused on the development of a quantitative index for comparing developmental toxicity across species, taking into account concurrent maternal toxicity. Underlying this approach is the perceived need to distinguish between compounds that are uniquely toxic to the embryo and those that induce developmental toxicity at exposure levels that are also toxic to the mother. Agents in the latter category should be regulated on the basis of their maternal toxicity, whereas those in the former would be regulated on the basis of their unique toxicity to the embryo.

Johnson (1980) has developed a testing system for addressing this issue quantitatively. He defined teratogenic hazard potential as the ratio of adult to developmental toxicity, or the A:D ratio, i.e.,

$$\text{Log} \ \frac{\text{lowest adult toxic (lethal) dose}}{\text{lowest developmental toxic dose}}.$$

He has calculated this ratio for more than 70 compounds using data from an *in vitro* system of *Hydra attenuata* adult and embryonic tissues. The A:D ratio from the hydra assay has been 0.1 to 10 times greater than the mammalian A:D ratio. Most compounds had ratios near 1, several had ratios larger than 5, and very few had ratios larger than 10 (Johnson and Gabel, 1983). This system has been proposed for setting priorities for further testing of agents in mammalian developmental toxicity studies.

Fabro et al. (1982) have explored the quantitative characteristics of a similar type of index in mammalian studies. Dose-response data for adult mortality and fetal malformations were fitted (probit of response against log of dose) for eight compounds. The observed log-probit dose-response lines for lethality and teratogenicity were not parallel, and there was not a constant ratio between the slopes for the two lines. Consequently, a simple ratio between the median lethal dose and the median effective dose (i.e., LD_{50}:ED_{50}) could not be used. To calculate a relative teratogenic index (RTI), Fabro and colleagues established a ratio between one point on each dose-response line. The LD_{01} value was chosen to represent adult mortality on the basis that a low LD value is necessary to guard against compounds with a shallow dose-response curve for adult mortality. The teratogenic dose tD_{05} was chosen for teratogenicity—that is, the dose causing a 5% elevation of the malformation rate above background. The investigators believed that the $tD0_5$ could be estimated with confidence for most teratogens, because induced malformations often occur at a frequency between 1% and 20% in animal studies. The committee concluded that this approach appears to be satisfactory for ranking the candidate compounds according to teratogenic potency, provided the relationship of dose to teratogenic response is not complicated by significant adult mortality.

This ranking system was developed to evaluate structure-teratogenicity relationships between structurally related compounds. For this purpose, the RTI seems adequate. The potential usefulness of this index for interspecies comparisons and risk estimation, however, has not been established. In their evaluation of the RTI, Hogan and Hoel (1982) argued that because of the lack of parallelism between the probit lines for lethality and teratogenicity, the index will not be invariant in the selection of other LD and tD values; e.g., if a ratio of LD_{10}:tD_{05} were chosen instead of LD_{01}:$tD0_5$, a different ranking order for the RTI would be obtained. In addition, the index would be subject to the established deficiencies of the probit model, which tends to be insensitive in the low dose region near the origin of the dose-response curve.

Therefore, until the RTI has been more extensively applied and evaluated, it should not be used for risk assessment. It is apparent, however, that a uniform method for ranking agents according to their selective toxicity to the conceptus needs to be established. Such a method would provide a yardstick against which all agents could be compared and would standardize the selection of the NOEL or LOEL for the risk assessment equation. Selection of the safety factor could then be based on the severity of the end point.

Existing models for quantitative risk assessment do not appear to be adequate for developmental toxicity data. Multistage models used for

mutagenicity and carcinogenicity data are based on a no-threshold assumption (Anderson and CAG, 1983), whereas it is generally accepted that thresholds do exist for developmental toxicity (Wilson, 1973). A more thorough discussion of models can be found in Chapter 8. The Environmental Protection Agency (EPA, 1983) used a number of models to evaluate developmental toxicity data on PCBs and found that the safe dose varied by a factor of 7,000 for one set of data, depending on the model used. The EPA and Oak Ridge National Laboratory concluded that existing mathematical models are inappropriate for assessing developmental toxicity data and that the safety factor approach is appropriate for establishing exposure levels expected to yield acceptable levels of risk (EPA-ORNL, 1982).

The FDA has also indicated that it will use the safety factor approach in developmental toxicity risk assessment but has not given specific details on how the safety factors will be chosen. It is likely that safety factors between 100 and 1,000 will be applied to NOELs identified in developmental toxicity studies of drug residues in human food. Smaller factors may be used when the prenatal effect can be ascribed to nonspecific maternal toxicity (Norcross and Settepani, 1983). Thus, due to the absence of other widely accepted approaches, the use of safety factors seems to be the only suitable approach to the quantitative assessment of developmental toxicity data (see Chapter 8).

Selection of the Safety Factor

The preceding discussion forms the basis of the committee's proposal that the following criteria be considered when selecting a safety factor:

- Minimum quality and quantity of data are required to perform a quantitative risk assessment. Compounds without a sufficient data base should be qualitatively assessed for high, moderate, and low potential to cause developmental toxicity in humans.
- The committee has concluded that humans should be considered at least 50 times more sensitive than animals to agents causing well-defined developmental toxicity in animal studies. This is in keeping with the fact that safety factors of 100 and 1,000 are most commonly applied to NOELs for developmental end points.
- Compounds causing developmental toxicity at levels well below those causing maternal toxicity constitute a greater level of risk than compounds causing developmental toxicity only at maternally toxic doses. This greater degree of risk should be reflected by application of a larger safety factor.
- The degree of risk associated with a compound is determined by the severity of response in animal tests and the conditions of time and route

of exposure under which the response occurs. The greatest degree of hazard is presented by compounds causing serious effects under conditions of exposure that are relevant to humans.

Ultimately, criteria for determining an acceptable risk will have to be developed (Bass and Neubert, 1980). This will involve defining the terms *acceptable* and *risk*. What magnitude of a risk is acceptable for a person or for a given population? Is, for example, the doubling of a background rate for an adverse response of 1 in 1,000 acceptable? To what extent should reversible effects and common variants be taken into consideration in risk assessment? These questions cannot be answered from a scientific point of view alone but will require public policy decisions that take into account the benefit of the chemicals under consideration and priorities for protecting public health. Decisions at this level will greatly influence the requirements for safety testing and risk assessment.

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3

Reproductive Toxicology

Reproductive dysfunction is broadly defined in this chapter to include all effects resulting from paternal or maternal exposure that interfere with the conception, development, birth, and normal growth of offspring. Chapter 3 is a broad discussion of the end points included under the heading of reproductive toxicology with the exception of embryo (fetal) death, growth retardation, and malformations, which are covered in Chapter 2. The relationship between exposure and reproductive dysfunction is highly complex because exposure of the mother, the father, or both may influence reproductive outcome. In addition, these exposures may have occurred at some time in the past, immediately before conception, or during gestation. For some specific dysfunctions, the relevant period of exposure is limited; for others, it is not. For example, chromosome abnormalities detected in the embryo can arise from lesions in the germ cells of either parent before conception or at fertilization, or from direct exposure of embryonic tissues during gestation. Major malformations, however, usually occur when exposure occurs during a discrete period of pregnancy, extending from the third to the eighth week of human development.

Many cases of infertility can probably be attributed to postfertilization reproductive failure, i.e., repeated early spontaneous abortion. Such peri-implantation embryonic mortality may not be clinically apparent, since abortion could occur before the expected time of menstruation. Approximately 15% of clinically recognized pregnancies terminate in spontaneous abortion. Embryonic death rates in humans may be substantially higher. In recent studies subclinical spontaneous abortion rates were found to be 21% (Chartier et al., 1979) and 34% (Overstreet, 1984). Nonetheless,

methods to assess early, subclinical spontaneous abortions are currently inadequate.

Although there are extensive data on reproductive performance in human populations, most have been collected for routine surveillance—not for environmental monitoring. Even though the effects of specific agents on reproductive function cannot be discerned from such data, useful information on trends and patterns in the frequency of various reproductive outcomes can be derived. For example, an estimated 11 million married couples in the United States are infertile (i.e., not capable of having children); 3 million of these couples have at least one partner who is noncontraceptively sterile (Mosher, 1985). Although early spontaneous abortions often go unreported, especially among pregnancies of less than 20 weeks duration, they are estimated to result in the termination of 15% of all pregnancies (Warburton and Fraser, 1964). This is generally regarded as an underestimate of the true rate insofar as most spontaneous abortions occur early in gestation, often before the mother is clinically recognized as pregnant. Approximately 7% of all babies are born prematurely (before the 37th week of gestation). Of those born at full term, an estimated 7% have low birth weights (2.5 kg or less) (Niswander and Gordon, 1972). Of the approximately 4 million infants born alive in the United States each year, 10.5 per 1,000 die within the first year (NCHS, 1985), and 2% to 3% of the 4 million infants have major congenital malformations that are recognized within that year (Edmonds et al., 1981). When defects that become apparent later in life are included, the frequency of major and minor malformations increases to about 16% (Chung and Myrianthopoulos, 1975).

In very few cases has it been possible to separate a specific chemical exposure's impact on human reproduction from the background rate of spontaneous genetic defects or from other causes, such as radiation, infection, nutritional deficiencies, or maternal metabolic imbalance. There are also ethical limitations to conducting human studies, especially those concerning reproductive function. Consequently, the bulk of the information on specific exposures reported to affect reproductive function is derived from animal studies.

The standard toxicological testing procedures for acute, subacute, and chronic exposures are not appropriate for detecting reproductive effects either in humans or in animals. Therefore, a separate series of tests has been developed to monitor reproductive function. These tests can provide both qualitative and quantitative analyses of reproductive toxicants in animals.

This chapter contains brief descriptions of the biological development and function of the male and female reproductive systems. Stages particularly susceptible to chemical insult are emphasized. This material is

presented to provide a context in which animal data can be applied to humans in estimating the risk of reproductive toxicity.

Toxicity to the embryo, fetus, or placenta, resulting in spontaneous abortion, teratogenicity, or other reproductive anomalies, has long been of concern. Other areas not as extensively studied are toxicities affecting the male and female reproductive systems, resulting in sexual dysfunction and infertility. Direct damage to germ cells, neuroendocrine imbalances, and alterations in accessory reproductive organs, which can be involved in these toxicological processes, are described in the following sections as they have been elucidated in animal studies. Consideration is also given to the use of these data to predict reproductive risk to humans.

SUSCEPTIBILITY OF THE NONPREGNANT FEMALE TO REPRODUCTIVE IMPAIRMENT

Maturation of the Female Reproductive System

The development of the female genital tract and subsequent attainment of fertility are processes susceptible to disruption by chemical agents. Reduced fertility in offspring is one of the most sensitive indicators of prenatal exposure to reproductive toxicants. The female fetus is particularly vulnerable to germ cell toxicity, since the development of the oocyte occurs prenatally and the maximum number of oocytes available for subsequent ovulation is present at the time of birth. Damage to oocytes during the perinatal period may result in decreased reproductive capacity that will not be evident until sexual maturity is reached.

Early in embryonic development, the progenitors of the germ cells, called primordial germ cells, are segregated from somatic cells. At 3 weeks of human development, these germ cells are first detectable in the yolk sac. Thereafter, they undergo mitotic divisions and migrate to the urogenital ridge where they populate the so-called indifferent gonad. Primordial germ cells then differentiate into oogonia. The oogonial stage is characterized by active mitotic divisions; the daughter cells do not separate, but remain attached to each other by interconnecting cytoplasmic bridges. In the human fetal ovary, approximately 1,700 germ cells migrate to the gonads. By 2 months of gestation, the number of germ cells increases to about 6×10^5. Mitotic activity peaks by the fifth month at approximately 7×10^6 cells. Oogonia first begin to enter meiosis at the third month, and by the end of the fifth month, all the oogonia have entered early prophase I of meiosis and are called primary oocytes (Gondos, 1978). The timing of gonadal sex differentiation and of ovarian germ cell development in various mammalian species is presented in Table 3-1.

TABLE 3-1 Ontogeny of Ovarian Germ Cell Development in Mammals[a]

	Events in Germ Cell Development in Days of Gestation (or Postnatal Age)				
Test Animal	Length of Gestation	Gonadal Sex Differentiation	Initiation of Meiosis	Completion of Oogenesis[b]	Arrest of Meiosis
Mouse	19	12	13	16	(5)
Rat	21	13-14	17	19	(5)
Hamster	16	11-12	(1)	(5)	(9)
Rabbit	31	15-16	(1)	(10)	(21)
Rhesus monkey	165	38	56	165	Newborn
Human	270	40-42	84	150	Newborn

[a] Adapted from Gondos, 1978

[b] Completion of oogenesis refers to the time when all oogonia have been transformed to primary oocytes.

Replicative DNA synthesis occurs during the final interphase before the oogonia enter meiosis. The primary oocyte in prophase I of meiosis thus contains two sets of chromosomes; that is, it is diploid (2N) but contains four strands of DNA. The process of meiosis consists of two cell divisions. First, the number of chromosomes is halved, resulting in the formation of secondary oocytes, each containing one chromosome (1N), i.e., haploid, and two strands of DNA. In the second meiotic division, the chromosome number remains the same but the amount of DNA is halved. Thus, the ovum contains one chromosome and one strand of DNA. Figure 3-1 depicts the process of oocyte maturation in the fetus and adult.

Each meiotic division has four stages: prophase, metaphase, anaphase, and telophase. The first meiotic division is initiated late in fetal life and progresses into early prophase during the fetal or neonatal period. By 8 weeks after birth, human oocytes have entered a resting phase of oocyte maturation, where meiosis remains blocked until the beginning of puberty (Biggers, 1980). Given the long duration of prophase I, this stage has been subdivided into five subphases: leptotene, zygotene, pachytene, diplotene, and dictyate (resting phase). Each substage is characterized by cytogenetic criteria of chromosome configuration.

Extensive physiological degeneration of germ cells occurs during the oogonial and primary oocyte stages of development. In humans, an estimated 70% of the germ cells present in a 5-month-old fetus are lost before birth (Biggers, 1980). Three distinct waves of degeneration occur in the human ovary, affecting oogonia in mitosis or in the final interphase, oocytes in the pachytene stage, and oocytes in the diplotene stage of prophase. Oogonia connected by cytoplasmic bridges undergo atresia in

synchrony, which accounts for the majority of germ cell loss. After completion of the meiotic prophase, groups of oocytes no longer appear to undergo atresia simultaneously, but individual oocytes may degenerate at all stages of development. It is not understood why some oocytes degenerate while others mature.

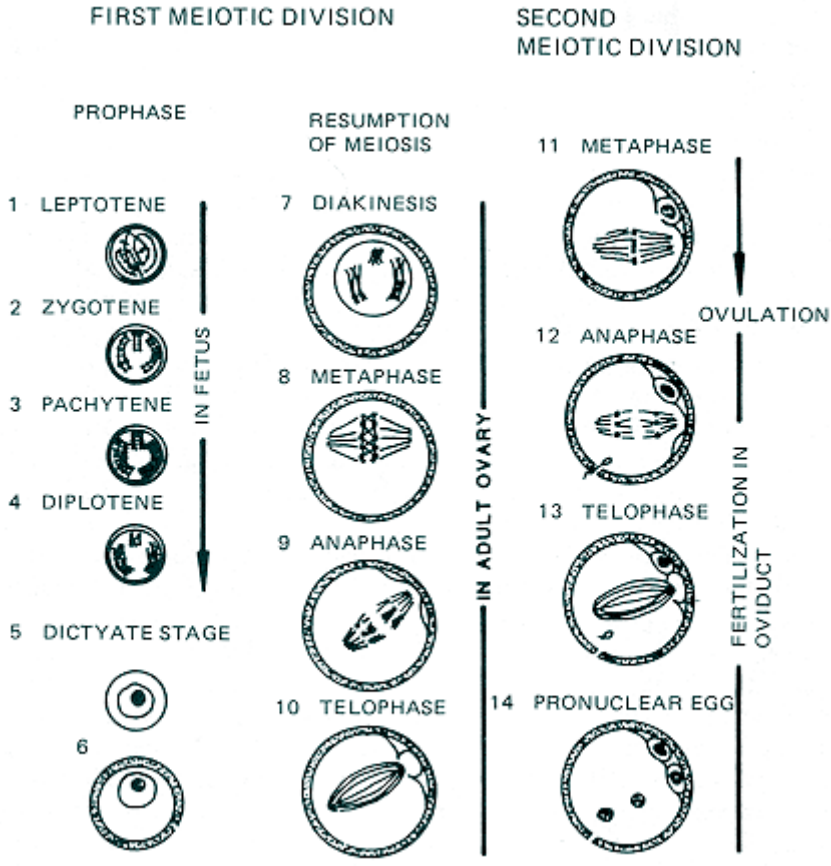

Figure 3-1
Oocyte maturation. Prophase of the first meiotic division (1-4) occurs during fetal life. In the zygotene stage, homologous chromosomes pair; in the pachytene stage, they form bivalent chromosomes. Genetic material is interchanged by a crossover process. At the diplotene stage, the chromosomes remain united at the points of interchange, the chiasmata. The meiotic process is arrested at the dictyate stage. When meiosis is resumed, the first division is completed (7-11). Ovulation occurs at metaphase of the second division (11), and maturation of the oocyte occurs in the oviduct (12-14) following sperm penetration. Adapted from Tsafriri, 1978.

At some point during oogonial proliferation, all the oocytes within a syncytial mass will recruit granulosa cells from the surrounding ovarian

stroma and enter meiosis. The mechanism of this process, termed folliculogenesis or the formation of follicle complexes, is unknown. Once meiosis is initiated, germ cells lost to physiological atresia cannot be replaced. The maximum number of germ cells potentially available for ovulation in the offspring is fixed in the fetal period when oogonia mature into primary oocytes; the number continues to decrease due to physiological atresia and ovulation (Hertig and Barton, 1973).

During the prepubertal and reproductive periods, the majority of germ cells remain as primary oocytes enclosed within unilamellar follicles. These resting follicles comprise the pool from which a select number of oocytes are recruited for further maturation to preovulatory or graafian follicles. In those follicles selected for maturation, a zona pellucida forms and separates the oocyte from the follicle cells. Thereafter, the follicular cell layer increases in size and the oocyte undergoes tremendous growth. Once a follicle embarks on this maturation process, it either reaches a preovulatory stage or it undergoes atresia (Tsafriri, 1978).

At puberty, release of gonadotropins, particularly luteinizing hormone (LH) and to a lesser extent follicle-stimulating hormone (FSH), initiates the resumption of meiosis (see Figure 3-2). Following the rise in gonadotropin levels, the primary oocytes in preovulatory follicles progress through the rest of the first meiotic division and form secondary oocytes that are blocked in metaphase of the second division. The first polar body is extruded; this body contains half the chromosomes (1N) present in the primary oocytes (2N). As the time of ovulation nears, the follicle becomes more vascular and swells out from the ovarian surface. It is macroscopically visible as a blisterlike protuberance. The secondary oocyte is released at metaphase of the second meiotic division, and it stays in this stage pending fertilization. At fertilization, the second meiotic division is completed, the second polar body is extruded, and the female pronucleus is formed. The male and female pronuclei combine to reestablish the diploid state (Espey, 1978).

In the absence of fertilization, the secondary oocyte degenerates. When the menstrual cycle is established in humans, ovulation occurs on the average of every 28 days, during which time those follicles recruited from the resting follicle pool (graafian follicles) are stimulated to ovulate by elevation in gonadotropin levels. This process continues throughout the reproductive life until the population of primordial follicles is depleted or menopause occurs.

Toxic effects on oocytes and effects on oogenesis are discussed in the following section. Germ cell mutagenesis is the subject of a separate section toward the end of this chapter.

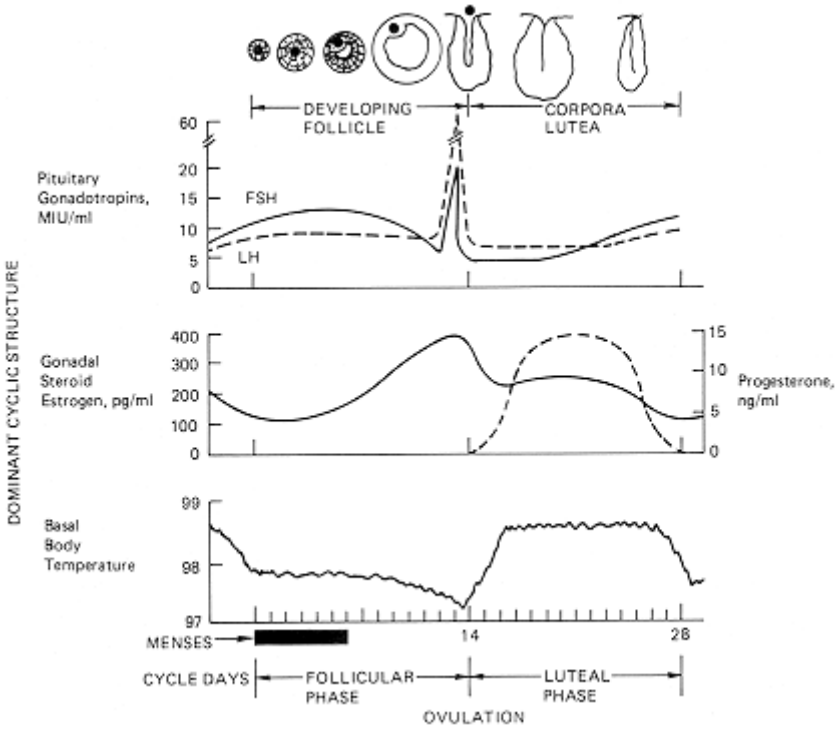

Figure 3-2
Endocrinology of the menstrual cycle in humans. From Haney, 1985, with permission.

Oocyte Toxicity

The ovary, as a repository of oocytes and as a source of steroid hormones that control the functional development of reproductive organs, plays a major role in fertility and initiation of pregnancy. As indicated in the preceding section, when folliculogenesis is complete in the female during the perinatal period, oogonial cells no longer persist.

The ovary cannot replace oocytes destroyed by toxicants. Complete destruction of oocytes prepubertally will result in primary amenorrhea and failure of pubertal onset. Complete oocyte destruction after puberty will produce premature menopause (Mattison, 1983).

Follicle growth can be separated into two phases: gonadotropin independent and gonadotropin dependent. Recruitment of follicles from the resting pool and the initial phase of follicle growth to the preantral stage is gonadotropin independent and may be controlled by an intraovarian

regulatory mechanism. Further growth and development to the preovulatory stage requires the support of gonadotropins. Follicle growth is initiated at all ages, but in the absence of gonadotropins, growing follicles undergo atresia (Mattison, 1985).

If the dominant follicle is destroyed, fertility will be immediately interrupted. Follicles in the growing pool can repopulate the preovulatory pool, followed by a resumption in fertility. If a toxicant destroys growing, gonadotropin-independent follicles, but spares preovulatory follicles, the delay in the onset of infertility will be proportional to the time required for follicles to reach the preovulatory stage. Destruction of resting follicles has the greatest delayed effect on fertility, and the results will not be evident until the end of the reproductive life. Partial destruction of the resting follicle pool is manifested as premature onset of menopause. Menopause generally occurs between 45 and 55 years of age; when it occurs before age 35, it is usually regarded as premature (Mattison, 1985).

In a mathematical model of functional ovarian life span, Mattison (1985) has estimated that menopause occurs when there are fewer than 3,500 oocytes per ovary. Calculations based on this model indicate that the age of menopause is weakly dependent on the number of oocytes at birth. When 75%, 50%, or 25% of the normal complement of oocytes are present at birth, menopause is estimated to occur at 47, 44, or 37 years, respectively. Mattison reported, however, that varying the normal rate of atresia, or oocyte half-life (9.2 years), had a strong influence on age at menopause. When oocyte half-life was 75%, 50%, or 25% of the normal rate, the age at menopause was 38, 25, or 12 years, respectively.

The results of this model are consistent with data on humans suggesting that most forms of premature ovarian failure, both genetically and xenobiotically determined, are due to an increased rate of atresia. Surgical procedures such as unilateral oophorectomy or bilateral wedge resection that decrease resting oocyte number without altering the rate of atresia do not appear to influence the age of menopause (Mattison, 1985).

Effects of Radiation on Oogenesis

In rodent species, female germ cells are extraordinarily sensitive to killing by exposure to ionizing radiation, especially during neonatal life. Primordial, or resting, follicles in juvenile mice have an LD_{50} of only 6 rads (Dobson and Felton, 1983), whereas typical LD_{50} s for most other cell types range from 100 to 300 rads. The entire primordial follicle pool in female squirrel monkeys is destroyed by prenatal exposure to only 0.7 rad/day throughout pregnancy. Histopathological examination of other tissues failed to yield evidence of cytotoxic effects at any other site (Dobson and Felton, 1983). High oocyte radiosensitivity has been demonstrated

in relatively few species, most notably in the neonatal mouse (Dobson and Cooper, 1974), the prenatal pig (Erickson, 1978), and the prenatal squirrel monkey (Dobson et al., 1978). In Swiss-Webster mice, oocyte radiosensitivity appears shortly after birth, increases rapidly to peak sensitivity from days 5 to 17 of life, and decreases moderately to adult levels (Dobson and Felton, 1983). The rat displays a similar pattern but is considerably less radiosensitive (Mandl and Beaumont, 1964). In contrast, maturing oocytes in the guinea pig are more radiosensitive than primordial oocytes (Oakberg and Clark, 1964).

The magnitude of prenatal germ cell loss in squirrel monkeys has led to examinations of loss in other nonhuman primates. In one such study, exposure of rhesus and bonnet monkeys to radiation during pregnancy failed to yield evidence of oocyte radiosensitivity (Dobson and Felton, 1983). In another, Baker (1978) found that oocytes in humans are resistant to radioactivity, reporting that LD_{50}s from x-ray exposure have reached 400 rads. X-ray exposures of the human ovary have most often been examined at prepubertal and adult stages, however, and a critical period during late fetal to early neonatal life would most likely have been missed.

In adult human females, the growing follicles appear to be most sensitive to ionizing radiation, partly because of the rapid rate of granulosa cell proliferation. The effects of ionizing radiation on the ovaries of women of reproductive age have been tabulated by Ash (1980). Exposure to less than 60 rads had no deleterious effects at any age. At 150 rads, women over 40 were at risk of becoming sterile. From 250 to 500 rads, women under 40 had temporary amenorrhea and 60% of the women became permanently sterile. All women over 40 had become permanently sterile at this level of radiation.

With the onset of preovulatory oocyte maturation and resumption of meiosis after the dictyate stage (see Figure 3-1), susceptibility to the lethal effects of radiation decreases but sensitivity to heritable genetic damage increases. Preovulatory oocytes in multilayered follicles are relatively resistant to radiation-induced death, but they are sensitive to induction of both recessive and dominant mutations. In irradiated preovulatory oocytes, the incidence of dominant lethal mutations is highest at the first metaphase, slightly less at the second, and low at other stages (Baker, 1978).

Effects of Xenobiotic Compounds on Oogenesis

Polycyclic Aromatic Hydrocarbons (PAHs)

These compounds have been demonstrated to cause ovarian tumors, chromosome aberrations during oocyte meiosis, and decreased fertility in laboratory animals (see Mattison et al., 1983, for a review). Several

investigators have also demonstrated that PAHs destroy oocytes in resting follicles in mice and rats at a rate depending on strain, species, age, dose, and metabolism (Felton et al., 1978; Mattison and Thorgeirsson, 1978, 1979; Mattison et al., 1983). The following PAHs are known to have this effect: benzo (a)pyrene, 3-hydroxybenzo(a)pyrene, 4,5-dihydroepoxybenzo(a)pyrene, *cis*-4,5-dihydrodiolbenzo(a)pyrene, *trans*-4,5-dihydrodiolbenzo(a)pyrene, 7,8-dihydrodiolbenzo(a)pyrene, 7,12-dimethylbenzo(a)pyrene, 7,12-dimethylbenzanthracene, and 3-methylcholanthrene (Chapman, 1983; Dobson and Felton, 1983; EPA-ORNL, 1982; Haney, 1985).

The oocytes are actually destroyed by reactive intermediates formed from the parent compound in the ovary by enzyme action. Although this metabolic process is necessary for oocyte destruction, inducibility at the *Ah* locus is not as highly correlated with this effect as is the rate of metabolism along the pathway leading to formation of the dihydrodiol epoxide (Felton et al., 1978; Mattison and Thorgeirsson, 1978, 1979; Mattison et al., 1983).

There are indications that cigarette smoking causes a toxic ovarian response in humans, resulting in premature onset of menopause. The incidence of infertility, defined as a woman never being pregnant throughout her reproductive life, was approximately 12% among white nonsmokers compared with 18% among white smokers from a total of 1,728 women in the study (Tokuhata, 1968). There are more than 3,000 identifiable compounds in cigarette smoke, and the specific agents responsible for this effect are not known, although PAHs and nicotine have been implicated (Surgeon General, 1981).

Antineoplastic Agents

A variety of antineoplastic agents have also been associated with ovulatory dysfunction and destruction of oocytes (Haney, 1985). Included among these are adriamycin, 5-fluorouracil, L-asparaginase, 6-mercaptopurine, bleomycin, methotrexate, busulfan, nitrogen mustard (mechlorethamine), chlorambucil, prednisone, corticosteroids, procarbazine, cyclophosphamide, vinblastine, β-cytosine arabinoside, and vincristine (Chapman, 1983; Dobson and Felton, 1983; EPA-ORNL, 1982; Haney, 1985).

These agents destroy rapidly dividing granulosa cells in growing follicles as well as in the resting primordial follicle. When prepubertal girls are treated with antineoplastic drugs, complete loss of germ cells is unlikely. In young, postpubertal women, however, fertility may be impaired despite the onset of normal menstrual cycles. As a general rule, the greater the number of chemotherapeutic agents used, and the older the woman,

the higher the likelihood of gonadal injury and permanent sterility (Haney, 1985).

Dobson and Felton (1983) reviewed data on the oocyte toxicity of 77 chemicals in 11 chemical classes. Of the 77 chemicals tested, 21 caused destruction in resting primordial follicles in mice. Positive compounds were found in 7 of the 11 classes, notably among the PAHs, alkylating agents, esters, epoxides and carbamates, fungal toxins and antibiotics, and nitrosamines. The four negative classes were the aromatic amines, aryl halides, metals, and steroids.

Alterations in Reproductive Endocrinology

In addition to direct effects on the survival of oocytes, exposure to xenobiotic substances can impair female fertility through alterations in the function of the hypothalamic-pituitary-uterine-ovarian axis. The central nervous system (CNS) component of the female reproductive system functions in a permissive, integrating role. Hypothalamic neurons synthesize and secrete gonadotropin-releasing hormone (GnRH). These hypothalamic neurons adjoin a portal vascular system that transports GnRH, which is secreted in a pulsatile pattern to the anterior pituitary gland. GnRH functions at this site in a permissive capacity, allowing the release of FSH and LH. The *pattern* with which these gonadotropins are released is controlled by the circulating levels of sex hormones (Knobil, 1980).

FSH and LH stimulate follicular maturation from the preantral to the preovulatory stage. In addition, they influence the synthesis and secretion of estrogen by thecal and granulosa cells in the follicle. Estrogen is critical to the viability of follicles because it is mitogenic to granulosa cells. During the surge in gonadotropins at midcycle, a series of events is set into motion that culminates in ovulation. These events include intrafollicular prostaglandin synthesis, terminal oocyte maturation, a shift in steroidogenesis from estrogen to progesterone production by granulosa cells, morphologic luteinization, and, finally, rupture of the follicle and release of the oocyte (Takizawa and Mattison, 1983). Without sustaining factors secreted by a conceptus, the corpus luteum undergoes regression. Peripheral progesterone levels begin to rise with the initiation of the LH surge and continue to increase until the midpoint of the luteal phase, when they begin a gradual decline that results in menses. In humans, human chorionic gonadotropin appears responsible for maintenance of the corpus luteum during early pregnancy.

There are a number of endocrine processes in which xenobiotic compounds can interfere with ovarian function aside from any direct injury of the oocyte. It is difficult, however, to separate direct injury to the follicle from alterations in hypothalamic-pituitary-gonadal function. In

terference with specific endocrine functions critical to follicular development produces the same end points as direct oocyte toxicity-ovulatory dysfunction and reproductive failure.

Endocrine Alterations in the Perinatal Period

Steroid hormones have been the most thoroughly studied agents in female reproductive toxicology and have served as model agents for the effects of xenobiotic substances. It has been well established that exposure of female rodents to pharmacological doses of androgens or estrogens during fetal or neonatal life results in disruption of the mechanisms that control cyclic secretion of gonadotropins (Kraulis et al., 1978). In addition, several contaminants in drinking water, such as Kepone, exhibit estrogenic activity and disrupt control mechanisms (Hudson et al., 1984).

The critical period for exposure to steroid hormones extends from day 18 of pregnancy to days 8 to 10 of neonatal life, in the rat. During this time, the hypothalamic centers believed to be involved in control of cyclic hormone secretion undergo neuronal maturation. Disruption or alteration of hypothalamic maturation has a permanent effect that results in an acyclic (tonic) pattern of hormone release like that found in males, and a persistent estrous syndrome characterized by infertility-disrupted periodicity (MacLusky and Naftolin, 1981).

In the rat and other rodent species, the persistent estrous syndrome appears to result from the action of estrogens on hypothalamic development during the neonatal period. The effect of androgens is also believed to stem from their aromatization to estrogens in the CNS. Neonatal exposure to estrogenic substances stimulates uterine growth and early vaginal opening. These two responses are good indicators of estrogenic action, and when they occur during the neonatal period they can be predictive of persistent estrous and reproductive tract anomalies in the adult (Sheehan et al., 1980). Physiological estrogens (such as estradiol, estriol, and estrone), nonphysiological estrogens [such as diethylstilbestrol (DES), Kepone, dichlorodiphenyltrichloroethane (DDT), and methoxychlor], and triphenylethylene drugs (such as nafoxidine, tamoxifen, and clomiphene) are known to cause these effects in the rat (Clark, 1982).

In addition to effects on the reproductive cycle, exposure of rodents to steroid hormones during neonatal life also causes abnormalities in the reproductive system. Neonatal or chronic exposure of adult hamsters to estrogens results in preneoplastic and neoplastic changes in the vagina, uterus, and pituitary gland (Leavitt et al., 1982; Lin et al., 1982). Neonatal treatment of mice with androgens causes persistent estrous as well as squamous metaplasia and alterations of stromal collagen in the uterus (Takasugi, 1976). These may result from conversion of the androgens to

estrogens during the neonatal period or from tonic release of gonadotropins, which would result in continuous exposure to ovarian estrogens during adult life. Nonphysiological compounds with estrogenic activity such as DES and clomiphene also cause reproductive tract abnormalities in rodents (Leavitt et al., 1982).

Endogenous physiological estrogens are prevented from exerting these toxicities because of their extensive binding to serum proteins. Consequently, the level of free hormone is low, leaving relatively little to bind to cellular estrogen receptors. This scenario has been well established in the rat, which has substantial quantities of α-fetoprotein (AFP) in the blood during fetal and neonatal development. AFP binds estradiol with high affinity and thus reduces the level of free hormone in the blood of rodents. DES, a nonphysiological estrogen, is weakly bound by AFP, however, permitting more interaction of this estrogenic substance with cellular receptors. Those estrogens not extensively bound to AFP tend to be potent estrogenic agents capable of disrupting normal reproduction in the rat (McEwen, 1981).

Although these concepts have been validated in rodents, there is controversy about their applicability to humans. AFP does not bind physiological estrogens well in humans, and it cannot be equated with AFP in rodents. Other proteins, such as steroid hormone-binding globulin, along with the high levels of progesterone during human pregnancy may protect against estrogen action (Clark, 1982).

The mechanisms that control the sexual development of the reproductive system in primates, including humans, also appear to act differently or to be less sensitive to toxic hormonal influences than they are in rodents. In the rat, it is generally accepted that androgens secreted by the testes during development are converted to estrogens in the hypothalamus (McEwen, 1981). These estrogens act to defeminize the hypothalamus and to produce an acyclic, male pattern of gonadotropin secretion. However, these mechanisms do not appear to operate in primates; instead, testosterone is converted to dihydrotestosterone, which is the active agent. Insofar as aromatization of androgens to estrogens is not involved in masculinization of the primate hypothalamus, exposure to estrogenic agents is not likely to lead to abnormal patterns of gonadotropin release or male infertility. The primate hypothalamus appears to be insensitive to androgens; pharmacological exposure of female fetuses to androgens can masculinize the external genitalia without influencing the periodicity of the adult menstrual cycle (Clark, 1982).

Even though the evidence suggests that hormonal insult from steroids during human development will not influence hypothalamic maturation or cyclic gonadotropin release, it is clear that masculinized behavior patterns are produced in female primates exposed to androgens during pregnancy.

Also, maternal exposure to DES during pregnancy has been associated with menstrual irregularity and subfertility in the daughters (Rosenfeld and Bronson, 1980), although the evidence for these effects is not as strong as for the structural and preneoplastic lesions in the reproductive tract.

We can conclude, therefore, that exposure to steroid hormones during the perinatal and adult periods is associated with a number of reproductive tract abnormalities in females. Moreover, although there may be differences in the critical period and in the mechanism of action between rodent and primate species, pharmacological exposure to sex steroids early in life predisposes the adults of all mammalian species to subsequent effects on their reproductive system (Clark, 1982).

Cns-Mediated Endocrine Alterations in the Adult

Certain exposures can cause reversible disruption of hypothalamic pituitary function and gonadotropin release in the adult. In laboratory animals, these effects are seen as temporary suppression of the estrous cycle, ovulation, and fertility in females or as inhibition of androgen production and suppression of spermatogenesis in males (Smith and Gilbeau, 1985). The evidence indicates that the major pathways involved in hypothalamic control of gonadotropins are adrenergic and dopaminergic (Smith and Gilbeau, 1985). There is profuse catecholaminergic innervation in the hypothalamus, and catecholamines play an important role in gonadotropin release. The surge type of gonadotropin release associated with preovulatory release of LH and FSH is under noradrenergic control and is stimulated by dopamines, norepinephrine, and epinephrine. These catecholamines stimulate the release of GnRH, which in turn controls the release of LH and, to a lesser extent, FSH. Other experiments have demonstrated that α-but not β-adrenergic blocking drugs can suppress GnRH release (McCann et al., 1982).

There is growing evidence that the endogenous opioid peptides may also be involved in GnRH release by a reduction in endogenous inhibitory tone at the time of the preovulatory surge in LH and FSH. Injections of morphine or opioid peptides inhibit LH secretion, and injections of naloxone given to block opioid receptors augment the magnitude of the preovulatory LH surge (McCann, 1982). The precise mechanism by which opioids modulate neuroendocrine function is unknown. Preliminary observations indicate that opioids may affect secretions of biogenic amines; i.e., they may decrease dopamine turnover and norepinephrine concentrations.

A variety of pharmacological agents can modify catecholamine levels by altering synthesis, release, receptor activation, and uptake. Drugs and

environmental contaminants that produce actions of this type are neuropharmacological agents that either inhibit CNS activities (e.g., anesthetics, analgesics, pesticides, sedatives, solvents, and tranquilizers) or that stimulate them (e.g., the antidepressants, hallucinogens, natural products, and stimulants). In addition, drugs of abuse are increasingly implicated in disruption of the hypothalamic-pituitary system, leading to reproductive dysfunction (Smith and Gilbeau, 1985).

Marijuana and its principal psychoactive ingredient, Δ9-tetrahydrocannabinol (THC), inhibit secretion of FSH, LH, and prolactin in rodent and nonhuman primate models. In primates, acute administration of THC results in 50% to 80% reductions in serum FSH and LH for up to 24 hours (Smith et al., 1979). Prolactin levels are maximally suppressed between 30 and 90 minutes after drug treatment (Smith et al., 1980). At blood THC levels comparable to those found in regular human marijuana users, nonhuman primates experienced disruption of the menstrual cycle and inhibition of ovulation (Asch et al., 1981). An 18-day exposure to THC resulted in disruption of the menstrual cycles that persisted until 6 months after treatment (Asch et al., 1979). In both rodent and primate models, the antifertility effects of THC could be reversed by treatment with GnRH, suggesting that the primary lesion occurred at the hypothalamic level.

Narcotic drugs have been found to cause reproductive dysfunction in human addicts. Clinical manifestations of decreased sexual desire and performance, menstrual irregularities, and infertility have all been attributed to altered hypothalamic-pituitary function (Gaulden et al., 1964; Hollister, 1973; Mintz et al., 1974). Acute doses of morphine inhibit ovulation in rats and rabbits (Barraclough and Sawyer, 1955; Sawyer, 1963). Chronic doses of morphine or heroin disrupt estrous cyclicity in rodents and the menstrual cycle in women (Gaulden et al., 1964; Packman and Rothchild, 1976). Evidence for a primary hypothalamic involvement has come from studies in male nonhuman primates, in which GnRH treatment prevented or reversed opioid-induced decrease in plasma testosterone levels (Scher et al., 1983).

Barbiturates are sedative-hypnotic agents that have been used as anesthetics in laboratory animals for many years. Their general effect is an inhibition of both LH and FSH release and a subsequent depression in steroid hormone levels. Nansel et al. (1979) found that phenobarbital inhibits gonadotropin secretion and thus blocks the rise in serum gonadotropin levels that would normally follow castration (Nansel et al., 1979). They also found that LH secretion and ovulation can be restored in these animals by treatment with GnRH, indicating a hypothalamic site of action (Nansel et al., 1979; Wedig and Gay, 1973).

Phencyclidine hydrochloride was developed as a tranquilizer for animals, but it has been used by humans as a drug of abuse. Many areas of

the CNS are affected by phencyclidine hydrochloride, which alters several neurotransmitter systems. Acute administration of phencyclidine hydrochloride to male rats at dosage levels equivalent to those used by human drug abusers produced slight depressions in serum testosterone and LH levels. Marked depression occurred after nine daily treatments. After treatment, LH and testosterone levels were significantly elevated over controls, and they did not return to normal levels until 60 days after withdrawal of the drug (Harclerode et al., 1984). Juvenile male rats receiving an identical treatment regimen during sexual maturation had severalfold higher elevations in hormone levels after withdrawal than did adult male rats, and the period of elevation persisted for 80 days. Other tranquilizers known to alter the hypothalamic GnRH level through effects on endogenous catecholamines are reserpine, chlorpromazine, and perphenazine (McLachlan et al., 1981).

In both human males and laboratory animal males, acute exposure to alcohol primarily affects testicular synthesis and secretion of testosterone (Mendelson and Mello, 1984). Both ethanol and acetaldehyde inhibit enzymes involved in gonadal testosterone synthesis (Johnston et al., 1981). Multiple endocrine abnormalities, including hypogonadism and gynecomastia, sometimes occur along with alcoholic cirrhosis (Gordon et al., 1978). Levels of estrogenic steroids increase as a result of altered hepatic metabolism and clearance of androgens. Women and nonhuman primate females do not appear to be as sensitive to the direct gonadal effects of alcohol and may be less vulnerable to antifertility effects with chronic alcohol abuse (Van Thiel et al., 1977, 1978).

Most neuroactive drugs produce transient effects on CNS pathways necessary for normal gonadotropin secretion. The disruptive effects of these drugs on sexual and reproductive function are likely to be transient and completely reversible. Adults with compromised reproductive function and prepubertal adolescents may be at greater risk to long-term impairment due to lack of hypothalamic-pituitary-gonadal homeostasis.

Test Systems for Detecting Female Infertility

Female fertility can be disrupted at a number of points during life and at a number of levels in the hypothalamic-pituitary-uterine-ovarian axis. Laboratory tests used in safety studies, however, tend to measure apical end points of estrous cyclicity and the ability to conceive and bear offspring. Entry-level tests can include exposure of males, females, or both sexes.

Two basic designs have been developed for reproductive toxicity testing, one involving exposure during one generation and the other based on exposure across several generations. (For details of the tests discussed in this section, see the appendix to this chapter.) The reproductive toxicity

of therapeutic drugs is usually evaluated in single-generation studies, on the premise that most are taken for relatively short periods and have comparatively short half-lives in the body. Multigeneration studies are used for compounds likely to concentrate in the body as a result of long-term exposure, such as exposures to pesticides and food additives. In these studies, animals are continuously exposed to the test compound, usually in the food or drinking water, for three generations. A new test procedure entitled Fertility Assessment by Continuous Breeding (FACB), under development in the National Toxicology Program, may provide a more accurate assessment of long-term, multigeneration effects on fertility.

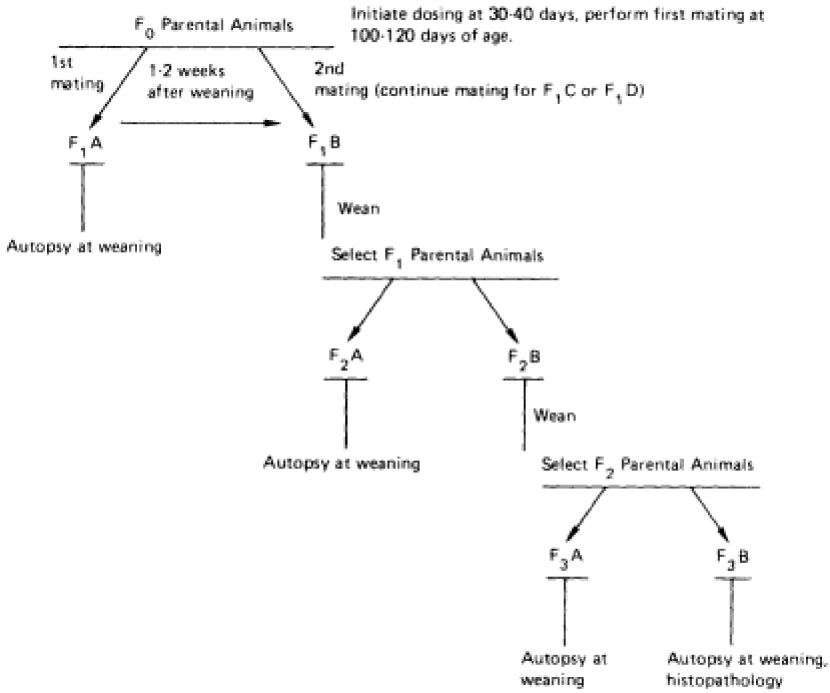

Figure 3-3
Multigeneration study encompassing three generations. From Manson et al., 1982, with permission.

The basic designs of the multigeneration and FACB tests are shown in Figures 3-3 and 3-4. These tests are designed to give an overview of the reproductive process. As such, they cast a broad net over a spectrum of reproductive end points to determine if any are impaired. Impairment often takes the form of an inability to conceive and to bear viable litters. If parental weight gain is approximately within a 10% range of control values, such impairments are indicative of a specific effect on the repro

ductive system. As time-consuming and laborious as these tests are, they rarely provide any insight into the mechanism of action or critical period of exposure, nor should they be expected to do so. In the context of safety testing for persistent environmental agents, a multigeneration-type test is used at initial stages to determine if an agent has any overall effect on reproduction. Consequently, these tests give a qualitative, yes-or-no signal about reproductive toxicity. If well performed, they also provide information of use in the selection of doses for single-generation studies.

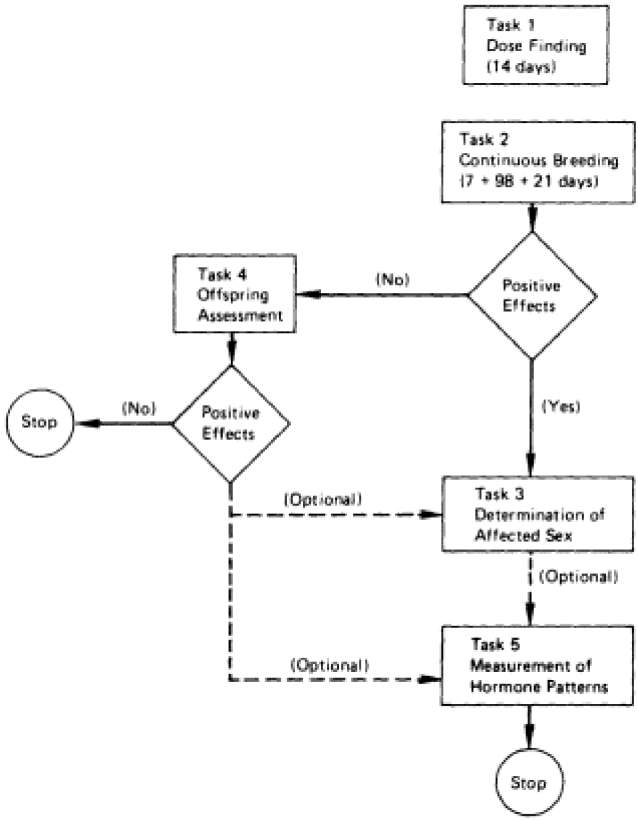

Figure 3-4
Flow chart for National Toxicology Program Fertility Assessment by Continuous Breeding protocol. From Lamb et al., 1984, with permission.

In Segment I studies, which are entry-level fertility tests for drugs, the effects of the test agent on gonadal function, estrous cyclicity, mating behavior, conception rates, and development are assessed. In addition, an overall view of the reproductive process within one generation is obtained.

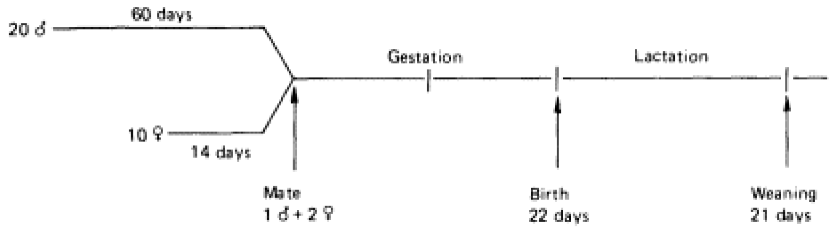

Figure 3-5
Segment I (general fertility and reproductive performance) test. From Manson et al., 1982, with permission.

The basic design of such tests is illustrated in Figure 3-5. If adequately performed, Segment I studies can yield information on the critical period of exposure, but they very seldom give information on the site or mechanism of action.

Although lowest-observed-effect levels (LOELs) and no-observed-effect levels (NOELs) can be identified from these studies, it should be understood that mating performance in rodents is not a sensitive indicator of the integrity of the reproductive system. Extensive germ cell loss, particularly of resting follicles, can occur in female rodents before fertility is measurably disrupted. When an agent tests positive in safety studies (i.e., in multigeneration or Segment I studies), additional studies to pursue the site and mechanism of action should be conducted. Information on some of these tests (e.g., protocols for identifying estrogenic activity and primordial oocyte toxicity) is given in the appendix to this chapter. These tests are more likely to yield the most accurate and quantitative data on reproductive toxicity. When available, results from these tests should be used in identifying LOELs and NOELs.

SUSCEPTIBILITY OF THE MALE TO REPRODUCTIVE IMPAIRMENT

The proliferation of human and laboratory studies conducted over the past 5 years to determine the impact of environmental exposures on male reproductive function reflects a growing concern about such effects. This section describes the susceptible stages in the development of the male reproductive system and the types of reproductive toxicities that are associated with exposure to xenobiotic compounds.

Maturation of the Male Reproductive System

In the early fetal period, prespermatogonial germ cells undergo mitotic proliferation and migration to the gonad, as described earlier in this chapter

for the female. When the gonad differentiates into a testis, Sertoli and Leydig cells form, androgen secretion begins, and the basic pattern of the male reproductive tract is established. Sertoli cells are epithelial cells lining the seminiferous tubules of the testis; they provide the cellular matrix within which germ cells develop. Leydig cells appear in the interstitial regions between Sertoli cells and are the site of *de novo* androgen synthesis. As a direct consequence of androgen synthesis in the fetal testis, the male reproductive tract grows and differentiates, and the accessory glands and external genitalia are formed (Gondos, 1980).

A major difference in male and female germ cell development is that male germ cells do not enter meiosis until puberty. Rather, in the late fetal, early neonatal period, when female oogonia enter prophase I of meiosis, the male prespermatogonial cells enter a period of mitotic arrest. Mitotic activity of prespermatogonial cells is reinitiated at about 10 years of age, and active spermatogenesis begins some 3 years later (Hafez, 1977). Sertoli cells undergo extensive changes during the postnatal period under the influence of FSH. Cell division and growth continue until the onset of puberty, at which time the Sertoli cells become nonproliferating. FSH stimulates the production of androgen-binding protein by Sertoli cells, resulting in a local accumulation of androgen necessary for the initiation of spermatogenesis. Intercellular bridges formed between Sertoli cells under the influence of FSH constitute the blood-testis barrier. This divides the seminiferous tubules into basal and adluminal compartments and isolates the spermatid cell stages from blood-borne chemicals during its development from the spermatocyte stage. Leydig cells undergo a regression in late fetal, early neonatal life, and testosterone production declines. In the postnatal period, Leydig cells redifferentiate, and testosterone and androstenedione synthesis increases, reaching a maximum shortly before puberty (Gondos, 1980).

At puberty, prespermatogonial cells are converted to spermatogonial cells, and meiosis is initiated. In this process, stem cells, the type A spermatogonia, pass through a complex series of transformations to give rise to spermatozoa. During the first phase, type A spermatogonia undergo six mitotic divisions to form type B spermatogonia. After the last mitotic division, preleptotene primary spermatocytes form and pass through the blood-testis barrier into the more protected environment of the adluminal compartment of the seminiferous tubule. These spermatocytes replicate their DNA, enter meiosis, and proceed to the leptotene stage. During the subsequent zygotene stage, the pairing of homologous chromosomes occurs. Cells with completely paired chromosomes are termed pachytene primary spermatocytes. The progression from early pachytene through the middle and late pachytene stage to form secondary spermatocytes is the longest process in meiotic prophase and is also the point at which cells

are especially susceptible to damage. The secondary spermatocytes have a short life span, and without replication of their DNA, they enter the second meiotic division and form haploid spermatids.

The mitotic divisions of spermatogonia into primary spermatocytes are not hormone dependent, but the meiotic divisions of primary spermatocytes into spermatids are believed to be testosterone dependent. The second stage of spermatogenesis, known as spermiogenesis, is characterized by morphological and functional maturation of spermatids to form spermatozoa. Acrosome formation, nuclear condensation, and tail formation take place, accompanied by the loss of cytoplasm.

Even after completion of spermatogenesis, significant changes occur in the sperm of mammals during epididymal transport. Mammalian sperm are incapable of fertilization when they enter the epididymis, but acquire this capacity as they pass through the epididymal duct. They undergo changes in metabolism and exhibit alterations in motility, acrosome shape, degree of cross-linking of nuclear chromatin, and surface charge. Sperm retain their fertilizing capacity in the epididymis for 20 to 35 days in most mammalian species (Bedford, 1966). They attain their full capacity for fertilization after they enter the female reproductive tract in the process of capacitation, when the pattern of energy metabolism changes and motility increases. Capacitation is believed to involve the removal of seminal plasma factors from the surface of the sperm (Aonuma et al., 1973). Differences between species in various aspects of spermatogenesis are given in Table 3-2.

Sperm Toxicity

An estimated cell loss of 35% occurs from the early spermatocyte to the spermatid stage due to physiological atresia (Salisbury et al., 1977). Unlike germ cells in the female, however, cells lost to atresia in the male can be replaced by continual production of spermatocyte cells from division of stem cells. Thus, physiological or drug-induced destruction of germ cells does not necessarily result in a shortening of the fertility span in the male, although it may cause temporary reduction in sperm count. This is true as long as some stem cells are spared; if the insult completely destroys the stem cell population, then permanent infertility will occur.

Testes function is controlled by the gonadotropins LH and FSH. LH stimulates Leydig cells to synthesize androgens, which control the functional activity of accessory sex organs and the development of secondary sexual characteristics. Prolactin, in the presence of other pituitary hormones, has a stimulatory effect on steroidogenesis in Leydig cells. Elevated prolactin levels have the opposite effect and can lead to impotence and infertility. FSH acts on Sertoli cells and is important in the initiation

of spermatogenesis at puberty and in the maintenance of optimal testicular function in the adult. The binding of FSH to Sertoli cells results in a general increase in protein synthesis through a cyclic adenosine monophosphate-mediated process. One protein synthesized is inhibin, which, when secreted, acts as a negative feedback control on the release of FSH. Another is androgen-binding protein, which maintains the high testosterone levels in Sertoli cells needed for maintenance of spermatogenesis. FSH also influences the biosynthesis and interconversions of steroid hormones (Phillips et al., 1985).

TABLE 3-2 Species Differences in Spermatogenic Parameters[a]

Characteristic	Mouse	Rat	Rabbit	Dog (Beagle)	Monkey (Rhesus)	Man
Duration of seminiferous epithelium cycle (days)	8.9	12.9	10.7	13.6	9.5	16.0
Life span (days) of: Type B spermatogonia	1.5	2.0	1.3	4.0	2.9	6.3
Leptotene spermatocytes	2.0	1.7	2.2	3.8	2.1	3.8
Pachytene spermatocytes	8.0	11.9	10.7	12.4	9.5	12.6
Golgi spermatids	1.7	2.9	2.1	6.9	1.8	7.9
Cap spermatids	3.6	5.0	5.2	3.0	3.7	1.6
Fraction of life span as: Type B spermatogonia	0.11	0.10	0.08	0.19	0.19	0.25
Primary spermatocyte	1.00	1.00	1.00	1.00	1.00	1.00
Round spermatocyte	0.41	0.40	0.43	0.48	0.35	0.38
Epididymal transit time (days)	—	8.1	12.7	—	10.5	12.0
Testes wt (g)	0.2	3.7	6.4	12.0	49	34
Daily sperm production Per gram testis (106/g)	28	24	25	20	23	4.4
Per male (106)	5	86	160	300	1,100	125
Sperm reserves in cauda at sexual rest (106)	49	440	1,600	—	5,700	420

[a] Adapted from Amann et al., 1976, and Amann, 1982.

Effects of Radiation on Spermatogenesis

The effects of radiation on spermatogenesis have been studied in a variety of species with different model systems and indices of measure

ment. Although the majority of information has been collected in mice and humans, some investigators have studied the effects of irradiation on the fruit fly, *Drosophila melanogaster* (Eeken and Sobels, 1985; Miyamoto, 1983; Sinclair and Grigliatti, 1985), the Amazon molly, *Poecilia formosa* (Woodhead et al., 1984), rats (Dedov, 1980), and male dogs (Fedorova et al., 1985).

Spermatogenesis in the mouse has been proposed as an *in vivo* test system with utility as a biological dosimeter (Hacker et al., 1980, 1981). Manikowska-Czerska et al. (1985) demonstrated that radiation interfered with spermatogenesis in the mouse, with chemical protection against these effects having low efficacy (Pomerantseva and Ramaija, 1984). Not all stages of spermatogenesis are affected equally in this species, however. Furthermore, cross-species and even cross-strain comparisons of the various stages of spermatogenesis and their susceptibility to radiation should be carefully examined before general conclusions can be drawn (Leenhouts and Chadwick, 1981). Thus, for example, the repair capacity of stem cells and spermatogonia in mice is remarkably high (Hacker-Klom, 1985), in sharp contrast to that observed in man (Greiner, 1982). Similarly, significant strain-specific differences in DNA repair of ultraviolet-induced lesions have been demonstrated in early mouse embryos (Bennett and Pedersen, 1984).

Several investigators have reported that spermatogenesis is considerably more sensitive to the effects of radiation in humans than in mice (Clifton and Bremner, 1983). This difference can be observed in the effects still present 2 to 9 months after exposure (Meistrich and Samuels, 1985). Martin et al. (1985) noted a progression to azoospermia and poor egg penetration in humans following exposure to radiation. All patients recovered from the azoospermia 36 to 48 months after exposure. Thus, while man may be more sensitive than mice to the effects of radiation on spermatogenesis, in terms of duration of observable effect, it appears the species are comparable in their sensitivity to longer-term, irreversible damage (Meistrich and Samuels, 1985).

Test Systems for Evaluating Male Reproductive Toxicity in Laboratory Animals

Entry-Level Testing

The study of male reproductive toxicity encompasses scientific approaches ranging from general toxicity testing to examination of the mechanism of action. The first step in assessing the potential action of an agent on male reproductive function should be to conduct tests in the intact animal so that the spectrum of physiological processes involved can be examined at one time, regardless of the target organ, cell, or molecule.

A comprehensive test system would include the varying susceptibility of the stages of spermatogenesis, the endocrine control of reproduction, the effects on libido, the ability of mature sperm to reach and fertilize the ovum, and, finally, the ability of the conceptus to complete development successfully.

Current approaches to evaluating reproductive function in laboratory animals are reviewed in the appendix to this chapter. The decision whether to conduct a multigeneration or a single-generation (Segment I) study depends on the length of human exposures and the type of agent. In single-generation studies, male rodents are exposed to the test agent for 60 to 80 days and then mated with control females to determine their mating ability (i.e., the number of females inseminated compared with the number of females that become pregnant). After mating, the males are killed and their reproductive organs are weighed and histologically examined. Mated females are killed at midpregnancy or at term, and their uterine contents are examined for preimplantation and postimplantation death. When mated females are killed at term, the fetuses can be weighed and examined for malformations.

Although this type of test is comprehensive, there is growing concern that rodent mating trials may be too insensitive to assess even dramatic effects on spermatogenesis and epididymal functioning. The insensitivity of rodent mating trials is due to the vast excess of sperm ejaculated over the amount required for normal fertility. Males of conventional laboratory animal species produce and ejaculate from 10 to 100 times more sperm than are necessary for normal fertility and litter size (Aafjes et al., 1980; Amann, 1982). Thus, the number of sperm available for ejaculation in rodent species can be reduced by as much as 90% before sterility occurs (Meistrich et al., 1978).

Evaluations of human semen and testicular function have led to the conclusion that "in comparison to laboratory animals, human testes are often functioning at the threshold of pathology" (Amann, 1982). For many men, the number of sperm ejaculated is only two to three times higher than the level at which fertility might be expected to decline (MacLeod and Wang, 1979), whereas in animals the number is many times higher (Amann, 1982). Median counts from four studies of fertile men ranged from 38 million to 68 million sperm per milliliter of ejaculate, while the potential for male infertility is believed to increase when counts fall below 20 million/ml (reviewed in MacLeod and Wang, 1979). Furthermore, the percentages of progressively motile sperm and of morphologically normal sperm in human semen are lower than those typical of males in other species. Consequently, measures of fertility and fecundity in animal models may be insensitive criteria for monitoring the integrity of the male reproductive system, and for identifying agents with the potential for causing reproductive toxicity in men (Amann, 1982).

A major difference between humans and laboratory animals is in the daily sperm production rate per gram of testis (see Table 3-2). The species of choice for reproductive toxicity testing are the rat and the rabbit. Rats are preferable to other rodents because of their well-characterized reproductive processes and general use in toxicological studies. As in all rodents, however, semen quality in rats cannot be evaluated in longitudinal studies. Rabbits are good in such studies because of the large amount of background information on their reproduction and because their semen can be quantitatively collected in longitudinal studies (Amann, 1982).

Current recommendations for entry-level testing are to expose rats to an agent for the complete period of spermatogenesis, which extends over approximately 77 days and includes 6 cycles of the seminiferous epithelium. After 77 days, treated males are mated with control females to evaluate fertility. After mating, males are given 6 to 8 days of sexual rest to allow restoration of sperm reserves in the cauda epididymis. If combined with a 90-day subchronic toxicity study, fertility testing would thus be postponed until days 78 to 84. At the time of sacrifice, reproductive organs are weighed and placed in fixative for histological evaluation. One testis and epididymis are saved to evaluate testicular sperm production rate and epididymal sperm numbers, morphology, transit time, and motility (Amann, 1982; EPA-ORNL, 1982).

This testing procedure is comprehensive and sensitive. Disruption of any of the reproductive processes in the male could be detected by decreased testicular or epididymal characteristics or by lowered fertility or fecundity. On the basis of results from this entry-level test, decisions could be made to conduct secondary-level testing to pursue positive results. If an elevation in epididymal sperm with morphological abnormalities was identified, for example, secondary testing could be conducted to identify germ cell mutations, which is described later in this chapter. Similarly, agents that did not affect sperm production but that caused failures in mating could be evaluated for effects on the physiology of ejaculation.

Human Test Systems

According to Overstreet (1984), more than 15% of all recently married couples will have major difficulties in conceiving a child. Approximately one-third of these cases of infertility result from a pathological condition in the male, one-third are attributed to the female, and one-third are due to a combination of factors in both partners. More than 75% of female infertility problems can be diagnosed and treated, but the biological bases of male infertility are not well understood. Few if any therapies can be offered to most infertile men. This clinical inadequacy can be attributed to a lack of basic research on male infertility, as the brief review in this

section indicates, and to the inadequacy of current diagnostic procedures (Overstreet, 1984).

Sperm Morphology

Extensive studies have been performed on the induction of abnormal sperm shape by physical and chemical agents in laboratory animals (Wyrobek, 1977). The visual assessment of sperm morphology is very subjective and critically dependent upon the classification scheme used. It is relatively easy to detect subtle deviations in the shape of the highly angular sperm heads in laboratory rodents. The more ovoid shape of the typical human sperm head, however, makes subtle differences in shape difficult to detect. There are also large interlaboratory differences in sperm morphology criteria (Wyrobek et al., 1984).

Recent work in mice suggests that generalized toxicity in the whole animal can result in an increased incidence of abnormal sperm forms (Komatsu et al., 1982), indicating that sperm abnormalities may not always be the direct effect of chemically induced gonadal damage. In the human, the link between semen quality and adverse pregnancy outcome is ambiguous. In some studies (Furuhjelm et al., 1962), poor sperm quality (count and morphology) was linked to the frequency of early pregnancy loss. Fathers of 201 spontaneous abortions had significantly more sperm shape abnormalities and lower sperm counts than did fathers of 116 normal pregnancies. Although several studies support a link between sperm defects and abnormal reproductive outcome, others found no correlation (see Wyrobek et al., 1984, for a review). More studies in humans are needed to compare exposures of male parents with resulting sperm defects and reproductive outcomes.

The morphology of sperm in the semen of fertile humans is stable. Approximately 60% of the sperm heads have the normal oval shape, and 40% have some type of abnormal form. The percentage of abnormally shaped sperm in an ejaculate remains relatively constant for one person but varies considerably between men. To detect relatively small changes in the percentage of abnormally shaped sperm following an exposure, a preexposure baseline should be established for each male. A procedure entailing repeated sampling and analysis of the ejaculate is the most accurate way to detect increases in abnormally shaped sperm.

Sperm morphology has gained popularity as a human surveillance technique because, unlike sperm number and motility, it is unaffected by frequency of ejaculation. In a study of six repeated measurements of semen samples from 100 males, large variations in volume, sperm number, and motility were found, but sperm morphology was identified as the most predictive and stable parameter for diagnosing infertility (Sherins et al., 1977). As indicated in Table 3-3, a relatively small population (at least

26 men) is needed in each group to conduct statistically valid studies of changes in sperm morphology.

TABLE 3-3 Relative Sensitivities of Three Assays of Human Sperm[a]

	Type of Assay (and Population Size)[b]		
Sensitivity Measure	Counts (N = 214)	Morphology, % (N = 26)	2F Bodies, % (N = 41)
Mean values[c]	132 x 10^6/ml	41.9	0.8
Standard deviation	160 x 10^6/ml	12.4	0.7

[a] Adapted from Edmonds et al., 1981, pp. 47 and 69, with permission of the copyright holder, the March of Dimes Birth Defects Foundation.
[b] Sample size for 5% level test with 80% power.
[c] Mean sperm counts, percentage of morphologically abnormal sperm, and percentage of sperm with 2F bodies in semen samples from fertile men.

Figure 3-6 contains drawings of morphologically normal and abnormal human sperm cells. The abnormal shapes are relatively easy to identify from stained preparations of semen smears. Other abnormal shapes, especially those involving sperm with small heads, are difficult to detect, and classification is usually subjective. Recent attempts to use computer-assisted technology for morphometric analysis of head length, maximum head width, head area, head circumference, and maximum midpiece width should greatly reduce the subjectivity of sperm shape assessment (Overstreet, 1984).

Since some morphologically abnormal sperm lack motility, the extent to which malformed sperm participate in fertilization is unclear. In the mouse, the proportion of sperm with abnormal head shape remains unchanged during transit from the testis, through the epididymis and vas deferens, and into the uterus. Around the egg, however, the proportion of abnormally shaped sperm is substantially lower (Krzanowska, 1974). Other studies have suggested that sperm with abnormally shaped heads but with normal motility and intact acrosomes can still fertilize (Clavert et al., 1975). In a review of this topic, Salisbury et al. (1977) contend that sperm take part in fertilization in numbers proportional to those reaching the egg. Selective pressures against sperm with grossly imbalanced chromosome complements and abnormal morphology are exerted during spermatogenesis and in the female reproductive tract. If genetically damaged sperm do fertilize an egg, a major selective pressure is embryo death; relatively few if any embryos carrying the genetic damage survive to term.

Thus, although abnormal sperm shape is clearly indicative of a disruption in spermatogenesis, it is not necessarily predictive of adverse pregnancy outcome. A more definitive conclusion about the role of these

abnormalities in determining the nature of the offspring will be important, because semen analysis is particularly adaptable to human reproductive risk assessment. As this method gains wide acceptance, it will be necessary to determine how predictive it is of male reproductive insult versus the potential for adverse pregnancy outcome.

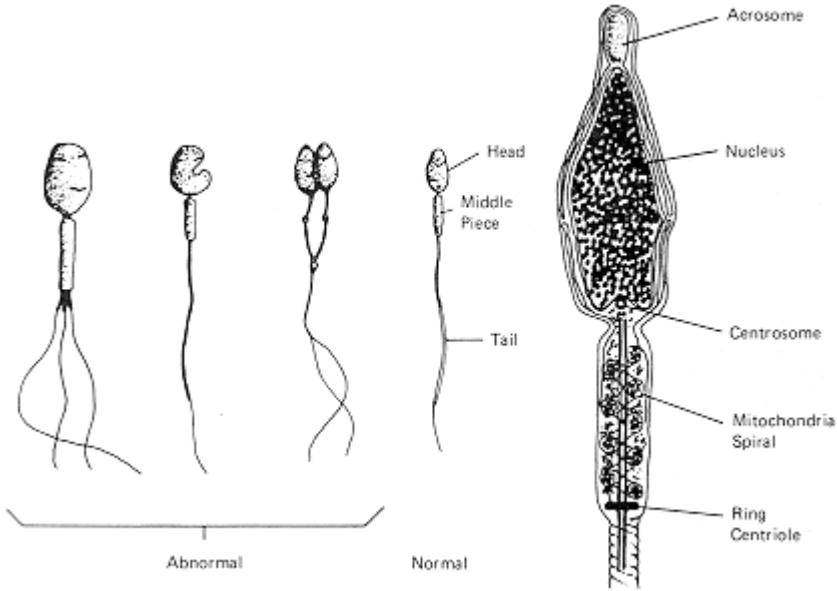

Figure 3-6
Normal and abnormal morphologies of human sperm cells.

Sperm Motility

The motility of sperm in human semen has been used for many years to predict male infertility (MacLeod and Gold, 1951). It is usually rated in two ways: by percentage of motile cells and by the quality of sperm movement (e.g., how fast and how straight the sperm swim). Sperm movement has traditionally been rated on a scale of 0 to 4 +, 2 + being regarded as normal. The normal percentage of motile cells in human semen is at least 50% to 60% (Eliasson, 1975).

The subjectivity of this assessment has greatly limited its usefulness. More recent, less subjective methods are based on photomicrographic techniques to obtain objective measurements of human sperm movement. Time-exposure photomicroscopy of moving sperm produces tracks on the negative that can be measured to calculate swimming speed. A mean swimming speed of 25 µm per second is considered adequate (Overstreet,

1984). In more recent procedures, videotapes of semen have been used to measure sperm swimming speeds. Videomicrography is more convenient and easier to interpret than time-exposure photography, since the sperm themselves rather than their tracks can be visualized (Katz and Overstreet, 1981).

Semen Analysis

At present, semen analysis is the primary method used to assess male fertility in humans. Induction of pregnancy is the only other test of this potential, but this end point is subject to highly confounding and variable factors such as female infertility, frequency of sexual contact, and contraceptive use. The strong differences in opinion on what constitutes a normal ejaculate are based on variations in methods for collecting semen as well as on the wide range of normal variability in the human population.

Methods for analyzing semen from humans and laboratory animals have undergone extensive development in the past 5 years. The evolution of basic research in mammalian sperm physiology has resulted in several new techniques for assessing human sperm functions. The following parameters can be measured from semen samples:

- gross parameters, including sperm concentration, sperm motility, and sperm morphology;
- functional parameters, including sperm-cervical mucus interaction, sperm-oocyte interaction, and seminal fluid content;
- genetic parameters, such as sperm chromosome complement (as measured with the YFF, or 2F bodies test, as described below).

Sperm Concentration

Sperm concentrations in the ejaculates of fertile and infertile men have been studied with the goal of identifying a range of sperm concentrations that would be predictive of fertility. Traditionally, subfertility was believed to occur when the sperm concentration was less than 20 million/ml of ejaculate (MacLeod and Gold, 1951). More recent studies have indicated that this level may be too high for an accurate assessment of fertility (Sherins et al., 1977; Smith and Steinberger, 1977). The division between fertility and subfertility has more recently been set at 10 million/ml, or 50 million sperm/ejaculate, a figure supported by conception rates as well as by plasma gonadotropin levels. Significant elevation in plasma FSH, indicative of germinal epithelial damage, occurs in men with a sperm concentration less than 10 million/ml and with total sperm counts below 25 million/ejaculate (Smith and Steinberger, 1977). It is important that

semen donors have 2 to 3 days of abstinence from ejaculation before providing a sample for sperm count determination.

Although sperm concentration is one of the most frequently examined semen parameters, it is not a sensitive indicator of early spermatogenic insult. Since sperm counts vary considerably, even among fertile and presumably healthy men, many cooperative subjects are needed to establish differences between control and exposed groups in studies where each male is sampled only once. As indicated in Table 3-3, at least 214 men are needed in each group (control and exposed) to detect a 25% change in mean sperm count values. With mean sperm count values of 132 million/ ml, men in the infertility range of less than 10 million/ml have most likely already experienced a considerable degree of spermatogenic insult. Thus, sperm count measurements alone can be used to detect severe cases of potential subfertility. When coupled with other semen parameters, a more sensitive indication of early or marginal insult can be obtained.

Sperm Chromosome Complement

The Y chromosome in human sperm can be differentially stained with quinacrine dye so that it becomes a visible fluorescent spot in individual sperm cells. This procedure, called the YFF or 2F bodies test, is based on scoring the frequency of sperm with two fluorescent (F) bodies and thus presumably two Y chromosomes in an ejaculate (Kapp and Jacobson, 1980). Although 50% of the sperm would be expected to have one spot, in practice, frequencies range from 30% to 40%. Approximately 1% to 2% of the sperm in ejaculates from fertile men contain two Y chromosomes, or two F bodies. Sperm with two Y chromosomes are believed to be formed as a result of meiotic nondisjunction, when homologous chromosomes are not equally partitioned to secondary spermatocytes during metaphase of the first meiotic division. An elevated frequency of sperm with two Y chromosomes has been interpreted as indicating nondisjunction of the Y and other chromosomes, and thus of chromosomal abnormality of the sperm (Kapp and Jacobson, 1980). Given the tight packing of nuclear material in sperm heads, it is not possible to visualize any chromosomes other than the Y for cytogenetic analysis. Although Kapp and Jacobson suggested that sperm with two spots contain two Y chromosomes, major uncertainties remain in this interpretation.

Validation of this test has been hampered by the lack of fluorescent Y chromosomes in the sperm of common laboratory or domestic animals. This quality of fluorescence seems to be unique to the Y chromosomes of humans and the higher apes. Nonetheless, this system may provide a useful model of chemically induced nondisjunction in male germ cells. Relatively small test populations (at least 41 men in each treatment group)

are needed for statistically valid application of the YFF test (see Table 3-3).

Measurement of chromosome aberrations in decondensed human sperm DNA may also be useful as an assay for human germ cell mutagens (Preston, 1982). But interindividual variation in background aberration rates must be thoroughly understood before this test can be meaningfully applied.

The chemical induction of a positive response in an *in vitro* genetic toxicity assay cannot be considered an indication of the chemical's potential as a germ cell mutagen *in vivo*. Numerous compounds that exhibit genetic toxicity *in vitro* and many compounds that are carcinogens *in vivo* in animals fail to produce measurable genotoxicity in germ cells *in vivo*. Dimethylnitrosamine, for example, is a potent genotoxic carcinogen (IARC, 1978); however, it does not produce detectable alkylation of mouse sperm heads *in vivo* (Stott and Watanabe, 1980), it does not induce DNA repair in spermatocytes *in vivo* (Doolittle et al., 1984; Working and Butterworth, 1984), it does not alter sperm number or the incidence of morphologically abnormal sperm in hamsters (Wyrobek et al., 1978), and it is uniformly negative in mammalian germ cell mutation assays (Epstein et al., 1972). A variety of pharmacokinetic and adaptational factors in germ cells are probably the cause of this apparent mutagenic selectivity.

Pharmacokinetic parameters that can modify the mutagenic potential of xenobiotic compounds in the gonads include, but are not limited to, those that control the absorption and detoxification of xenobiotic compounds within the exposed animal as well as the biotransformation capability of the gonads. Evidence indicates that the gonads contain relatively low levels of biotransformation activity with respect to monooxygenation reactions and, in contrast, that detoxifying enzymatic activities predominate in both ovaries and testes (see review by Heinrichs and Juchau, 1980). Epoxide hydratase, glutathione-*S*-transferase, and aryl hydrocarbon hydroxylase activities have been measured in the testes and ovaries and in some cases are differentially distributed between germ cell and interstitial cell compartments (Mukhtar et al., 1978; Oesch et al., 1977). Gonadal biotransformation is an important factor in the chemical induction of germ cell mutagenesis, since highly reactive electrophilic metabolites have extremely short biological half-lives and, thus, are not likely to be transferred from one organ to another (Nelson et al., 1977). Hence local, i.e., extrahepatic, bioactivation of promutagens plays an important role in the mediation of chemically induced genotoxicity within the gonads.

Accurate information about the mutagenic hazards of chemical exposure has been difficult to obtain and is therefore limited. The few assays suitable for detecting germ cell mutagens *in vivo* generally fall into one of two broad categories: (1) those that detect effects presumed to be related to

DNA alterations and that may be useful as predictors of mutagenic potential, and (2) those that directly measure heritable mutagenic events. Assays in the former category measure germ cell effects but not necessarily heritable mutation. Assays for germ cell effects include the measurement of DNA damage (Skare and Schrotel, 1984) or repair (Pedersen and Brandriff, 1980; Sega, 1982; Working and Butterworth, 1984), the assessment of sperm morphology (Wyrobek et al., 1982), the measurement of mutagen-induced chemical modification of DNA (Stott and Watanabe, 1980), the detection of chemically induced chromosome aberrations in the zygotes (Adler and Brewen, 1982), and the dominant lethal test (Ehling, 1977) which scores all genetic effects in early embryos that cause death of offspring.

Positive results in any of these assays should not be considered as final evidence that a chemical is a germ cell mutagen but, instead, that it is an indicator of possible mutagenicity. A positive response can, however, be taken as presumptive evidence that a suspected mutagen or its metabolites did reach the germ cells. Assays of this type detect actual genomic alteration, measured as the effects of gene mutation or gross alteration or damage of chromosomes. The heritable translocation test (Generoso et al., 1980) detects primary reciprocal translocations; the specific locus test (Russell et al., 1981) measures intragenic lesions at marked loci, which are recovered in F_1 offspring.

Many factors must be weighed when extrapolating data from these *in vivo* animal test systems to humans. Differences in background mutation rates have been observed between different strains of mice (Generoso et al., 1983), between different animal species (Lyon and Smith, 1971), and even between humans from urban and nonurban populations (Neel and Rothman, 1981). Nevertheless, an indication of mutagenic activity in the germ cells of test animals must be taken as a warning of potential mutagenic activity in humans.

The most relevant animal models are those in which germ cell mutations can be measured directly by the heritable translocation test and the specific locus test. Assays that measure germ cell DNA effects, including dominant lethals, DNA damage and repair, chemical alterations in germ cell DNA, and alterations of chromosome structure in zygotes, may be less relevant but can provide information relating to the accessibility of the gonads to the putative mutagen.

Sperm-Cervical Mucus Interaction

Sperm functions related to transport in the female can be assessed by observations of sperm-cervical mucus interaction. Sperm deposited in the vagina must migrate through cervical mucus en route to the site of fer

tilization. A postcoital test to examine sperm in the mucus is an important part of any infertility evaluation (Overstreet, 1984).

Sperm penetration of midcycle cervical mucus in humans significantly correlates with fertility (Kunitake and Davajan, 1970; Ulstein, 1973). In one study, the relative fertility of sperm donors in artificial insemination programs correlated more strongly with cervical mucus penetration than with any other semen parameter (Ulstein, 1973). Katz and Overstreet (1980) developed a new quantitative technique for evaluating this interaction through the use of flat capillary tubes. In this assay, the expected number of collisions between sperm cells and mucus is determined and the proportion of those collisions that result in successful penetration is calculated. In addition, the vitality of sperm that enter the mucus is measured by comparing their swimming speeds there with their speeds in the semen. The difficulty of obtaining midcycle human cervical mucus for this test, however, limits its immediate application to human reproductive risk assessment.

Sperm-Oocyte Interaction

Yanagimachi et al. (1976) have developed a test of human sperm fertilizing capacity that involves the incubation of human sperm with hamster ova. In this system, the zona pellucida of the hamster ova, which constitutes a barrier to cross-species fertilization, is enzymatically removed. This permits penetration of human sperm into the hamster ooplasm. Both capacitation and the acrosome reaction are required for this interspecies fertilization, and the hamster egg system is a suitable assay for these sperm functions. The penetration of the treated hamster ova by sperm from infertile men was significantly lower and was not correlated with sperm concentration, motility, or morphology. Although tests of sperm-oocyte interactions appear to be sensitive in detection of the early stages of functional infertility (i.e., not associated with abnormal counts, morphology, or motility), they are technically difficult to perform. At present, sperm-oocyte interaction tests are most appropriately applied in settings such as artificial insemination clinics rather than in surveillance studies of human populations.

Examination of Seminal Fluid

The average ejaculate from a fertile man comprises approximately 3 ml of semen containing 50 million to 120 million sperm per milliliter of seminal plasma. Approximately 60% of the volume of the seminal plasma is contributed by seminal vesicle secretions, 30% by the prostate gland, and the remainder by the urethral and bulbourethral glands, ampullae,

epididymis, and testes (Lewin, 1977). Sperm cells, along with accumulated rete testis fluid, pass into the caput epididymis, which absorbs most of the rete testis fluid and secretes certain components of it—principally carnitine. Mature sperm move from the epididymis into the vas deferens and then to the ampulla. Upon ejaculation, sperm are mixed with secretions from the seminal vesicle and prostate, which together provide the bulk of seminal fluid volume.

The biochemical composition of the seminal plasma is complex and differs in many respects from that of blood plasma and other body fluids. The measurement of components in seminal fluid specific to the secretions of each accessory gland in the male reproductive system can aid the clinician in evaluating the functional status of those glands. To evaluate prostate function, the levels of acid phosphatase, citric acid, inositol, zinc, and magnesium can be monitored. Fructose levels are used as a marker for seminal vesicle function, and carnitine levels for the epididymis (Eliasson, 1982). Alterations in the levels of these seminal plasma constituents can indicate functional alterations in the accessory organs that are related to male fertility.

The excretion of drugs into semen is a phenomenon that is not well understood. In addition to influencing sperm motility and capacitation, drugs in semen have been reported to cause allergic reactions in women after intercourse. Cases of vaginitis have been reported in women whose sexual partners were taking vinblastine therapeutically (Paladine et al., 1975), presumably due to excretion of this drug in the semen. Various drugs excreted into the semen of rats have caused pharmacological responses in female rats after mating (Ericsson and Baker, 1966). A number of chemical agents have been shown to be rapidly absorbed through the vaginal mucosa and to enter the systemic circulation of the female (Hartman, 1959).

Lutwak-Mann et al. (1967) found thalidomide or its metabolites in rabbit semen after giving them ^{14}C-thalidomide by oral intake. When added to semen *in vitro*, thalidomide bound to rabbit sperm. Progeny of thalidomide-treated male rabbits exhibited low birth weights, elevated incidences of birth defects, and poor neonatal survival.

Similar findings have been obtained with methadone. Male rats given methadone sired offspring with low birth weights and depressed neonatal survival. High levels of methadone are excreted in the semen, giving a semen:blood ratio of 0.82 to 4.72 (Gerber and Lynn, 1976).

A number of antibiotics have also been measured in human semen in studies to develop improved treatments for venereal disease and chronic prostatitis (Malmborg et al., 1976). Thus, a spectrum of drugs are excreted in the semen and have the potential of being spermatotoxic and of entering the systemic circulation of the female after intercourse. The impact of this route of exposure on sperm and on the female has not been fully explored.

TABLE 3-4 Chromosome Macrolesions in Germ Cells

		Outcome			
Type of Lesion	Stage of Induction	Germ Cell Death	Embryonic Death	Late Fetal Effect	Postnatal Survival
Chromosome break	Gonial	+			
	Postgonial		+		
Balanced chromosome rearrangement	Gonial		+	+	+[a]
	Postgonial				+[a]
Missegregation	Premeiotic	+[b]	+[c]	+[d]	+[e]
	Meiotic		+[c]	+[d]	+[e]

[a] Roughly 50% of survivors will carry the balanced rearrangement. Some of these may show organismic disorders. All will have some chromosomally unbalanced offspring.
[b] Possibly some monosomies.
[c] Monosomies.
[d] Most autosomal trisomies die.
[e] Sex chromosome numerical anomalies and a few autosomal trisomies.

GERM CELL MUTAGENESIS IN MALES AND FEMALES

Genetic toxicants must be regarded as potential germ cell mutagens and, thus, as threatening irreversible damage to the human gene pool. Although there is as yet no documented case of chemically induced heritable genetic disease in humans (ICPEMC, 1983b; Mohrenweiser and Neel, 1982), epidemiological studies suggest that the association found in animal studies may also apply to humans (Strobino et al., 1978). This potential association is of particular concern when chemical exposure occurs through contaminated drinking water, since exposure may be chronic throughout the reproductive years.

Test Systems for Germ Cell Mutations

Two types of mutagenic damage can be examined in germ cells: macrolesions and microlesions. A macrolesion is a microscopically visible change in either chromosome number or structure. The types of macrolesions that have been observed, their transmissibility, and their effects on reproductivity are described in Table 3-4. Macrolesions may be detectable in the germ cells of the parental animal or human in any cell of any offspring to which they have been transmitted. Certain macrolesions observed in specific germ cell stages of the parent are likely to lead to germ cell death, and these will not be transmitted to offspring. For example, chromosome breakage in spermatogonia can produce a dicentric chromosome and an acentric fragment during spermatogonial replications,

leading to loss of the affected chromosome and probable cell death. On the other hand, chromosome breakage in a meiotic or postmeiotic germ cell does not lead to chromosome loss until after the resulting gamete has participated in fertilization. This produces early embryonic death, i.e., an effect transmitted from the exposed parent to his or her offspring.

Chromosome arrangements such as translocations or inversions are not lethal to cells, but segregation during meiosis produces chromosomally unbalanced as well as balanced gametes, all of them capable of participating in fertilization. Conceptuses resulting from the former type of gamete usually die during prenatal life. Those resulting from balanced gametes survive postnatally and have roughly a 50-50 chance of being chromosomally normal, or carrying the balanced rearrangement and in turn transmitting it to some of their offspring. (They also transmit unbalanced chromosome complements to others of their offspring, which will die as early embryos). Although in the past it has been assumed that carriers of balanced rearrangements are themselves normal, there is increasing evidence that some balanced translocations can be associated with certain organismic disorders (Russell and Shelby, 1985; Rutledge et al., 1986).

Heritable numerical chromosome anomalies result from missegregation of chromosomes during mitotic or meiotic divisions of the germ cells. Offspring can be trisomic or monosomic for the chromosome that has missegregated. If the affected chromosome is an X or Y, the offspring often survive to adulthood. Monosomies for any of the other chromosomes lead to early embryonic death, but trisomies do not kill until late fetal stages and are associated with organismic defects. In humans, trisomies for chromosomes 21 (Down's syndrome), 18, and 13 survive postnatally.

Microlesions, such as frameshifts and base-pair substitutions, are chemical alterations in DNA that are transmissible without cell death as dominant or recessive mutations. Dominant mutations are expressed in the F_1 generation and can be transmitted to all subsequent generations in up to 50% of the offspring. They are often recognizable, however, so that the possibility of transmitting this effect further could be reduced by knowledgeable genetic counseling. Recessive mutations probably represent the greatest long-term hazard in that they can be accumulated in the population in the heterozygous state (Brusick, 1978).

Mutations can and probably do occur both in coding and noncoding regions of the genome. Many of these will be unnoticed as silent mutations. Their silence is often a reflection of the accuracy and sensitivity of analytical techniques, which range from morphologic analysis of offspring to a search for alterations in protein structure or function and in DNA sequence. Any change induced at a single genetic locus is a monogenic or Mendelian mutation that can be inherited as a recessive or dominant

trait. A recessive mutation is not expressed in an individual when the normal allele is present at the same locus in the homologous chromosome. However, its presence can often be detected by molecular techniques.

Other abnormalities are transmitted by polygenic inheritance in which more than one genetic locus is responsible for their expression. Examples of these are seen in some adult diseases (diabetes, coronary artery disease, hypertension), psychiatric diseases (schizophrenia, depression), and congenital defects (cleft lip, club foot, neural tube defects, congenital heart defects) (Dean, 1983).

A number of studies have been conducted to determine if heritable mutations occur in the human germ line as a result of exposure to ionizing radiation or various chemicals. To date, studies of the offspring of large populations of atomic bomb survivors in Japan have shown no increase in the frequency of heritable genetic diseases that can be attributed to the radiation exposure of the parents, in whom significant somatic cell effects were observed. The genetic end points monitored in the progeny have included a variety of easily observable phenotypic changes. Smaller-scale studies of patients who received medical radiation for testicular cancer or lymphomas provide further evidence that genetic effects, if any, are not easily detectable in human populations, which are very heterogeneous genetically. A number of occupational and environmental exposures to various chemicals have been associated with heritable genetic defects induced in the male germ line. These results were obtained from highly controversial studies, however, and have not been uniformly replicable (reviewed by Dean, 1983).

Studies on exposures to agents resulting in adult male reproductive dysfunction are summarized in Tables 3-5 and 3-6. In general, agents have been examined for their effects on fertility, testicular histology, and neuroendocrine balance. Information on the mechanism of action whereby these agents exert their effects on the reproductive system is scarce; it is inferred mostly from studies of mechanisms in other organ systems. Most of the agents listed cause reversible or short-term effects; permanent sterility rarely occurs under the exposure conditions described. The paucity of observations in humans (Table 3-5) is not a result of negative results but a reflection of the absence of studies. The concordance in response between humans and laboratory animals to those agents studied in both species is apparent, implying that much more experimentation in humans is needed.

The findings from studies in laboratory animals do, therefore, show that germ cell mutations induced by chemical exposure can result in passage of genetic diseases to the offspring. The dominant lethal, heritable translocation, and specific locus tests are the methods most commonly used in such studies to assess the mutagenic potential of chemicals in

TABLE 3-5 Results of Studies in Human Males Demonstrating Reproductive Effects from Environmental and Occupational Exposures[a]

Agents	Type of Exposure Conditions	Group Exposed	Type of Study	Effects
Anesthetic gases	Occupational	Male workers	Reproductive history	Increased incidence of congenital anomalies in offspring
Carbon disulfide	Occupational	Male workers	Semen analysis; reproductive history	Impotence; loss of libido
Chloroprene	Occupational	Male workers	Semen analysis; reproductive history	Decreased motility and number of sperm; threefold excess of miscarriages in partners
Dibromochloropropane	Occupational	Male workers	Semen analysis; reproductive history	Decreased sperm count; infertility
Hydrocarbons	Occupational	Male workers	Reproductive history	Twofold increased incidence of cancer in children of fathers occupationally exposed to hydrocarbons
Kepone	Environmental	Males	Reproductive history	Decreased fertility
Lead	Occupational	Male workers	Semen analysis	Decreased sperm count and motility; increase in number of abnormally shaped sperm
Microwaves	Occupational	Male workers	Semen analysis; reproductive history	Decreased libido; decreased sperm count and motility; increase in number of abnormally shaped sperm
Cigarette smoke	Environmental	Males	Semen analysis	Increase in number of abnormally shaped sperm
Irradiation	Occupational	Male workers	Gonadotropic hormone and semen analysis	Depression of gonadotropic hormone levels; alterations in spermatogenesis
Oral contraceptives	Occupational	Male workers	Reproductive history; physical exam; blood analysis	Gynecomastia; decreased libido; infertility
Vinyl chloride	Occupational	Male workers	Reproductive questionnaire	Adverse pregnancy outcome; excess fetal loss in partners

[a] Adapted from Manson and Simons, 1979.

mammalian germ cells (Russell and Shelby, 1985). They have been extensively used in studies of radiation exposure and, to a lesser extent, chemical mutagens. The genetic risk evaluated in these tests is based on an increase in embryonic death and inherited alterations in the F_1 generation. Since analogous studies cannot be conducted in humans, results from these tests are extrapolated to determine the potential risk to humans.

Dominant Lethal Test

The dominant lethal test is the conventional entry-level test for assessing clastogenic damage to the germ cell. It can be used to measure germ cell macrolesions in both male and female animals, although typically it is conducted in males, since embryonic death following exposure of females might be the result of adverse effects on the maternal environment, rather than of genetic damage in the conceptus. Male animals are treated for 1 to 5 days and then sequentially mated with groups of control females for the duration of spermatogenesis (Green et al., 1985).

The sensitivities of different stages of spermatogenesis to mutation vary widely, in some cases by as much as 100- to 1,000-fold. Therefore, male rats are conventionally bred on a weekly basis for 10 consecutive weeks after exposure. This timing permits sampling of germ cells exposed in the mature sperm, spermatid, spermatocyte, spermatogonia, and stem cell stages of maturation. The mating index (the number of females inseminated divided by the number of females caged with males) and the fertility index (the number of females pregnant divided by the number of females inseminated) are calculated. Females are killed at midpregnancy and examined for the number of living or dead embryos, implants in the uterine horns, and corpora lutea in the ovary. The rates of preimplantation death (the number of corpora lutea divided by the number of implantation sites) and postimplantation death (the number of resorption sites divided by the number of implantation sites) are calculated. Mutation of sperm is measured as increased mortality among embryos conceived with treated sperm (Green et al., 1985).

The dominant lethal assay has been the test most frequently used to measure germ cell mutations. One disadvantage is that a high spontaneous background of embryonic mortality, on the order of 7% to 10%, occurs in most rodent species, which reduces the sensitivity of the test. Only dominant mutations that are lethal to the embryo are measured, and the genetic origin of these macrolesions cannot be fully determined because only one generation—the F_1—is examined. Advantages of the assay are that it measures the most prevalent reproductive outcomes of germ cell damage, i.e., infertility and embryo death, and thus requires fewer animals than other assays that may be more sensitive but measure rarer outcomes.

TABLE 3-6 Results of Studies in Animals Demonstrating Reproductive Effects in Males from Exposures to Environmental Agents[a]

Agents	Exposure Conditions	Species	Type of Study	Effects	References
Metals					
Cadmium chloride	0.1-8 mg/kg,[b] s.c., i.m., i.v.,[c] intratesticular, single dose	Dog	Histological, semen analysis	Damage to testicular blood vessels; disruption of blood-testis barrier; destruction of seminiferous tubules; reduced fertility	Donnelly and Monty, 1977
Lead-cadmium interaction	25 µg/day, i.m., i.p.,[d] 70 days	Rat	Histological	Absence of spermatogenesis; synergistic effect of simultaneous exposure to lead and cadmium	Der et al., 1976
Methylmercury hydroxide and mercuric chloride	1 mg/kg, i.p., single dose	Mouse	Serial mating	Inhibition of early stages of spermatogenesis; reduced fertility	Lee and Dixon, 1975
Nickel sulfate	25 mg/kg/day, oral, 120 days	Rat	Histological	Damage to testicular parenchyma; reduced fertility	Waltschewa et al., 1972
Pesticides					
Carbaryl	7, 14, and 70 mg/kg/day, oral, 12 months	Rat	Endocrinological, semen analysis	Reduced motility of sperm; alterations in spermatogenesis	Shtenberg and Rybakova, 1968
Chlordecone (Kepone®)	200 ppm diet, 42 days	Japanese quail	Histological	Fluid-filled and atrophic testes; disruption of germinal epithelium	Eroschenko, 1978
Dibromochloropropane	100 mg/kg, oral, single dose	Rat	General toxicological	Reduced weight of testes; alterations in spermatogenesis	Torkelson et al., 1961; Reznik and Sprinchan, 1975

Agents	Exposure Conditions	Species	Type of Study	Effects	References
DDT	200-500 mg/kg, oral, 2-19 days	Juvenile rat	Histological, dominant lethal	Reduced weight of testes; alterations in spermatogenesis; small litters	Krause et al., 1975
DDVP (Dichlorvos)	10-20 mg/kg, oral, 2-19 days	Juvenile rat	Histological, dominant lethal	Reduction of spermatogenic cells and Leydig cells; reduced fertility	Krause et al., 1976
Malathion	20-40 mg/kg, oral, 2-19 days	Juvenile rat	Histological	Reduction of spermatogenic cells and Leydig cells	Krause et al., 1976
Herbicides					
Diquat	76 mmol/kg, i.p., single dose	Mouse	Dominant lethal	Reduced fertility of exposed males	Pasi et al., 1974
Paraquat	66 mmol/kg, i.p., single dose	Mouse	Dominant lethal	Reduced fertility of exposed males	Pasi et al., 1974
Fungicides					
Captan	2.5-200 mg/kg, gavage, i.p., 5 days	Mouse	Breeding	Reduced fertility; decreased survival of offspring	Collins, 1972
Physical Agents					
Elevated carbon dioxide (CO_2)	1.8/1.0 mixture of air/CO_2, inhalation, 6-26.5 hours (over 6 days)	Mouse	Semen analysis	Abnormally shaped sperm; low conception rate	Mukherjee and Singh, 1967
Elevated temperature	Various	Multiple species	Histological, hormone profile	Atrophy and pathology of testis, prostate, and seminal vesicles; lowered testosterone levels; seminiferous tubule pathology; abnormally shaped sperm	Lipshultz and Howards, 1983
High oxygen tension	Various	Various	Review	Gonadal injury; testicular atrophy; disorganization of germinal cells; arrest of spermatogenesis	Balentine, 1966

Agents	Exposure Conditions	Species	Type of Study	Effects	References
Physical Agents (Continued)					
Irradiation	200-1,000 rads	Mouse	Histological and general toxicological	Reduction in testes weight; alterations in spermatogenesis; embryo death	Cattanach et al., 1977; Ehling, 1971
Food Additives and Contaminants					
Aflatoxin B_1	5 mg/kg, i.p., single exposure	Mouse	Dominant lethal	Reduced fertility	Leonard et al., 1975
Dibutyl phthalate	2,000 mg/kg, oral, 3-14 days	Rat	Histological	Histopathology and reduction in testes weight	Cater et al., 1977
Diethyl adipate	0.43-1.44 ml/kg, i.p., single dose	Mouse	Dominant lethal	Reduced fertility; increased numbers of early fetal deaths	Singh et al., 1975
Glutamic acid (monosodium glutamate)	25 mg/kg, oral, 1 month	Rabbits	Histological and general toxicological atrophy of testes	Inhibition of spermatogenesis; reduced fertility;	Tugrul, 1965
Nitrofuran compounds	10 mg/100 g, oral, 7 days	Rat, guinea pig	Histological	Atrophy of testes; degeneration of seminiferous tubules	Miyaji et al, 1964; Albert et al., 1975
Other Agents					
Carbon disulfide	Inhalation	Rat	Histological	Alterations in spermatogenesis	Artamonova and Klishova, 1972
Carbon tetrachloride	0.3 ml/100 g, i.p., 10, 15, 20 days	Rat	Histological	Atrophy and necrosis in testes	Kalla and Bansal, 1975

Agents	Exposure Conditions	Species	Type of Study	Effects	References
Chloroprene	Various	Mouse; rat; *Drosophila*	Dominant lethal; semen analysis cytogenetics; recessive lethal	Testicular atrophy; abnormal semen profile; reduced fertility; increased embryo mortality; chromosome anomalies	Infante et al., 1977
Cyclosiloxanes	2-200 mg/kg, oral, 2-40 days	Rabbit; dog	Histological	Testicular pathology; depression of spermatogenesis	Palazzolo et al., 1972; Nican-der, 1975
Ethoxyethanol	100-800 µl/kg, s.c., oral, 28-59 days	Rat	Histological	Testicular edema; absence of germ cells	Stenger et al., 1971
Ethylene oxide	1,000 ppm in air, inhalation, 4 hours	Rat	Dominant lethal	Increased postimplantation fetal death; decreased fertility	Embree et al., 1977
Hair dyes	Oral, 3 days	*Drosophila*	Recessive lethal	Mutagenic primarily to spermatids and spermatocytes	Blijleven, 1977
Methoxyethanol	50-200 mg/kg, oral, 5 days	Rat	Reproduction	Sperm abnormalities and reduced fertility	Chapin et al., 1985
Nitrous oxide (N_20)	20% N_20, 20% 02, 60% N_2, 35 days	Rat	Histological	Damage to seminiferous tubules; suppression of spermatogenesis	Kripke et al., 1976
Phthalate esters (mono-*n*-butyl phthalate and di-*n*-butyl phthalate)	2,000 mg/kg, oral, 4-6 days	Rat	Histological	Testicular pathology	Gangolli, 1982
Tris (flame retardant)	2.27 g/kg/week, dermal, 13 weeks	Rabbit	Histological	Testicular atrophy; degeneration of spermatozoa	Osterberg et al., 1977

[a] Adapted from Manson and Simons, 1979.
[b] Per kilogram of body weight per day, unless otherwise noted.
[c] s.c., subcutaneous; i.m., intramuscular; i.v., intravenous.
[d] i.p., intraperitoneal.

TABLE 3-7 Cell Divisions in the Formation of Germ Cells[a]

	Females			Males			
	No. of Cell Divisions			No. of Cell Divisions			
Species	Before Germ Cell Formation	During Oogenesis	Maximum No. of Oocytes	Before Germ Cell Formation	To Mature Sperm	Division Cycle (days)	No. of Stem Cells
Humans	Unknown	21	3.4×10^6	Unknown	380-540[b]	16	5×10^8
Mice	10-13	13	1×10^4	10-13	40-80	8	1×10^6

[a] Adapted from Lyon et al., 1979.
[b] Between 28 and 35 years of age.

In addition, cell stage specificity can be detected through the sequential mating design. Overall, the sensitivity of the dominant lethal assay is such that germ cell mutagens can be identified with certainty if large numbers of animals are used (50 rodents per group) and if the compound is a potent mutagen (Green et al., 1985).

The increased sensitivity of male germ cells to dominant lethal or other types of mutations is based on the fact that many mutations occur predominantly in replicating cells. As indicated in Table 3-7, the stem cell spermatogonia of male mammals continue to replicate throughout the breeding life, which confers susceptibility to replication-dependent mutations. In female germ cells, as noted earlier, mitosis ceases at the fetal stage, and the oocyte remains in a resting stage of meiotic prophase from birth until puberty. Thus, since the oocyte spends a greater proportion of time in a nonreplicating state, the incidence of replication-dependent mutations will be lower in females than in males (Lyon et al., 1979).

A dominant lethal assay in females would be based on a similar design, except that exposed females would be mated with control males at weekly intervals up to 7 weeks after exposure to sample the different stages of oocyte maturation. Dominant lethal tests are not routinely performed in females, however, because it is difficult to discriminate between nonspecific systemic toxicity (uterine toxicity resulting in failure to implant) and true genetic alterations in the germ cells (Badr and Badr, 1974). The sex chromosome loss test can be used to detect clastogenic damage in female germ cells. This test utilizes genetic markers on the X chromosome, so that it can be identified by a marker phenotype rather than by a reduced number of survivors as in the dominant lethal test (Russell and Shelby, 1985). The presumed XO-females detected by the marker phenotype can be verified cytogenetically by the presence of only 39 chromosomes.

The end point observed in the dominant lethal test is embryonic death, presumably due to chromosome aberrations in sperm. Thus, a tremendous

inherent selectivity is involved in the identification of mutagenic events to be monitored; i.e., there is a focus only on aberrant germ cells that survive the meiotic sieve, that are competitive with normal germ cells, and that result in early embryonic death. Somatic cell assessments (metaphase analysis) can detect a wider spectrum of chromosome-damaging events, including the type that could lead to a dominant lethal event. In a study comparing the two types of assays for 76 compounds, the total incidence of concordance (positive-positive or negative-negative) between somatic and germ cell assays was 75%. Eighteen compounds gave a positive response in one or more somatic cell assays but were negative in germ cell assessment; no compounds were positive in germ cell assays but negative in somatic cells (Holden, 1982). In a comparison of available published data, the International Commission for Protection against Environmental Mutagens and Carcinogens (ICPEMC, 1983) found 88% concordance between germ cell assays (mouse specific locus, heritable translocation, and rodent dominant lethal tests) and cytogenetic in vitro tests, 83% concordance between germ cell assays and sister chromatid exchange tests, and 64% concordance between germ cell assays and the Ames test.

This leaves open the question of whether germ cells are equipped with more effective mechanisms for removing mutagenic lesions. These mechanisms may take the form of DNA packaging, DNA repair capability, or the meiotic sieve (Holden, 1982), which confer selective disadvantage to genetically abnormal cells. Each of these would be expected to reduce the mutagenic response of germ cells relative to that of somatic cells. The important application of these assays is to determine whether an agent that causes genetic toxicity in somatic cells also causes heritable mutations in germ cells, or if testicular lesions or reductions in male fertility identified in reproductive toxicology studies are due to germ cell mutations.

Heritable Translocation Test

The heritable translocation test is also used frequently to assess germ cell mutations. Male mice are exposed and mated to control females, which are allowed to deliver their litters. The F_1 male progeny are reared to reproductive maturity, mated to control females, and then examined to estimate their sterility or semisterility. Criteria for F_1 male sterility include an absence of embryos in females with vaginal plugs; for a classification of semisterility, there must be a decrease in the number of live embryos per litter relative to controls. The germ cells of males exhibiting varying degrees of sterility are analyzed cytogenetically at meiotic metaphase for translocations (Brusick, 1978; Generoso et al., 1980).

TABLE 3-8 Relative Inducibility of Dominant Lethal Mutations and Heritable Translocations in Male Germ Cells[a]

Mutagen (and Dose)	Germ Cell Stage	Percentage of Dominant Lethal Frequency	Percentage of Heritable Translocation Frequency
X ray (700 rads)	Spermatozoa and spermatids	67	27.0
Ethyl methane sulfonate (200 mg/kg[b])	Spermatozoa	69	32.0
Triethylenemelamine (0.2 mg/kg)	Spermatids	75	29.0
Isopropyl methane sulfonate (75 mg/kg)	Spermatozoa	82	2.7
	Spermatids	58	0.4
Benzo[*a*]pyrene (500 mg/kg)	Spermatozoa	27-50	0.18

[a] Adapted from Generoso, 1982.
[b] Per kilogram of body weight in each case.

Carriers of translocations have reduced fertility—a criterion used to select F_1 progeny for cytogenetic analysis. Complete sterility is caused by certain types of translocations (X-autosome and c/t type). Translocations are cytogenetically observed in meiotic cells at metaphase I of males and in either F_1 males or male offspring of F_1 females (Russell and Shelby, 1985).

The background rate of heritable translocations is relatively low: maximum of one carrier per 1,000 control animals (Generoso, 1973). In the heritable translocation test, only one type of chromosome aberration is measured—a balanced translocation, which can occur only through genetic alteration of the germ line but not through nonspecific cytotoxicity. More animals are needed for this test than for the dominant lethal assay because the control rate is very low. At least 500 male progeny should be tested per dose group (ICPEMC, 1983).

The levels of dominant lethal and heritable translocation mutations induced in male germ cells by x rays, by several direct-acting alkylating agents, and by benzo[*a*]pyrene are listed in Table 3-8. For a number of mutagens, including ethyl methane sulfonate, triethylenemelamine, and x rays, there is a positive correlation between the induction of dominant lethal mutations and the occurrence of heritable translocations. The frequency of heritable translocations induced by the maximum tolerated dose of any of these three agents is about 30% among live progeny in each case. This figure is reached when the frequency of dominant lethal mutation is approximately 60% or more. Studies of isopropyl methane sul

fonate and benzo[a]pyrene have indicated, however, that these compounds differ markedly in that few or no heritable translocations were induced, even at doses that caused high levels of dominant lethal mutations. Thus, some compounds can induce both types of genetic changes, whereas others induce only one (Generoso, 1982).

Specific Locus Test

As discussed above, the dominant lethal and heritable translocation assays measure dominant mutations that are macrolesions in the chromosomes of germ cells. In the specific locus test, recessive point mutations, or microlesions, can be measured, and mutations to recessive alleles at a small number of specified loci can be detected in the first generation. The test method consists of mating treated, wild-type male mice to a test stock of untreated females that are homozygous for recessive markers at several loci. When there is no mutation at these loci in the treated male germ cells, none of the recessive markers contributed by the mother would be expressed in the F_1 generation because they would be in the heterozygous state. In the presence of mutation, alterations in coat color, eye color, and other visibly apparent morphological features occur (W. L. Russell, 1951).

To measure rare events occurring at only a few targets, one must examine a large population in order to observe a significant change in mutation frequency. Since only seven loci are available for monitoring in the specific locus tests with mice, many animals must be used. Thus, this test can be used to measure frequencies of recessive mutations but not to evaluate large numbers of chemicals.

Several investigators have developed methods to detect electrophoretic variants of proteins as measures of point mutations (Johnson and Lewis, 1981; Valcovic and Malling, 1975). These methods increase the total number of loci assessed in the specific locus test and thus require observation of fewer F_1 animals, although the time required for examination of each F_1 animal is considerably longer. Combining the specific locus test with the scoring for cataracts in F_1 progeny quadruples the number of loci that can be scored, since dominant mutations at approximately 20 loci can produce cataracts (Ehling, 1980). Examination of F_1 skeletons can detect dominant mutations at over 100 loci (Selby, 1983).

The specific locus test can also be applied to females to study recessive mutations in oocytes. In this case, the treated, wild-type parent would be female, and the untreated parent would be a homozygous recessive male. Although relatively few studies have been conducted in females, it appears that specific locus mutations, like macrolesions, are predominantly induced in the first 7 weeks after treatment. Experiments with the mutagens

procarbazine (Ehling, 1980) and ethylnitrosourea (Russell et al., 1979) indicate that oocytes may be less sensitive than spermatogonia for induction by chemicals of specific locus mutations.

RISK ASSESSMENT IN REPRODUCTIVE TOXICOLOGY

In light of the 15% infertility rate among married couples, it is surprising that risk assessment is infrequently based on reproductive toxicity data and that safety testing programs do not always include measurements of adverse effects on reproduction. For example, the Toxic Substances Control Act, P.L. 94-469, identifies four progressive levels of testing, depending on the extent, frequency, and nature of chemical use, but information on reproductive effects is not required until level IV, when the product is already on the market. At level II, the most comprehensive test is a subchronic study designed to predict effects on humans exposed during production or industrial use.

As demonstrated by Koëter (1983), there is concern that the acceptable daily exposure level estimated from the subchronic study may be too high with respect to effects on reproductive function. Koëter evaluated toxicity data on 37 compounds tested both in subchronic studies and in one or more reproductive toxicity studies to determine the impact of the latter in identifying the NOEL and the LOEL. For the LOEL, reproductive toxicity studies were more sensitive than the subchronic studies for 35% (13) of the compounds. They were equally sensitive for another 35% (13). For 65% (17) of these 26 compounds, effects related to reproduction and development were factors in the determination of the LOEL. For 30% (11) of the total number of compounds tested, the reproductive toxicity studies were less sensitive than the subchronic studies. For 8 of the 37 compounds tested (>20%), fertility or reproduction end points were the most sensitive, and this solely determined the LOEL. Koëter (1983) concluded that reproductive function is highly sensitive to impairment and should be examined at earlier stages of safety testing.

Even when data from reproductive toxicity studies are available for use in risk assessment for a particular compound, there is much confusion about how to apply results from animal studies to humans. The confusion is due partly to the fact that considerably less is understood about the underlying events leading to reproductive toxicity than is known about corresponding processes in mutagenesis and carcinogenesis. There are currently no agreed-upon standard quantitative methods for cross-species extrapolation. Some of the conditions under which reproductive toxicity data from animal studies can be applied to predict human risk and the methods currently used are examined in Chapter 8.

APPENDIX: DETAILS OF TEST PROTOCOLS FOR REPRODUCTIVE TOXICOLOGY TESTING

MULTIGENERATION TEST

The classic multigeneration study encompasses three generations. Figure 3-3 provides the basic outline of the procedure but does not encompass all possible variations. First, weanling animals (usually rats or mice, 30 to 40 days of age) are randomly assigned to control or test groups, and the test compound is administered continuously in the diet or drinking water or via inhalation for the duration of the study. After at least 60 days of exposure, or when these parental, F_0 generation animals are 100 to 120 days old, males and females within a treatment group are mated to produce the F_1A generation. Within 24 hours after birth, each pup is weighed, sexed, and examined for gross abnormalities. On day 4, litters of more than 10 pups are culled to 10 or some smaller constant number by random selection. Separate weights for male and female offspring and their survival are recorded at birth and on days 4, 7, 14, and 21 of life. After weaning (21 days of age), F_1A offspring are sacrificed and autopsied to detect internal abnormalities.

After weaning of F_1A pups, 1 to 2 weeks are allowed to pass before the second mating of the parental generation takes place for production of the F_1B offspring. The same procedure is followed for observation and data collection, except that a sufficient number of animals are selected at weaning to serve as the parents of the F_2 generation. Males and females are randomly paired within a treatment group, but brother-sister matings are avoided. F_1B weanlings remaining after selection of parents are sacrificed and autopsied. The F_2A and F_2B litters are generated in the same manner as the F_1A and F_1B litters, and the same observations are made from birth to weaning. The F_3 generation litters are produced from parents selected from the F_2B litters. The pups are weighed, sexed, and weaned in the same manner, but at sacrifice, tissues from at least 10 animals are preserved for histological examination. Complete necropsies are performed on all animals that die spontaneously during the study. At routine necropsy, observations are made on all major organ systems, and weights are obtained.

Although the protocol described in Figure 3-3 still serves as a model for multigeneration studies, several modifications are often conventionally applied (Collins, 1978a,b). A teratology study can be added by mating F_1A offspring instead of sacrificing them at weaning. Likewise, the F_3B offspring can be mated and their progeny used for teratology studies. After resting, the parental animals may be mated to produce an F_1C or an F_1D

generation to study the effects of exposure on reproduction in aging animals. The F_2A generation can be maintained for chronic studies to examine the carcinogenic effect of the exposure.

According to current recommendations, three dose levels and one control group are used. The highest dose level may be a multiple of the human exposure level or it may be one-tenth of the test species' LD_{50}. The low dose is determined from subchronic or chronic toxicity studies and is expected to be a no-observable-effect level. The intermediate dose level is logarithmically spaced between the high and low levels. When rodents are used, enough females should be started at each dose level so that at least 20 pregnant females per dose level for each generation are obtained. All doses are reported as mg/kg body weight per day, which necessitates measurement of food and water intake.

Rodent species are used most frequently, so that a three-generation study can be conducted within 20 months. Although other species are acceptable, rats and mice are the species of choice because they are inexpensive and there is a large amount of historical data on testing in these animals.

The following indices are calculated from data derived from multigeneration studies:

- The fertility index: (the number of pregnancies/the number of matings) x 100. This index represents the percentage of matings that result in pregnancies.
- The gestation index represents the number of live fetuses per litter or the number of live-born per total born.
- The sex ratio is calculated at birth and at 4, 7, 14, and 21 days of age.
- The viability index: (the number of pups alive at 4 days/the number of pups born alive) x 100. The number of pups remaining after culling (10 or 8) is used in the denominator on days 7, 14, and 21 of age.
- The weaning index: (the number of pups alive at 21 days/the number of pups maintained at 4 days) x 100.
- The growth index represents the average weights of male and female offspring at birth and at 4, 7, 14, and 21 days of age.

To obtain results that are statistically sound, it is critical that at the beginning of the study, parental animals be randomly assigned to control and treatment groups. This procedure should also be followed in culling litters to a constant size and in selecting parental animals from each generation for mating.

A number of variants of this procedure, based on regulatory and international guidelines, have been described by Palmer (1981). For further

elaboration on multigeneration tests, consult the review prepared by Collins (1978a,b).

FERTILITY ASSESSMENT BY CONTINUOUS BREEDING

The National Toxicology Program is validating its new reproductive toxicity test, Fertility Assessment by Continuous Breeding (FACB) (Lamb et al., 1984). This test provides an alternative to multigeneration studies, producing similar comprehensive reproductive toxicity data at a lower cost and within a shorter time. In addition, it provides for multiple, sequential breeding (up to five mating cycles) of one parental generation, which permits identification of latent toxic effects on immature germ cells (resting follicles, spermatogonia).

During the first task, a vehicle control and five dose groups (eight/sex/group) of 8-week-old CD-1 mice are repeatedly dosed for 14 days, during which time dose levels for the second task are identified. The high dose, or maximal tolerated dose, may produce some significant signs of toxicity, but it should not suppress body weight gain to levels more than 10% below that of the controls and it should allow 90% or greater survival. The intermediate dose should produce minimal or no toxic effects, and the low dose should ideally be a no-effect level. An LD_{10} calculation is made, clinical signs are observed, body weight gain during the 14-day period is measured, and either food or water consumption is measured, depending on the route of exposure.

The continuous breeding phase occurs in task 2, which consists of a vehicle control group (40/sex) and three dose groups (20/sex/group). Eleven-week-old male and female CD-1 mice are exposed to the test agent during a 7-day premating period, during which time clinical signs are observed and measurements are made of weight changes and water consumption, if water is the route of exposure. The mice are then randomly paired (1:1) within each group and housed together for 98 days (14 weeks). Newborn litters are evaluated and immediately sacrificed. Exposure of the parents is continuous during the 98-day cohabitation period and the subsequent 21-day period of segregation. Observations made throughout these two periods include number of litters produced per breeding pair; number and percentage of fertile pairs; number and percentage of live newborns per litter; mean body weight of newborns; length of time between litters; parental body weights at weeks 2, 5, 9, 13, and 18; and water consumption at weeks 1, 2, 5, 9, 13, and 18. At the end of the 98-day cohabitation period, data are evaluated to determine whether to proceed to task 3 or task 4. The final litters born to the control and high-dose pairs during the segregation period may be weaned, reared to maturity, and evaluated for

reproductive performance in task 4. Chemical exposure of the offspring is continued.

If significant effects on reproductive performance (fertility or litter size) are found in task 2, then the affected sex is identified in task 3. Task 3 is a continuation of the 119-day mating trial. On day 120, the following cross-matings (20/sex) are performed: high-dose male with control females, control males with high-dose females, and control females with control males. All controls are assigned new cagemates. Treatment is discontinued during the 7-day cohabitation period; after mating has occurred, parental animals are placed back on their original treatments for 21 days.

To determine reproductive performance, the following observations are made and evaluated: vaginal cytology in females not mating, percentage of fertile pairs, mean litter size, number and percentage of live newborns, and mean litter weight.

If males are identified as the affected sex, they are killed and bled by cardiac puncture. The following observations are then made and evaluated: body weight, liver weight, fixed brain weight, fixed pituitary weight, right testis weight, ventral prostate weight, seminal vesicle weight, right epididymal weight, number of cauda epididymal sperm per milligram of tissue, and sperm morphology and motility.

If females are the affected sex, they are also killed and bled by cardiac puncture. The following observations are then made and evaluated: body weight, liver weight, fixed brain weight, fixed pituitary weight, and weight of the reproductive tract (ovaries, oviducts, uterus, and upper half of vagina).

Task 4 is a continuation of the 119-day mating trial. If no adverse effects on fertility are found during continuous breeding, offspring obtained between days 98 and 119 from high-dose and control groups are reared to reproductive maturity. At 70 ± 10 days of age, 20 male offspring and 20 female offspring from the same treatment groups are paired. Chemical exposures continue throughout task 4, until litters are born.

If possible, all high-dose and control task 2 litters are sampled and sibling matings are avoided. The pairs are housed together for 7 days, or less if a copulatory plug is observed, and treatment is continued during cohabitation. Reproductive performance is determined by evaluating the litters. If it is found to be adversely affected in the high-dose pairs, then task 3 can be carried out with these same mice to determine whether one or both sexes are affected. In this instance, the high-dose animals are bred to control animals of the opposite sex.

If treatment has an adverse effect on fertility or reproductive performance in parental mice (task 3) or in their offspring (task 4), radioimmunoassays for reproductive hormones can be performed on plasma collected at necropsy. Hormones evaluated in the male are testosterone, FSH, and

LH; in females, they are estradiol-17 (or total estrogens), prolactin, FSH, and LH.

The following end points are evaluated in task 4: number and percentage of fertile pairs, litter size, litter weight, and number and percentage of live-born animals.

SEGMENT I STUDIES: GENERAL FERTILITY AND REPRODUCTIVE PERFORMANCE

Segment I studies can be performed with exposures of males alone, females alone, or both, depending on the background information available. According to Food and Drug Administration guidelines, 20 male rodents are exposed for 70 days to cover the duration of spermatogenesis. A minimum of 20 females are exposed for 14 days to cover three estrous cycles. Treated animals are mated, and exposure of inseminated females is continued throughout pregnancy and lactation. Either at midpregnancy or just before delivery, half the females per test group (at least 10) are killed, and their uterine contents are examined for preimplantation and postimplantation death. The remainder (a minimum of 10) deliver spontaneously and wean their offspring. Weanlings are killed and autopsied for gross observations and examination of viscera.

Common variations in Segment I testing are to mate treated males and females with untreated counterparts to determine sex-specific effects on fertility and pregnancy outcome. Also, the midpregnancy sacrifice can be delayed to day 20 so that fetuses can be examined for malformations. Recommendations have been made that weanlings be spared and raised to maturity to detect latent effects on behavior, physiological development, and reproductive capacity (Collins, 1978b).

The following observations are made in Segment I studies:

- Preimplantation death: number of corpora lutea in ovaries/number of implantation sites in uterine horns. Most control dams have a slight excess of corpora lutea, which appear as highly vascularized bulges on the surface of the ovary.
- Postimplantation death: (the number of resorption sites in the uterus/ number of implantation sites) x 100. One to two resorption sites are conventionally found in control litters.

Statistical procedures based on the assumption of a normal distribution are not preferable for Segment I studies in that the occurrence of embryo death in litters more closely follows a Poisson distribution. Variance analysis with nonparametric tests, such as the Mann-Whitney U-test for comparison of two groups and the Kruskal-Wallis test for comparison of more than two groups, are appropriate (Gaylor, 1978). The fertility of the parental animals and viability of the offspring can be expressed in the

manner used for findings of the multigeneration test, i.e., fertility index, gestation index, sex ratio, viability index, weaning index, and growth index.

Experience with this test procedure has led some investigators (Palmer, 1978) to conclude that the most common outcome observed with positive agents is infertility. Thus, mating success is a critical factor that should be carefully assessed. When nonpregnancy is encountered, additional studies that include examination of paternal contribution at lower exposure levels should be performed to ensure that infertility does not mask effects such as delayed implantation or later developmental toxicities. With 10 animals per treatment group for each observation (10 for midpregnancy examination, 10 litters for weanling autopsies), only highly active reproductive toxicants can be detected. A 40% to 50% difference between control and treatment groups must be obtained to achieve statistical significance at the 0.05 level by the Fischer exact test. Thus, compounds with borderline toxicity are not likely to be detected by this test unless large numbers of animals are used (Palmer, 1978).

For Segment I studies, dosages should be extrapolated from subchronic studies in adult animals. The choice of dose should be made with an understanding of the complex relationship between maternal toxicity and embryonic toxicity. Adverse effects on the embryo may occur secondarily and nonspecifically from adverse effects on the mother. The highest dose used should not cause frank maternal effects (e.g., sedation, hyperactivity, and respiratory distress) or death. Treatment should be regulated so that no more than a 10% reduction in maternal weight gains compared with controls occurs in the highest dose group. Dosages causing minimal signs of maternal toxicity, such as increased liver weight, can be selected from subchronic studies in adult animals. The choice of low and intermediate doses should be made only after a suitable high dose has been selected. The lowest dose should cause therapeutic or physiological effects similar to those intended for humans or, in the case of environmental agents, should lead to measurable tissue levels or enzyme induction without toxic effects. The intermediate dose should lie logarithmically between the lowest and highest level.

TIME OF VAGINAL OPENING IN THE RAT PUP

This test can be used to identify estrogenic activity of test agents such as the estrogen agonists DES, clomiphene, and tamoxifen, which cause premature vaginal opening in the rat. First, newborn litters are culled to a constant size at birth (not less than six pups). The neonates are injected on days 1, 3, and 5 of postnatal life, and the time of vaginal opening is

noted. Vaginal opening should occur by day 7 in controls (Clark and McCormack, 1980).

UTERINE EPITHELIAL HYPERTROPHY

Rat pups are treated as described above, and uteri are removed, weighed, and processed for histological evaluation on days 7 to 10 after birth. Compounds such as Kepone, DES, DDT, and zearlenone have been shown to stimulate uterine growth and cause epithelial hyperplasia (Clark and Peck, 1979).

PRIMORDIAL OOCYTE DESTRUCTION IN JUVENILE MICE

Female mice are injected intraperitoneally at 7 to 21 days of age with six graded doses of the test agent. There are five animals per treatment group. The highest dose should be at the adult LD_{50} for the agent. The day after the last dose, ovaries are removed, fixed, serially sectioned (5 μm), and stained. Oocytes and follicles are classified according to the procedure of Pedersen and Peters (1968) by histological examination of every twentieth section. The percentage of primordial follicles surviving relative to controls is plotted against dose, resulting in sigmoidal dose-response curves for positive agents. Quantitative comparisons between agents can be made in terms of the oocyte toxicity index. This is the ratio of the LD_{50} for the juvenile mouse to the oocyte LD_{50} (Dobson and Felton, 1983).

SPERM PRODUCTION RATES IN RATS

One testis (freed of the epididymis and spermatic cord) from each male is weighed. Absolute testis weight should be reported rather than weight per gram of body weight. In normal adult males, testis weight and body weight are independent variables ($r = 0.24$ for 125-day-old rats; Robb et al., 1978).

Relative changes in sperm production rates can be established by direct comparison of testicular spermatid reserves, i.e., the number of spermatids per testis, per pair of testes, or per unit weight of testicular parenchyma. The technique for determining testicular spermatid reserves involves homogenization of testicular tissue and subsequent enumeration of elongated spermatids by hemacytometry. Because the time required for these cells to be transformed into sperm is relatively constant for members of the same species, relative rates of sperm production can be determined and the rates in control and experimental animals can be compared. These procedures are much simpler and less tedious than histological methods,

and a larger sample of the testis can be analyzed. Since elongated spermatids are the last cells to be formed in the testis, alterations in spermatogenesis at any stage should ultimately be reflected in changes in spermatid reserves (Berndtson, 1977). Also, ejaculation frequency does not influence daily testicular sperm production (Amann, 1982).

Rat testicular parenchyma is homogenized in 20 to 30 ml of a cold 0.9% sodium chloride solution containing 0.01% Triton X-100. The tissue is homogenized at low speed in a laboratory blender for 2 minutes, and spermatids are concentrated by low-speed centrifugation. This procedure destroys the nuclei of somatic cells and all germinal cells except elongated spermatid nuclei with shapes characteristic of step 17 to 19 spermatids (Clermont, 1972). Resistant spermatid nuclei are counted in a hemacytometer (at least six chambers per sample), and counts are expressed per testis and per milligram of parenchyma. These values are divided by 6.10 days (the time required for spermatids to form spermatozoa in the rat) to obtain the daily sperm production rate.

In an alternative procedure, homogenization-resistant spermatids are counted with a Coulter counter. This requires sonication of the sample after homogenization and filtration through a screen for removal of residual debris prior to counting (Mian et al., 1977). Likewise, linear gradients of 22% to 36% Percoll can be used to obtain highly purified preparations of rat spermatids (Meistrich et al., 1979).

ASSESSMENT OF EPIDIDYMAL SPERM NUMBERS AND TRANSIT TIMES

Epididymal sperm numbers can be determined from fresh or frozen samples. The epididymis is weighed and divided into cauda and corpus plus caput sections. Each section is minced and homogenized separately in 20 ml of sodium chloride-Triton X-100 solution, and the number of spermatozoa in each section is enumerated in a hemacytometer, as described above. Epididymal transit times are calculated by dividing the total number of spermatozoa per whole epididymis by the sperm production rate of the associated testis on a per animal basis (Robb et al., 1978).

ASSESSMENT OF SPERM MOTILITY

The cauda epididymis is excised immediately after sacrifice and placed in a petri dish containing 10 ml of Dulbecco's calcium-and magnesium-free phosphate-buffered saline and a 10-mg/ml concentration of bovine serum albumin. The solution is kept at 37°C. The tissue is finely minced with scalpels for approximately 1 minute. Then the dish is placed in a 37°C incubator for 15 minutes before assessing sperm movement. After

incubation, a 15-µl aliquot of the sperm suspension is placed on a microscope slide and a cover slip is added. At least nine different microscopic fields are then videotaped for 10 to 20 seconds using phase-contrast microscopy and a 16X objective. The videotapes are later analyzed for percentage of motile cells (50 cells per animal) and straight-line swimming speeds (25 cells per animal). The video system and methods for analyzing the tapes have been described by Katz and Overstreet (1981). The sperm specimen must be held at 37°C during taping in an air-curtain incubator.

HISTOLOGICAL EVALUATION OF THE TESTIS

The choice between histological or homogenization techniques for quantifying relative changes in sperm production depends upon the nature and severity of anticipated effects. One limitation of homogenization techniques is that they are not useful in establishing the relative severity of effects on earlier stages of spermatogenesis. Also, the recovery of spermatogonia, spermatocytes, and early spermatid stages cannot be evaluated in the absence of elongated spermatids. Neither histological nor homogenization techniques are clearly superior in all instances. Consequently, it is advisable to process testicular tissue so that both procedures can be utilized.

A number of excellent reviews have been published on procedures for preparing slides and carrying out histological analysis of the testis. Lamb and Chapin (1985) have reviewed in detail the different methods of fixation and embedment for rat testicular tissue. The most common procedures, i.e., fixation in 10% neutral buffered formalin followed by paraffin embedding, produces highly unsatisfactory results that preclude even qualitative evaluation of the testis. A major improvement occurs with fixation in Bouin's, Zenker's, or Helly's fixatives followed by paraffin embedment. When tissues are embedded in glycol methacrylate, formalin becomes a suitable fixative for the testis (although glutaraldehyde is better) and superior resolution of cellular detail is obtained. Moreover, the entire cross section of the rat testis can be obtained in one block, permitting examination of up to 400 tubules. Coupled with the range of usable stains and histochemical techniques, the water-based plastic embedding media are preferable for most light microscopic studies.

In histological procedures, cross sections of seminiferous tubules are considered to be representative of the testis as a whole. This is valid provided all seminiferous tubules are affected uniformly and the histological sections are separated by a reasonable distance. The seminiferous tubules of most mammals are highly convoluted, and cross sections of the same tubule may appear in a section of testicular tissue from a single location. Consequently, at least five tissue sections separated from each

other by a minimum distance of 1,000 μm should be used for histological evaluations (Amann, 1982).

Initial qualitative appraisal of the testis should take into account the appearance of Leydig cells, the occurrence of lymphoid cell or macrophage infiltration, the presence of each stage of germ cell (spermatogonia, spermatocytes, spermatids, sperm) in seminiferous tubules, and the presence of large numbers of degenerating, multinucleate, or abnormal cells (EPA-ORNL, 1982). In addition, the percentage of tubular cross sections with no evidence of spermatogenesis should be scored during brief examination of 250 cross sections per testis magnified 100 or 400 times. The percentage of tubules with spermatids lining the lumen should be approximately 30% for normal rats, and the percentage of those with no evidence of spermatogenesis should be less than 5% (Amann, 1982). Such examination can be performed 2 to 7 days after acute treatment or six cycles after initiation of chronic treatment. Most likely, these qualitative assessments will be highly correlated with measurements of daily sperm production rates.

Different types of information are obtained from observations of long-term and short-term exposures to (or sacrifice after administration of) an agent. In short-term studies, several hours or a few days are usually necessary to observe results after single or multiple exposures to an agent. The initial morphological changes are the primary indicators of a defect, and they provide the best clues to the mechanism of damage. Long-term administration of agents results in end-stage effects or in maturation-depletion effects, in that no further deterioration of the testis occurs with time. Experiments designed to show end-stage effects best reveal the extent to which maturation of germ cells has been arrested. Detailed descriptions of methods for precise staging of the seminiferous epithelium of the rat have been described by Leblond and Clermont (1952a,b), Berndtson (1977), and Russell (1983).

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4

Neurotoxic Effects

Interest in the toxicology of the nervous system has grown rapidly in recent years not only because of heightened public concern about the impact of toxic substances on human health but also because the nervous system has been shown to be especially vulnerable to chemical insult (Anger and Johnson, 1985). The potential effects of such toxicity are quite varied, reflecting the large role played by the nervous system in regulating vital body functions and in perceiving, assessing, and responding to the external environment. Interference with these processes by overexposure to chemical and certain physical agents can produce a variety of abnormal neurobehavioral responses, some of which may be life threatening, others resulting in short-term neurobehavioral changes that disappear without a trace, and still others causing permanent or even progressive neurological or psychiatric disorders. Of special concern is the possibility that environmental exposure to certain neurotoxic agents, such as lead, may irreversibly compromise the normal development and capacity of the human brain.

Although there are no reliable estimates of the number of persons who develop neurotoxic disorders in adult life, or of the impact of overexposure to chemical agents on the developing or aging nervous system, more than 850 chemicals are known to produce neurobehavioral disorders in humans or in animals (Anger and Johnson, 1985). The National Institute for Occupational Safety and Health (NIOSH) considers neurotoxic and psychological conditions 2 of the 10 leading work-related disorders, and the American Conference of Governmental Industrial Hygienists (ACGIH) has identified neurotoxicity as a basis for recommending threshold limit

values (TLVs) for 30% of the chemicals most frequently encountered in industry for which toxicological symptoms of occupational disease have been documented (Anger, 1984). Of the 197 industrial chemicals found in a 1974 NIOSH survey as having been implicated in exposure of more than 1 million people (NIOSH, 1977), 65 are neurotoxic at some exposure concentration. The magnitude of the worldwide occurrence of neurotoxicity in humans is also demonstrated by the recent World Health Organization estimate that 375,000 cases of pesticide intoxication occur annually (Almeida, 1984). Most pesticides exhibit some neurotoxic effects.

Neurotoxicology is concerned not only with the actions of chemical agents but also with biological and certain physical (e.g., radiation) agents that produce adverse effects on the developing, mature, and aging nervous system (including special sense organs as well as neuroendocrine and neuromuscular systems) and on the behavior of humans and other animals. Some neuroactive agents produce rapidly reversible changes, other compounds cause permanent damage, and a few may induce progressive and terminal neural deterioration. Toxic disorders of the nervous system follow overexposure to abused substances (e.g., ethanol, inhalants, narcotics), therapeutic drugs, toxic products or components of living organisms (e.g., bacteria, fungi, plants, animals), chemicals designed to affect organisms unwanted by humans (e.g., fungicides, herbicides, insecticides), industrial chemicals, chemical warfare agents, additives and natural components of foods, and certain other types of chemicals encountered in the environment (Spencer and Schaumburg, 1980). The mechanisms of action of chemical agents that lead to nervous system dysfunction are, in general, poorly understood. Some agents act directly on the nervous tissue; others induce neurological or behavioral dysfunction indirectly, for example, by inducing changes in electrolyte balance, in cerebral blood flow, in metabolism of glucose, or in levels of critical intermediary metabolites. Such pathophysiological changes are often expressed clinically as neurobehavioral disorders because of the peculiar sensitivity of neural tissue to disruption of body homeostasis.

Principles of neurotoxic response can be founded on the biology of the nervous system during development, at maturity, and during senescence. Experimental studies and clinical experience have demonstrated that the response of the developing nervous system to chemical insult may be either quantitatively or qualitatively different from the response of the mature system (Wilson and Fraser, 1977a, pp. 50-54). Some agents that induce developmental abnormalities of neural structure (neuroteratogens) or function (behavioral teratogens) may produce no or different abnormalities in the adult (Vorhees and Butcher, 1982). Very few studies have been conducted to examine the susceptibilities of aged subjects to toxic substances. Thus, our understanding of the biological processes that might

underlie differential responses in that population is limited. However, it seems reasonable to conjecture that deficits associated with old age are aggravated by those chemical agents that further impair neural centers and functions already compromised by the aging process. The House Select Committee on Aging has identified neurotoxicology as an important area of research likely to have a strong impact on the prevention and treatment of neurological disorders associated with old age (OTA, 1984).

In the following paragraphs, the committee has provided a rationale for using neurotoxicity as a basis for determining acceptable levels of exposure to chemicals in community drinking water. The discussion includes a review of the structure and function of the nervous system, an outline of the significant forms and end points of neurotoxicity or end points that may serve as a basis for regulation, and the identification of susceptibilities of special populations. Later in the chapter, the types of information and research that can contribute to a risk assessment based on neurotoxicity are described, and issues relating to the use of neurotoxicity as a basis for risk assessment are discussed.

THE HUMAN NERVOUS SYSTEM IN HEALTH AND DISEASE

Gross Structure and Function

By exploring different aspects of the vast body of knowledge on the human nervous system, one can attempt to learn how neural elements react to xenobiotic substances. Although the acquisition of such an understanding begins with a study of the structural and functional organization of the nervous system, the broad range of these neurotoxic effects is usually recognized first by the appearance of clinical symptoms or deficits in behavioral function.

At maturity, the nervous system is separated anatomically into central and peripheral divisions. The peripheral nervous system (PNS) is composed of nerve cells (neurons) and their processes (axons), which conduct information to muscles and between muscles, glands, sense organs, and the spinal cord or brain. PNS axons are ensheathed by Schwann cells to form nerve fibers, which run together in bundles in peripheral and cranial nerves (I and III-XII) (Thomas and Ochoa, 1984). These nerves contain afferent (sensory) and efferent (motor) fibers, both of which are represented in the somatic (voluntary) and visceral (autonomic) components of the nervous system. Somatic afferent fibers conduct information from special sense organs and sensory receptors in skin and muscles to the brain, whereas visceral afferent fibers convey impulses from the gut, heart and vessels, glands, and various organs. Somatic efferents innervate striated muscle, whereas visceral efferents supply smooth muscles of blood ves

sels, glands, heart, and gut. In toxic states involving the PNS, degeneration of sensory and motor fibers leads to peripheral neuropathies associated with sensory loss (e.g., decreased sensitivity to vibration, touch, or physical orientation) and motor (muscle) weakness. Dysfunction or breakdown of autonomic fibers may precipitate abnormal sweating, cardiovascular changes, or dysfunction of the gastrointestinal tract, the urinary tract, the genitals, or other organs or systems (McLeod, 1983).

Manifestations of toxic disorders of the central nervous system (CNS) depend largely on the site, nature, and extent of the induced functional or structural change. The CNS consists of those parts of the nervous system contained within the skull and vertebral column. The spinal cord receives information via afferent fibers from PNS sensory receptors in the skin, voluntary muscles, tendons, blood vessels, and glands. It transmits signals for motor function through efferent fibers and communicates information via specific pathways, which include coordination centers within the brain. The brain is responsible for initiating, receiving, and integrating signals needed to maintain internal homeostasis, cognition, awareness, memory, language, personality, sexual behavior, sleep and wakefulness, movement and locomotion, sensation, vision, audition, balance, taste, olfaction, and many other body functions (Kandel and Schwartz, 1981). The brainstem, consisting of the midbrain, pons, and medulla oblongata, receives and processes information from skin, muscles, and special sense organs (e.g., inner ear), and in turn controls motor and certain autonomic functions relayed to the periphery by way of the cranial nerves. The cerebellum and basal ganglia are required for the modulation, regulation, and coordination of muscle tone and activity. The diencephalon, including the thalamus and hypothalamus, is a relay zone for transmitting information about sensation and movement. It also contains important control mechanisms for maintaining the internal homeostasis of the body. The hypothalamus functions as the primary control center for the visceral organs and integrates the activity of the neuroendocrine and other systems. The cerebral hemispheres, capped by the cerebral cortex, are concerned with perceptual, cognitive, motor, sensory, visual, and other functions. The optic (cranial II) nerves and their radiations conduct visual information from the retina through the thalamus to the occipital cortex.

The cerebral cortex is divided into areas responsible for sensory reception, motor control, and integration or association. Visual input is received in the posterior (occipital) cortex, and auditory input is directed toward the temporal lobes. Tactile sensation is perceived in the parietal cortex. The frontal cortex is concerned with the control of movement and is associated with the parietal cortex in the unilateral (left or right) regulation of speech. The remainder of the cortex is devoted to integrative and associative functions. Beneath the cerebral cortex lies a number of other

structural areas, most with relatively clear functions. The basal ganglia, located at the base of the cortex, are important in the control of automatic and coordinated movement, as is the cerebellum. The limbic system consists of portions of the cortex and lower parts of the brain, which are involved in the control of eating, drinking, aggression, general arousal, motivation, and emotion.

The primary or initial sensory areas of the brain are characterized by a receptor-topical organization; that is, the sensory receptor is represented point by point. Areas adjacent to each initial reception area in the cortex provide for an integration of the simple sensory input into successively more complex sensory perceptual organizations. One of the most thoroughly explored of these areas is the visual system, whose initial reception area lies in the lateral geniculate ganglia of the thalamus, where a point-to-point map of the retina is organized. The somatotopic arrangement in a given portion of the retina is further organized by the orientation or angularity of the cell layers in the lateral geniculate ganglia and visual cortex as they appear in the retina. Specific neurons are responsive to edges of light and dark, angles, and corners—all of a particular size or strength. Such an organization appears well suited to the identification of complex visual stimuli (Kandel and Schwartz, 1981).

The motor section in the frontal cortex controls movement throughout the body: the foot and leg are controlled at the top of the motor strip; the hand, at the top of the lateral aspect; and the face, jaw, and mouth, in most of the lateral area. An entirely separate section of the frontal cortex is devoted to the precise control of eye movements. By stimulating specific neurons in the motor area, individual muscles can be activated, whereas stimulation of several neurons can produce movements of various body parts. The motor area is generally divided into the pyramidal and extrapyramidal systems. The pyramidal tracts descend from the frontal cortex to the spinal cord, where they regulate the activity of nerve cells controlling the skeletal muscles. The extrapyramidal pathways exert control over more general responses, such as postural adjustments, and do so through connection with other subcortical CNS structures, including the basal ganglia and cerebellum.

The largest and least well understood area of the cortex involves associative responses. The frontal association areas of the cortex govern delayed response or delayed-response alteration performance, mediated by memory. Disorders of the frontal lobe are often associated with euphoria, apathy, indifference, impaired judgment, recent memory, and immediate recall. When this area is removed, there are alterations of discrimination ability in a range of senses, especially when visual searching is involved. Hyperactivity, increased social aggressiveness, and inability to tolerate frustration generally occur when frontal lobe tissue is destroyed.

A different set of functions is mediated by posterior cortical association areas, which govern visual-pattern discrimination and some aspects of color vision, but not visual acuity and object recognition. Learning ability is also mediated by this cortical area. The parietal association area is concerned with perception of body image and spatial relations as well as with learning involving somatesthesia. Disorders of language and inability to perceive objects occur in patients with a damaged parietal lobe (right or left).

The two hemispheres of the brain commonly have structural differences and are functionally distinct with regard to higher functions. In a greatly oversimplified but didactically useful way, the human brain consists of two halves: a left hemisphere that tends to be an intellectual, rational, verbal, and analytical thinker, and a right hemisphere that tends to be a perceiver and an emotional, nonverbal, and intuitive thinker. In principle, each hemisphere is capable of independent function, but integration of function is normally maintained by an extensive network of interconnections known as the corpus callosum. Hemispheric dominance develops gradually, as evidenced by the ability of young children to develop virtually normal speech and language capabilities after complete removal of the left hemisphere. In adults, extensive damage to the dominant hemisphere results in severe functional deficits in speech functions, and recovery is slow or absent. Moreover, there are differences in the rates at which lateralization of higher functions develops in boys and girls. Hence, in preadolescents there are sex differences in vulnerability to left hemisphere damage. Speech becomes fixed at an earlier age in boys, who are therefore more likely to suffer more severe and enduring language deficits after cortical injury in childhood.

Cellular Structure and Function

The fundamental neurocellular components are neurons and glial cells, which are associated with blood vessels and other specialized epithelial and connective tissue cells (Jacobson, 1978). Neurons have relatively small cell bodies (perikarya) and multiple, relatively short processes (dendrites), which receive information transmitted from axons of other nerve cells. In addition to dendritic processes, neurons are characteristically equipped with a single, elongated axon that conducts electrical signals between neurons, or to muscles, skin, and glands. Signals are transmitted along axons by a longitudinal spread of changes in membrane potential—a difference in electrical potential that exists between the intracellular (axoplasmic) and extracellular environments. In the resting state, membrane potential is maintained by a high intracellular concentration of potassium ions and a high extracellular concentration of chloride and sodium

ions. This balance is maintained by an energy-requiring ion pump located in the cell membrane; failure of this pump in certain toxic states leads to a disturbance of transmission and intracellular edema—the accumulation of abnormal amounts of fluid inside the cell. Transmission of an electrical impulse along an axon is initiated by reversing the membrane potential locally; this is achieved by a rapid influx of sodium ions via membrane channels, cessation of sodium-ion flow, and an increase in potassium-ion conductance causing the potassium ions to move out of the cell along their concentration gradient. The resulting localized change in membrane potential—the action potential—moves along axons by reproducing these electrical events in adjacent areas of excitable membrane. Impulse propagation is accelerated in myelinated nerve fibers by the concentration of electrical events on the naked axon at sites between adjacent myelinating cells (nodes of Ranvier). Toxic substances that interfere with the function of ion channels in the excitable membrane or impair the function of Ranvier nodes produce transient alterations of nerve conduction that precipitate characteristic sensory or motor phenomena (Narahashi, 1984).

Axons terminate at synapses where chemically encoded information is conveyed to other neurons or effector organs. Proven chemical neurotransmitters (up to 50 are suspected) include acetylcholine, nonepinephrine, epinephrine, and dopamine; putative transmitters include glycine, glutamic acid, γ-aminobutyric acid, serotonin (5-hydroxytryptamine), histamine, substance P, enkephalin, and many other neuroactive peptides (Cooper et al., 1982). Enzymes responsible for the synthesis of transmitters are manufactured in neuronal perikarya. Subsequently, with membranous and cytoskeletal elements, they move toward the nerve terminal by energy-requiring axonal transport (Brady, 1984). Retrograde transport mechanisms return materials to the cell body for reprocessing (Kristensson, 1984). Toxic substances may be specific for one or more transmitter systems, acting by changing the rate of synthesis, synaptic release, reuptake, or degradation or by interfering with the interaction of the chemical signal with the receptor surface on the dendrite or other cellular membrane (muscle or gland). Such changes are usually short lasting and reversible. By contrast, chemical agents that induce loss of CNS neurons produce irreversible damage. Degeneration of CNS axons, initiated by blockade of axonal transport or by other methods, is also commonly irreversible, except for small-diameter unmyelinated monoaminergic axons that regenerate and elongate after injury.

Glial cells in the CNS include astrocytes, oligodendrocytes, and microglia (Varon and Somjen, 1979). Astrocytes are divisible into protoplasmic and fibrous forms. They are closely associated with neurons and blood vessels, play a nutritive role in maintaining neurons and other cells, and regulate the composition of the extracellular fluid. Astrocytes have

also been implicated in the metabolic activation of methylphenyltetrahydropyridine (MPTP), an agent that produces parkinsonism in humans and animals. In addition, they provide an outer cellular border around the brain and spinal cord. These two organs are surrounded by a delicate pial connective tissue and are suspended in cerebrospinal fluid (CSF), which circulates between the ventricular system of the brain and spinal cord and spaces beneath the arachnoid and dural connective tissues that encase and protect the CNS. Interruption of the normal flow of CSF during development or adult life leads to hydrocephalus, a condition resulting from the accumulation of fluid in enlarged brain ventricles or between the brain and the overlying dura (Weller et al., 1983). The oligodendrocyte, another type of glial cell, elaborates short lengths of myelin around clusters of adjacent CNS axons. Myelination begins in the fourth month of fetal life and continues until the first year of postnatal life (Pansky and Allen, 1980). Toxic substances that induce changes in oligodendrocytes or myelin in postnatal or adult states cause reversible myelin vacuolation, demyelination, and remyelination—pathological events that interrupt nerve-impulse transmission without disrupting the structural continuity of axons (Rasminsky, 1980). CNS injuries resulting in a loss of tissue are associated with proliferation of astrocytes, which form a glial scar.

In the PNS, Schwann cells envelop many small axons to form unmyelinated fibers, or associate with and elaborate lengths of myelin (internodes) around single axons. PNS myelination occurs over a time course similar to that in the CNS. Toxic conditions resulting in damage to mature PNS axons are usually reversible, since axonal regeneration, reconnection, and reactivation of denervated end organs usually follow injury. Motor denervation leads to neurogenic atrophy of voluntary muscle. Damage to Schwann cells or myelin causes localized demyelination and then remyelination—pathological changes associated with blockade and restoration of nerve-impulse activity, respectively (Rasminsky, 1980). Extensive demyelination may be accompanied by axonal loss and poor functional recovery.

The adult nervous system is provided with specialized regulatory interfaces, known as blood-brain, blood-nerve, blood-CSF, and blood-retinal barriers, among others. Such permeability barriers are formed by tight junctions between adjacent endothelial cells lining neural capillaries and their corresponding basal laminae (Jacobs, 1980). The barrier functions as a differential filter that controls the passage of various substances (including certain blood-borne toxicants) from the blood to the interstitial fluid. Certain areas of the brain and PNS are devoid of blood-nerve barriers, including the area postrema, hypophysis, pineal body, hypothalamic regions, subfornical organ, supraoptic crest, choroid plexus, dorsal root, and autonomic ganglia. Such regions are directly exposed to toxic and

other substances circulating in the blood. Experimental studies demonstrate that large doses of several glutamate analogs (excitotoxicants) induce neuronal degeneration in areas of the CNS lacking a blood-brain barrier (Olney, 1980) and that other agents (e.g., doxorubicin and large concentrations of pyridoxine) alter the integrity or cause degeneration of sensory neurons in dorsal root ganglia (Cho et al., 1980). The CNS and much of the PNS are also protected from exogenous agents by connective tissue sheaths, but the terminal regions of efferent PNS axons in muscle and glands may be directly exposed to noxious agents present in tissue fluid. Such agents may be selectively or nonspecifically taken up by nerve terminals and carried by retrograde transport to remote target sites in the neuronal perikaryon (for agents such as ricin) or associated synapses (for substances such as tetanospasmin) (Price et al., 1975).

Normal Developmental Structure and Function

Development of the human nervous system begins during the third week of embyrogenesis (see Chapter 2 on Developmental Toxicity) (Pansky and Allen, 1980). The CNS commences as an elongated neural plate of thickened ectoderm. The lateral edges of the plate elevate, abut, and from the fourth week onward, fuse to form a closed tubular structure (neural tube) with a long caudal portion (the future spinal cord) and a broader cephalic segment (the future brain). The latter displays three distinct dilatations corresponding to the fore-, mid-, and hindbrain vesicles. Differential growth and migration of cell populations then mold the vesicles into more complex forms. By the fifth fetal week, the forebrain consists of the diencephalon with primordial optic vesicles, pineal gland and posterior hypophysis, and two lateral expansions—the primitive cerebral hemispheres. The hemispheres expand rapidly and cover the brainstem, forming various specialized centers including the hippocampus.

During late fetal life, the surface of the cerebral hemispheres grows so rapidly that convolutions (gyri) develop on its surface. The diencephalon also gives rise to the thalamus, which contains the dorsal nuclei important for the reception and transmission of auditory impulses, and ventral nuclei, which serve as relay stations for transmitting the impulses to higher centers. These and other elements form the limbic system, which is concerned with the regulation of emotional activity, visceral and memory functions, and such other activities of the hypothalamus as the regulation of body temperature. The pituitary gland (hypophysis) develops from a downward extension of the diencephalon (becoming the posterior hypophysis or neurohypophysis) and an ectodermal outpocketing of the primitive oral cavity (Rathke's pouch, becoming the anterior hypophysis, or adenohypophysis). Each optic vesicle forms an optic cup, which grows out to form the optic

nerve and retina, and induces the surface ectoderm to invaginate and form the lens and cornea. Congenital abnormalities of the eye induced by chemical and other agents may lead to the development of a single eye (cyclops), no eye (anophthalmia), malformation of the iris, retinal dysplasia or herniation, and many other conditions (Wilson and Fraser, 1977b, pp. 329341). In the normal state, after the eye opens postnatally, visual impulses are conducted from the retina to the lateral geniculate body and then to the occipital cortex.

The remaining portion of the brain develops from the specialization of plates of cells. In general, cells located in the basal plate form motor neurons whose axons supply cranial nerves, while the more dorsal, alar plates form the sensory relay nuclei of the brainstem (Pansky and Allen, 1980). The midbrain develops the motor nuclei of cranial nerves and relay nuclei for visual and auditory reflexes. The hindbrain comprises the metencephalon, from which develops the pons and cerebellum, and the myelencephalon, the forerunner of the medulla oblongata. The roof plate of the myelencephalon forms the choroid plexus—tuft-like invaginations that produce CSF. The cerebellum develops from the metencephalon, and by the twelfth fetal week, forerunners of the midline vermis and paired hemispheres are visible. Cells migrate to the surface to form the external granular layer, and at the sixth developmental month, cells migrate from this layer inward toward the differentiating Purkinje cells.

The inner ear, which subserves both audition and equilibrium, develops after the third week from otic placodes, which are visible on each side of the hindbrain. During later development, each vesicle divides into a ventral portion that gives rise to the saccule and cochlear duct and a dorsal component that makes up the utricle, semicircular canals, and endolymphatic duct. The organ of Corti, the true organ of hearing, is visible by the tenth week. At maturity, impulses are transmitted by the spiral ganglion to the brainstem via cranial nerve VIII. The semicircular canals appear at the sixth fetal week, and at maturity, impulses generated in the sensory cells of the cristae and maculae as a result of position changes of the body are carried to the brain by the vestibular fibers of cranial nerve VIII. Other special sense organs include receptors for taste, which convey information via sensory ganglia to the brainstem and cortex, and receptors for olfaction, which send impulses directly to the primary and secondary olfactory cortex.

Development of the spinal cord is comparable to that of the brainstem. Basal plates on the ventral sides of the neural tube form motor neurons, notably anterior horn cells, while the dorsal, alar plates form sensory association areas. These receive the central axon processes of sensory neurons in dorsal root ganglia, which develop from neural-crest cells that

overlie the dorsal surface of the neural tube. The peripheral axons of the sensory neurons supply sensory receptors.

The motor neurons of the autonomic division of the PNS develop between the ventral and dorsal plates deep in the immature spinal cord. A sympathetic branch is formed in the thoracolumbar region, and a parasympathetic component is found in the craniosacral region. The efferent portions of both parts are composed of a chain of two neurons, whereas afferent impulses from pressure and chemoreceptors in the aorta and carotid arteries ascend via axons of visceral sensory neurons in dorsal root ganglia (Mayer, 1980). Some sympathetic neurons originate from the neural crest and migrate on each side of the cord to form a bilateral chain of segmentally arranged ganglia interconnected by nerve fibers; others form the celiac and mesenteric ganglia of the aortic branches and the organ plexuses found in the heart, lungs, and gastrointestinal tract. Preganglionic efferent axons of the sympathetic chain use acetylcholine as their neurotransmitter. Postganglionic efferent neurons, in conjunction with the adrenal medulla, release nonepinephrine directly or through the bloodstream to effector sites in the ciliary body of the eye, the salivary glands, bronchi, heart and blood vessels, liver, gut, kidney, bladder, and external genitalia. Preganglionic neurons of the parasympathetic system, located in the midbrain, medulla oblongata, and sacral spinal cord, also use acetylcholine as a neurotransmitter, as do their postganglionic counterparts located close to their effector sites in the organs listed above.

Functionally, the two divisions of the autonomic nervous system tend to counteract each other (Mayer, 1980): the sympathoadrenal system, which can discharge as a unit during rage or fright, accelerates heart rate; increases blood pressure and glucose concentration; shifts blood from the spleen, skin, and gut to the skeletal muscles; and dilates the pupils and bronchioles. By contrast, the parasympathetic system tends to conserve energy by slowing the heart rate, lowering blood pressure, stimulating gastrointestinal movements and secretions (thereby aiding nutrient absorption), protecting the retina from excessive light, and emptying the urinary bladder and rectum. Synchronized discharge of parasympathetic neurons does not occur normally, but is seen during poisoning with anticholinesterases, which inhibit the enzyme (cholinesterase) required to terminate the transmitter activity of acetylcholine (Mayer, 1980).

Neuroteratology and Psychoteratology

Experimental studies and clinical experience have demonstrated that the response of the developing nervous system to chemical substances may be either quantitatively or qualitatively different from the response

of the mature system. Some agents that induce developmental abnormalities of neural structure (neuroteratogens) or function (behavioral teratogens) may produce no abnormalities in the adult (Vorhees and Butcher, 1982). Classic examples are agents that interfere with cell proliferation: these agents can alter the number of nerve cells permanently, if exposure occurs during neuron production, but may be benign or produce different effects in the same tissue when rapid mitotic activity has ceased. For example, although the mitotic inhibitor colchicine blocks axon transport at any age, its disruption of mitosis is problematic only in the immature CNS (in the adult, colchicine produces axonal neuropathy). Finally, at different stages of development, the CNS may have varying sensitivity to agents, even though the same mechanisms may be active.

Classic teratology studies have demonstrated that the degree of responsiveness of the conceptus to the induction of congenital malformations by teratogens depends on, and varies with, gestational age (Wilson and Fraser, 1977a, pp. 50-54). As far as teratological susceptibility is concerned, the time from conception to birth is roughly divisible into three periods: before germ-layer formation, the period of embyro development, and the fetal stage. The first period, from fertilization to implantation, lasts approximately 6.5 days in humans and is generally resistant to the induction of congenital malformations. The embyronic period begins after implantation and lasts to the end of the second gestational month. During this period, differentiation, mobilization, and organization of cells and tissue groups take place to form individual organ systems, and toxic disturbances of normal development while these processes are occurring may cause gross structural abnormalities (malformations). Since the genesis of different organs occurs at different stages of development, the type of malformation is largely dependent on the susceptibility of the individual organs at the time of the insult. Teratogenic agents causing interruption of neural-tube closure lead to birth defects such as spina bifida and anencephaly. After the neural tube closes and early development of the brain is under way, toxic insults may result in grossly abnormal brain morphology. During this stage, developmental abnormalities have been produced in experimental animals exposed to vitamin A (Wilson and Fraser, 1977a, p. 270) and trypan blue (Wilson and Fraser, 1978, pp. 116-117), the former producing anencephaly, anophthalmia, spina bifida, and other defects.

Teratogenic susceptibility decreases in the third (fetal) stage, since cellular proliferation and differentiation is less marked and organogenesis may be complete (Wilson and Fraser, 1977a, pp. 50-54). However, some tissues, such as the cerebrum and cerebellum, continue active differentiation and thus remain susceptible to the action of certain chemical agents until term or even into the postnatal period. Experimental animal studies have demonstrated that neuroteratogenic changes can be induced in the

fetal stage by transplacental exposure to methylazoxymethanol (MAM), 5-azacytidine, ethylnitrosourea, and x-irradiation. MAM also induces changes in cerebellar development when administered to laboratory animals postnatally. Similarly, agents that inhibit myelination can have profound effects on oligodendrocytes when administered prenatally or postnatally (Le Quesne, 1980). Finally, the immaturity of the blood-brain, blood-retinal, and perhaps other barriers may lead to special susceptibilities in developing states. The high incidence of lead encephalopathy in the young has been attributed to increased access of the circulating toxicant to brain tissue.

Toxic agents act with some selectivity on the developing organism, inducing a characteristic pattern of teratogenic change within the framework of a specific developmental stage. However, an agent may produce different responses at identical levels of exposures occurring at different developmental stages. Differential reactions across species have also been recognized.

Dose is another important factor: large doses can produce fetal death; smaller doses, teratogenesis; and the smallest doses, no detectable gross malformation. The thalidomide tragedy demonstrated graphically that the dose required to induce phocomelia in humans (0.5 to 1.0 mg/kg/day) differed markedly from that required to produce comparable effects in common laboratory species (10 to 350 mg/kg/day) (Wilson and Fraser, 1977a, p. 314).

Nutritional status of the dam is also a critical consideration: both fasting and specific vitamin deficiency states can induce disorders such as hydrocephalus and eye defects in developing animals. Additive effects have been observed in fasting animals exposed to teratogenic substances such as trypan blue (Wilson and Fraser, 1977a, p. 451). Mineral deficiency (e.g., manganese and copper) in the dam reportedly can lead to congenital ataxia in the offspring (Shils and McCollum, 1943). However, maternal deficiency of certain other required substances, including vitamins C, D, and K, fatty acids, choline, biotin, and various amino acids, fails to produce congenital malformations of the nervous system.

Other experimental factors positively correlated with fetal susceptibility to the teratogenic effects of chemical substances include maternal weight and, probably, age, litter size, implantation site, route of administration, and season. Ionizing radiation, hypoxia, and certain infections supplement the long list of factors associated with neuroteratogenesis in humans and animals. Other neuroteratogens include alkylating agents, certain antimetabolites and alkaloids, hypoglycemic agents, ethanol, salicylates, antibiotics, antihistamines, neuroleptics, anticholesterolemic drugs, steroids, sulfonamides, and organomercury (Wilson and Fraser, 1977a, pp. 317-340).

Psychoteratology, or behavioral toxicology, is a more recent discipline concerned with the study of the more subtle effects of chemical substances that induce changes in the nervous system or behavior of the developing organism (Vorhees and Butcher, 1982). Many behavioral teratogens are simply lower doses of classical teratogens that at these levels of exposure fail to produce gross malformations in the nervous system. Some of these behavioral changes have been traced to abnormal development of neurocellular elements detectable with the light microscope. Others, the psychoteratogens, induce experimental behavioral changes without presenting detectable structural damage to the CNS and, even at higher doses, fail to induce malformations. The mechanisms underlying these changes are unknown. The most important example of human psychoteratogenesis is the fetal alcohol syndrome; other fetal syndromes have been reported in association with maternal exposure to methylmercury, barbiturates, phenytoin (diphenylhydantoin), trimethadone, and primadone.

Neurotoxic Responses after Birth and at Maturity

The nervous system of humans and other animals is also susceptible to toxic perturbations after organogenesis is complete, although the substances that induce these changes and the nature of the disorders are usually different from those that affect the developing organism. As before, factors such as species type, dose and duration of chemical exposure, and the cellular target of the toxic agent or its metabolites are critical in determining the nature and severity of the induced lesion. Different syndromes may appear in humans according to the rate and degree of intoxication; for example, acute overexposure to acrylamide causes a toxic encephalopathy with seizures, whereas prolonged, low-level intoxication produces a syndrome of peripheral neuropathy.

Evidence suggests that most direct-acting neurotoxicants induce structural or functional changes, as a result of either reversible or irreversible binding of toxic substances to receptors or other vital macromolecules in nervous tissue. There appears to be no good reason why neurotoxicity will not follow normal sigmoidal dose-response relationships. However, the dose-response curve for neurotoxic effects is likely to be steep, since similar degrees of qualitatively identical neurobehavioral effects commonly occur in the majority of people or animals exposed to the same dose of the neurotoxic agent. For example, most, if not all, patients undergoing a standard regimen of vincristine for the treatment of a malignant tumor develop peripheral (toxic) neuropathy (Schaumburg et al., 1983). Universal responses of exposed human populations are quite different from the experience with chemical carcinogens where, in most instances, only a small portion of a population is expected to develop

malignant growths. Whether these statements are true for low levels of exposure to neurotoxic agents is unknown, and much more research is required to gather this information.

Differential responses may appear in individuals of different sex, e.g., males are more susceptible to the Lathyrus toxin (Gopalan, 1950), and of different age. Structure-activity relationships are clear for only a few classes of compounds, such as the anticholinesterases, organophosphates, and hydrocarbon solvents with a common γ-diketone metabolite (Johnson, 1975a,b; NRC, 1982; O'Donoghue, 1985). For other compounds, prediction of neurotoxicity based on chemical structure alone is presently a hazardous venture. The neurotoxic potential of some substances can be altered as a function of metabolic state, such as the susceptibility of slow acetylators (who are homozygous for an autosomal recessive gene) to the antituberculosis drug isoniazid (Elis, 1984). Between 50% and 70% of the North American and Central European populations are slow acetylators. Fast acetylators (i.e., those who are either heterozygous or homozygous for a dominant gene) constitute approximately 90% of the Japanese population (Price Evans, 1978). The potency of other neurotoxic agents may be altered by concurrent exposure to another compound lacking the property. This principle is clearly illustrated by the ability of methyl ethyl ketone to accelerate the development and increase the severity of the peripheral neuropathy induced by repeated exposure to *n*-hexane or methyl *n*-butyl ketone (Altenkirch et al., 1982).

Developing countries are concerned that environmental compounds with neurotoxic potential may be especially hazardous to people with neurological susceptibility associated with malnutrition. The synergistic action of ethanol and thiamin deficiency exemplifies this concern. Finally, there are some agents (e.g., organoarsenicals) that do not obey classic dose-response laws and unpredictably induce rapid-onset neurological disorders in humans comparable to the Guillain-Barré syndrome. It seems likely that these will prove to be hypersensitivity reactions resulting in cell-mediated attacks on targeted nervous system components, notably myelin. Alteration of the immune system was also implicated in the 1981 Spanish toxic oil syndrome—a multiphasic, multisystem vascular disorder affecting some 20,000 people exposed to an adulterated cooking oil. Some victims of the syndrome eventually developed a devastating neuromuscular disorder in which muscles, nerves, and skin were invaded by fibrotic tissue.

Toxic chemicals are often grouped and studied on the basis of their commonplace occurrence, use, or physiochemical properties. For example, free reference is made to industrial, biological, and heavy metal neurotoxicants. This method of classification is a necessary but entirely misleading practice, since the neurotoxic properties of a substance are

TABLE 4-1 Classification of Neurotoxicants by Target

Target	Neurotoxicant
Prenatal period	
Neural tube	Excessive vitamin A intake
Developing nervous system	Ethanol, methylazoxymethanol
Postnatal period	
Neurons	
Excitable membrane	Channel agents, including pyrethroids
Neurotransmitter systems	
Cholinergic agonists	Anticholinesterases
Cholinergic antagonists	Tricyclic antidepressants
Structural integrity	
Neuron	Doxorubicin, mercury, trimethyltin
Axon	Acrylamide, *n*-hexane, methyl *n*-butyl ketone
Dendrite	Glutamate excitotoxins
Glial cells	
Myelinating cells and myelin	
Oligodendrocytes (CNS)	Isoniazid, triethyltin
Schwann cells (PNS)	Lead, diphtheria toxin
Astrocytes	6-Aminonicotinamide
Ependymal cells	Amoscoline
Special sense organs	
Olfactory/gustatory	Penicillamine, thiouracil
Optical	Methanol, chloroquine
Otic	Noise, toluene
Vestibular	Hydroquinone
Muscles	
Striated muscles	Anticholinesterases, dimethyl sulfoxide
Cardiac muscles	Diphtheria toxin
Neural vasculature	Cadmium
Neuroendocrine system	
Hypothalamus/hypophysis	Chlordecone
Immune system, with secondary effects on nervous system or musculature	Gold thiourea
Various cell types	
Malignant transformation, with primary or secondary growths affecting nervous system	Alkyl nitrosoureas

based on chemical structure and target site in the nervous system—not on the source of the agent, type of usage, or, for most elements, position in the periodic table. A more appropriate classification of chemical neurotoxicants is based on apparent target sites within the nervous system (Table 4-1), although one agent may have more than one site of action,

especially when different doses are under consideration (Spencer and Schaumburg, 1984).

This nosological approach, i.e., the classification by apparent target site within the nervous system, can yield useful information, a point well illustrated by a diverse collection of chemical substances that interferes with the opening and closing of sodium channels in excitable membranes, thereby producing a common initial clinical expression of rapid-onset circumoral and distal-extremity paresthesias. The agents in question—tetrodotoxin, batrachotoxin, ciguatoxin, dichlorodiphenyltrichloroethane (DDT), cyanopyrethroids, and scorpion toxin—originate from diverse sources and have disparate chemical structures, but because of their similar sites of action, they produce comparable effects. These so-called sodium-channel toxins, and those that perturb neurotransmitter function, usually produce rapidly reversible changes in nervous system function; although if intoxication is severe enough to impair vital centers, such as the brainstem respiratory center, death may ensue.

Since the amount and timing of neurotransmitter release at synapses are critical, toxicity is associated with agents that increase the amount or mimic the action of neurotransmitters (agonists) as well as those that impair their function (antagonists) (Spencer and Schaumburg, 1984). This principle is well illustrated by responses of the cholinergic system: cholinergic toxicity follows overexposure to direct agonists (e.g., muscarine) or agents that inhibit enzyme inactivation of acetylcholine (anticholinesterases), whereas anticholinergic toxicity is caused by agents such as β-bungarotoxin, which block acetylcholine reception at the neuromuscular junction (Mayer, 1980). Similarly, in the catecholamine system, some antidepressants inhibit monoamine oxidase, thereby increasing the duration of action of synaptic norepinephrine and circulating epinephrine, whereas certain hypertensives, such as guanethidine, interfere and displace catecholamines from presynaptic terminals. Other agents such as lysergic acid (LSD) and trimethyltin interfere with the action of serotonin, picrotoxin with γ-aminobutyric acid (GABA), ibotenic acid with glutamate, tetanospasmin with glycine, or antipsychotics or opiates with several transmitters concurrently (Damstra and Bondy, 1980). The type of neurological disorder associated with these different conditions usually reflects closely the functions subserved by the neurotransmitter systems that have been perturbed by the toxic substance or its metabolites. Most of the disorders are not known to be associated with detectable structural changes in the nervous system or elsewhere, and a large majority of them are rapidly reversible. Some, such as the tardive dyskinetic states associated with chronic use of phenothiazines and certain other neuroactive drugs, are usually attributed to increased sensitivity of receptor sites on synaptic membranes and are irreversible, or slowly reversible after cessation of exposure.

Toxic disorders associated with compounds that produce structural changes in neurons, with consequent degeneration of the nerve cell perikaryon or distal axon, often develop slowly and lead to long-lasting or permanent decrements in sensory, motor, and autonomic functions. Some agents, such as the methylated compounds of lead, arsenic, or mercury, readily penetrate brain tissue and produce rapid, widespread, and irreversible neuronal degeneration. Other inorganic compounds (e.g., salts of thallium, arsenic, lead, aluminum, and bismuth) also are capable of producing encephalopathies with permanent neurobehavioral deficits, although the precise pattern of damage in these conditions has not been adequately described. Delayed and then progressive deterioration of brain structure and function may occur in persons who recover from acute carbon monoxide intoxication (Ginsberg, 1980). Loss of neurons may also be observed in dorsal root ganglia after exposure to mercury or doxorubicin, whereas adjacent motor neurons protected by the blood-nerve regulatory interface in the spinal cord are usually spared. Retinal ganglion cells undergo degeneration in methanol intoxication, and other agents (e.g., quinine and lead) induce degeneration of photoreceptors (Merigan and Weiss, 1980). Aminoglycoside antibiotics (e.g., streptomycin) cause loss of the receptor cells of the organ of Corti, and certain solvents (e.g., toluene, styrene, and xylenes) recently have been implicated as ototoxins in experimental animals (Lane and Routledge, 1983; Rebert et al., 1982).

A particularly common type of neuronal change induced by toxic chemicals is central-peripheral distal axonopathy, i.e., degeneration of long and large-diameter axons in peripheral nerves, spinal cord, medulla oblongata, and cerebellum. Some agents (e.g., thallium and arsenic salts and organophosphates) can precipitate this type of change in humans after a single intoxicating event; however, most other recognized neurotoxic substances of this class, such as *n*-hexane (Spencer et al., 1980a), acrylamide (see Chapter 9), and carbon disulfide (Seppäläinen and Haltia, 1980), require prolonged intermittent or continuous exposure. Similar disorders are seen in common nutritional deficiencies (e.g., deficiencies of the B vitamins) and metabolic disorders (e.g., diabetes mellitus) in humans. Degeneration of axons begins distally and proceeds along affected tracts in a retrograde manner, a pattern referred to as dying-back. Stocking-and-glove sensory-motor neuropathy is the common clinical result of this pattern of damage, and considerable recovery usually follows cessation of exposure. However, if substantial degenerative changes have occurred in vulnerable ascending and descending spinal-cord tracts, people who recover from PNS changes may be left with residual sensory loss, ataxia, or spasticity associated with permanent CNS deficits. A small number of agents

(e.g., clioquinol) produce comparable disorders selectively in CNS tracts (e.g., central distal axonopathy) (Thomas et al., 1984).

Damage to myelinating cells may lead to life-threatening encephalopathy (e.g., from exposure to hexachlorophene) or neuropathy (e.g., from exposure to diphtheria toxin) (Cammer, 1980). If the damage is restricted to myelin, and does not involve neighboring cells, a reasonable degree of recovery usually accompanies remyelination. Agents that damage myelin are legion: some appear to precipitate CNS and PNS demyelination without damaging the myelin-producing glial cells, whereas others (e.g., diphtheria toxin and ethidium bromide) interfere with the metabolic machinery of cells directly. Triethyltin, musk Tetralin ®, hexachlorophene, isoniazid, and cyanide inhibit mitochondrial respiration (Cammer, 1980). Edematous swelling of the myelin sheath (often reversible without demyelination and remyelination) is the hallmark of these toxic myelinopathies. Widespread spongiform degeneration of white matter is typical, and signs of increased intracranial pressure (e.g., headache, vertigo, visual disturbances, behavioral changes, convulsions, and coma) and increased CSF protein are common clinical features (Lane and Routledge, 1983; Powell et al., 1980).

Changes in muscle induced by chemical agents (mostly drugs) are well known. Steroids commonly produce a proximal myopathy associated with weakness and wasting of the quadriceps muscle. Myopathies associated with muscle pain, tenderness, stiffness, and cramping in a proximal, largely symmetrical distribution are also relatively common (Lane and Routledge, 1983). This syndrome may be associated with a necrotizing myopathy (e.g., from exposure to clofibrate), an inflammatory myositis (e.g., in Spanish toxic oil syndrome), or a hypokalemic vacuolar myopathy (e.g., from exposure to barium salts). Rhabdomyolysis, an uncommon and occasionally fatal syndrome heralded by severe muscle pain and swelling, sometimes occurs in drug addicts, e.g., users of ethanol, opiates, or phencyclidine (PCP). Myatonia may develop from treatment with β-adrenergic receptor agonists.

Other types of neurotoxicity are less well characterized. Changes in astrocytes have been induced experimentally by intoxication with water, ouabain, and antimetabolites (Powell et al., 1980). Capillary damage with extravasation of blood cells occurs in humans or animals with hemorrhagic encephalopathy induced by lead, cadmium, indium, or terbium (Jacobs, 1980). Bismuth administered intramuscularly reportedly can precipitate spinal ischemia and spastic paraplegia (Sterman and Schaumburg, 1980). A few agents (e.g., alkyl nitrosoureas) can induce primary malignant tumors in brain or nerves. Finally, important changes are believed to occur in the neuroendocrine system in response to selected toxicants such as DDT and chlordecone.

Susceptibilities of Special Populations

The Aged

Aging is associated with significant changes in the nervous system (Katzman and Terry, 1983); age-related decline in neural function may allow previously silent neurotoxic disorders to reach clinical expression. Clinical experience with therapeutic drugs indicates that the elderly, especially those with metabolic abnormalities or with hepatic or renal impairment, are more susceptible to the toxic effects of xenobiotic substances. However, systematic studies of the effects of chemical neurotoxicants on laboratory animals of varying age have yet to be undertaken.

The most intensively studied and best documented aspects of normal human aging are changes in intellect and memory. Intellectual performance, as measured by tests of vocabulary, information retention, and comprehension, reaches a peak between ages 20 and 30 and is maintained throughout adult life, at least until the mid-70s, in the absence of disease. Perceptual processing and choice reaction time is slowed during aging. Learning, storage, and retrieval of information associated with short-term memory are consistently impaired in older subjects. Defective thermal regulation and decreased lacrimation may also occur. Sleeping patterns are altered. Motor tasks, including locomotion, handwriting, and other purposeful movements, are performed more slowly, weakly, or in an uncoordinated manner. Vibration sense is progressively impaired with advancing age, touch sensation is diminished, thermal discrimination is impaired, and pain threshold is mildly raised. Muscle wasting is common, strength is reduced, and tendon reflexes are difficult to elicit (Katzman and Terry, 1983). All these changes may also occur as a consequence of chemical intoxication (Silverstein, 1982).

Neurobehavioral changes accompanying human aging are associated with structural or functional alterations in the CNS and PNS. Some changes, such as alterations in vascular and cardiac reflexes, galvanic skin response, potency, micturition, and pupillary response, probably result from changes in the autonomic nervous system (Katzman and Terry, 1983). Sympathetic hyperactivity is commonly present. This may interfere with cognitive functioning in older subjects, especially under the stress of psychological testing. Such individuals would be expected to be more susceptible to chemical toxicants with sympathomimetic properties. Changes in cerebral blood flow, essential for the maintenance of normal brain function, may occur over the age of 80 years.

Cortical atrophy and ventricular enlargement have been documented in normal individuals during senescence. Certain regions of the brain are more susceptible to neuronal cell loss: the locus ceruleus and substantia

nigra, both of which undergo maximum reduction in the third and fourth decades and slowly decline thereafter, Purkinje cells, and putamen neurons, which decline in number at a linear rate (Katzman and Terry, 1983). On the other hand, several cranial nuclei and the olivary nucleus maintain stable populations. The number of cerebral cortical neurons may be reduced by one-half from age 20 to 80 years, and supplementary reductions and alterations occur in their dendritic arborizations and synaptic inputs. The volume of a yellow, insoluble pigment, lipofuscin, increases at a linear rate in most neurons with increasing age, but there is no evidence that this material is cytotoxic. Other types of neuronal pathology occur in normal aged brains, including neurofibrillary tangles, neuritic plaques, and granulovacuolar bodies. Neuronal changes and cell loss result in substantial local or more generalized alterations in the normal concentration of neurotransmitters, including dopamine, nonepinephrine, serotonin, GABA, and choline acetyltransferase—the enzyme required for the synthesis of the neurotransmitter acetylcholine (Katzman and Terry, 1983).

Morphological changes in the PNS include a probable reduction of sensory neurons, an increase in the normal incidence of demyelination in spinal roots and peripheral nerves, increased amounts of connective tissue, and a mild loss of myelinated fibers. The central processes of dorsal root ganglion cells typically undergo distal dystrophic and degenerative changes (Spencer and Ochoa, 1981). There may be a slight reduction in the number of motor neurons with age, and regressive changes have been reported in the terminals of motor axons. Changes in sensory and motor nerve conduction along with a progressive slowing of the nerve action potentials are also characteristic.

The Diseased

There are compelling theoretical reasons to suspect that people with certain diseases and under specific therapeutic regimens may be more susceptible than others to environmental neurotoxicants. This immense subject can only be considered briefly in this report.

Many therapeutic drugs induce neurological disorders, and some of these might be potentiated (or suppressed) by concurrent exposure to environmental toxicants acting at related sites in the nervous system. Exposure to chemical substances may also unmask a latent neurological or neuromuscular disorder in previously asymptomatic individuals. Some unfavorable responses to therapy increase in frequency and severity in direct proportion to dose and duration of treatment; others appear to be unassociated with dose and probably result from hypersensitivity reactions (Lane and Routledge, 1983; Silverstein, 1982). Some drugs have the potential to act on the cerebral cortex to produce coma, seizures, or strokes.

Headache occurs as a result of exposure to agents that stretch pain-sensitive blood vessels and meninges. The basal ganglia are a target of many psychoactive or neuroactive drugs that cause a range of disabling effects, including tremor, asterixis, myoclonus, dyskinesia, catatonia, and dystonia. Other drugs interfere with cranial nerve function, leading to anosmia, trigeminal neuropathy, extraocular movement disorders and ataxia, oculotoxicity, or ototoxicity. When ototoxicity is associated with therapeutic doses of aminoglycoside antibiotics, the extent of damage can be exacerbated by concurrent exposure to noise (Prosen and Stebbins, 1980). Finally, many widely used drugs produce neuromuscular disorders that are expressed as peripheral neuropathy, myasthenic syndromes, and various types of myopathy.

Neurological diseases of childhood and adulthood are numerous. Some of them are treated with therapeutic agents that also may have neurological or psychological side effects. Nutritional disorders and inadequate preventive medicine may compound the situation in developing countries. Among the most common conditions associated with neurological dysfunction in childhood are convulsive disorders. In adults, changes in neural structure or function commonly develop in association with such common syndromes as alcoholism, diabetes mellitus, and hypertension. Psychiatric disorders, such as schizophrenia, are primary dysfunctions of the brain, possibly associated with irregularities of CNS neurotransmission. Such individuals may be especially susceptible to chemical agents that further perturb affected transmitter systems.

The aging human is most likely to develop devastating degenerative changes in the nervous system. These include, for example, senile dementia of the Alzheimer type, a syndrome of progressive mental deterioration, loss of memory, and impaired cognitive function associated with primary degeneration of neurons predominantly in the cerebral cortex and hippocampus; parkinsonism and disorders of motor function associated with striatal degeneration of the substantia nigra and characterized by flexed posture, rigidity, tremor, and mental and autonomic deficits; and amyotrophic lateral sclerosis, a disorder predominantly affecting motor neurons in the motor cortex and spinal cord. Axonal syndromes, such as mild polyneuropathy, are also common among the elderly. Diseases of myelin increase in frequency after the third decade: multiple sclerosis, most likely to affect women, is a progressive but remitting demyelinating disorder associated with impaired vision, nystagmus, tremor, ataxia, paraplegia, bladder dysfunction, and altered emotional responses (Katzman and Terry, 1983). Special sense organs also undergo important degenerative changes during the aging process. For example, hearing impairment is a major affliction of the elderly, affecting more than one-fourth of those past 65 years of age (Katzman and Terry, 1983). Most cases are idiopathic,

often of genetic origin, and result from loss of sensory hair cells in the inner ear and involvement of the auditory nerve. Significant visual impairment occurs in 6% of individuals over age 65 and in 46% of those over 85. Senile macular degeneration, associated with changes in the retinal epithelium, accounts for a proportion of these cases; nonneuronal change (cataract, glaucoma) accounts for the balance. Age-associated declines also occur in taste sensation and olfaction.

Selected toxic agents are able to mimic many of the clinical and pathological features described in some of the disorders listed above. For example, the parkinsonian syndrome is observed in workers chronically exposed to manganese ore or carbon disulfide and in persons intoxicated for relatively short periods with MPTP (Langston, 1985). Certain types of motor neuron disorders have been associated with exposure to lead, and polyneuropathy follows overexposure to a number of occupational chemicals. Psychoses can be exacerbated by acute exposure to diisopropylfluorophosphate.

In summary, therefore, the nervous system appears to have a number of systems that are vulnerable both to the aging process and to toxic chemicals, and a limited repertoire of neurobehavioral responses. Therefore, it is likely that aging populations with compromised neural structure and function, as well as reduced capacity for liver metabolism and renal clearance, are more susceptible to certain neurotoxic substances than are their younger adult counterparts.

Other groups that may be especially susceptible to neurotoxic agents include those occupationally exposed to chemicals with neurotoxic properties, persons with renal dysfunction, and those with skin conditions that increase dermal absorption of chemical agents.

STUDIES IN HUMANS

Occurrence of Neurological Disease

Experience has demonstrated that direct and indirect chemical interference with the nervous system can perturb virtually any part of the neuraxis, and the resulting disorders can usually be classified in terms of the anatomical site affected and the clinical presentation. In general, the signs and symptoms of drug-induced neurological disorders are virtually indistinguishable from those seen in naturally occurring disease but are usually reversible if diagnosed early enough (Lane and Routledge, 1983; see Table 4-2). Neurotoxicity commonly accompanies prolonged therapeutic treatment with anticonvulsants, anticholinergics, neuroleptics, antiparkinson drugs, and antineoplastic drugs (Katcher et al., 1983). One of the most recently recognized iatrogenic neurotoxicities is the sensory neuropathy

TABLE 4-2 Some Common Clinical Manifestations of Human Neurotoxicity[a]

Function Affected	Manifestation	Chemical
Cognitive	Intelligence loss	Lead salts[b]
	Learning decrements	Lead salts[b]
	Memory dysfunction	Anticholinesterases
Sensory	Irritability	Carbon disulfide
	Apathy/lethargy	Carbon monoxide
	Attention difficulty	Anticholinesterases
	Illusions, delusions	Ergot
	Dementia	Aluminum
	Depression, euphoria	Ozocerite
	Stupor, coma	Dicyclopentadiene
Sensory, special	Abnormalities of:	
	Smell	Cadmium
	Vision	Organomercury
	Taste	Selenium
	Audition	Toluene
	Balance	Methyl nitrite
Somatosensory	Skin senses (e.g., numbness, pain)	Trichloroethylene
	Proprioception	Acrylamide
Motor	Muscle weakness, paralysis	Organophosphates
	Spasticity	β-*N*-Oxalylamino-L-alanine
	Rigidity	MPTP
	Tremor	Chlordecone
	Dystonia	Manganese
	Incoordination	Organomercury
	Hyperactivity	Lead salts
	Myoclonus	Toluene
	Fasciculation	Anticholinesterases
	Cramps	Styrene
	Seizures, convulsions	Acetonitrile
Autonomic	Abnormalities of:	
	Sweating	Acrylamide
	Temperature control	Chlordane
	Gastrointestinal function	Lead salts
	Appetite/body weight	Dinitrobenzene
	Cardiovascular control	1-Nitrophenyl-3-(3- pyridylmethyl) urea
	Urination	Dimethylaminopropionitrile
	Sexual function	β-Chloroprene
Immune system	Myositis, vasculitis, fibrosis	Spanish toxic oil
	Guillain Barré syndrome	Gold salts

[a] Adapted from Lane and Routledge, 1983.
[b] Data not conclusive.

syndrome associated with pyridoxine megavitamin therapy (Foca, 1985), prescribed for the treatment of premenstrual tension.

In developing countries, biological toxins (e.g., *Clostridium botulinum* , *C. tetani*, and *Corynebacterium diphtheriae*), neurotoxic agents naturally present in food (e.g., cassava and *Lathyrus* suppl.) or as contaminants (e.g., ergot and aflatoxin), and pesticides probably account for a large number of human neurotoxic disorders. Uncontrolled cholinergic crises, sometimes leading to death, are commonplace in certain regions among agricultural and pesticide workers (Almeida, 1984), and long-lasting changes in the electroencephalograms and behavior of surviving persons have been recorded (Duffy et al., 1979). Other pesticides contain tremor-and seizure-inducing organochlorines or synthetic pyrethroids that perturb neurotransmission (Narahashi, 1984; Taylor et al., 1979). The worldwide problem of substance abuse, particularly abuses involving ethanol, hallucinogens, narcotics, CNS stimulants, solvents, and nitrous oxide, leads to various types of short-or long-lasting neurological dysfunction. Many other substances encountered in the workplace (e.g., solvents, monomers, and catalysts) have been associated with neurological illnesses ranging from polyneuropathy to organic brain syndrome. Both of these disorders were observed in workers exposed for only a few weeks to one particularly potent industrial neurotoxicant, Lucel-7 (2-*tert*-butylazo-2-hydroxy-5-methylhexane) (Kurt and Webb, 1980).

Among the environmental pollutants with neurotoxic potential, lead and mercury each occupy a prominent position, although the number of people in North America with overt neurotoxic disorders attributable to these sources is probably low. Potent chemical toxins are secreted by or contained in numerous members of the animal kingdom. Many of these agents disturb nerve conduction, and one, ciguatoxin, is a major cause of acute neurotoxicity in the Pacific among those who eat contaminated fish (Kaplan, 1980). Others (e.g., α-bungarotoxin and α-latrotoxin) affect synaptic transmission. Both types can produce acute, life-threatening conditions. Some of these agents find their way into food and water consumed by humans. Some chemicals with experimentally proven neurotoxic potential in animals are used as food additives (e.g., monosodium glutamate), flavors and fragrances (e.g., 2,6-dinitro-3-methoxy-4-tert-butyltoluene), and antiseborrheic agents (e.g., zinc pyridinethione), but no cases of human neurotoxic disease from these sources have been reported.

The occurrence of subclinical neurological and behavioral disorders associated with chemical substances is unknown but is believed by some to be widespread. Examples include the unresolved controversies about childhood cognitive impairment from environmental lead contamination and the neurobehavioral effects attributed to prolonged occupational exposure to a variety of industrial solvents.

Methods for assessing neurotoxic diseases in humans fall outside of the scope of this document. For information on this subject, please refer to Geller et al. (1979) and Spencer and Schaumburg (1980, pp. 650-707).

Epidemiological Studies

There have been several epidemiological studies of outbreaks of human neurobehavioral disorders in which chemical compounds have been implicated and subsequently proved to be neurotoxic in animal studies. Few of these investigations have been focused on features important to the toxicologist, such as the exposure concentration and duration, route of entry, and individual susceptibility. A notable exception was the outbreak of neuropathy induced by methyl *n*-butyl ketone (MBK) in an Ohio factory in 1973 (Allen et al., 1975), where an analysis of the atmosphere and exposure conditions was conducted by NIOSH. This investigation was followed by a large number of experimental animal studies that demonstrated, for example, that dermal penetration was a significant route of human exposure to MBK, that oxidative metabolism yielded a number of metabolites with neurotoxic potency, and that other solvents played a role in potentiating the neurotoxic activity of MBK (Spencer and Schaumburg, 1980).

Other notable recent outbreaks subjected to epidemiological investigation include occupational injuries associated with exposures to chlordecone, dimethylaminopropionitrile, *n*-hexane, Lucel-7, and leptophos (Pestronk et al., 1979, 1980; Spencer et al., 1980a,b, 1985; Taylor et al., 1978; Xintaras et al., 1978). Other major outbreaks have involved consumption of foodstuffs contaminated with such neurotoxic substances as hexachlorobenzene, tri-*o*-cresyl phosphate, methylmercury, polybrominated biphenyls, or thallium salts (Schaumburg and Spencer, 1980). A small outbreak of neuropathy and encephalopathy in a Japanese family followed consumption of well water contaminated with acrylamide (Igisu et al., 1975).

Controlled and Other Studies

Controlled studies of the human response to environmental neurotoxicants have been limited to acute exposure associated with short-term and presumably reversible neurobehavioral dysfunction. Some of these studies have been focused on the effects of inhaled organic solvents on manual dexterity, performance on psychological test batteries, vestibular functions, and other functions. Another set of data from relatively controlled situations reflects clinical experience with therapeutic drugs. Although many of these compounds are not of immediate concern, some agents

(e.g., anticholinesterases) used in the therapeutic setting may also appear in different forms in drinking water.

STUDIES IN ANIMALS

Many methods have been used to assess neurotoxicity in animals, and many types of data have been produced. Experimental studies of the adverse actions of chemical agents on the nervous system include systematic observation and measurement of behavior, neural function, structure, and biochemistry in various laboratory animals. Behavioral toxicology and teratology are relatively recent introductions to the field, and the specific methods used in these studies are evolving rapidly. In this section, no attempt has been made to describe methodology in detail.

In broad terms, behavioral toxicology deals with two groups: respondent behaviors (e.g., auditory startle response), which are elicited by a specific observable stimulus and determined primarily by the properties of the stimulus presentation, and operant behaviors (e.g., exploratory activity), which occur in the absence of an eliciting stimulus and are determined primarily by their consequences. Operant behavior is frequently conditioned by reward or punishment to generate a reliable quantitative baseline. Some of the test methods used in behavioral toxicology, such as the psychophysical measurement of cortical blindness induced by methylmercury, have been successfully used to model visual neurotoxicity in humans, whereas the findings of other tests bear an unknown relationship to human disease. However, with further refinement of methods and the ability to evaluate specific changes in relation to biochemical or functional changes in the nervous system, behavioral toxicology and teratology should be especially useful in studying human neurobehavioral disorders (such as tardive dyskinesia), which appear to be unassociated with detectable neuropathological changes.

A variety of electrophysiological techniques have been used to assess the presence and extent of CNS and PNS injuries, to locate the target sites of action, and to determine the cellular and molecular mechanisms of action. These techniques include measurements of PNS sensory and motor conduction velocities, tests of sensory-and motor-evoked cortical potentials, electroencephalography, electromyography, and extracellular unit recordings from retinal ganglion cells. Intracellular recordings, voltage and patch clamping, and noise analysis have also been used (Fox et al., 1982; Narahashi, 1984). Several of these experimental techniques have been used in clinical situations, since they are noninvasive, sensitive, reliable, and relatively easy to use. These experimental and clinical techniques thus allow one to determine the contribution of pathophysiology to neurological and behavioral impairment.

In experimental animals, observation and measurement of changes in neural structure have provided a solid basis for understanding human neurotoxic syndromes associated with pathological changes in the developing or adult nervous system. This has been possible because the methods used to assess neurological disorders in humans have been exploited and further refined by experimental neurotoxicologists. The transmission electron microscope has greatly extended the resolution of tissue detail, which used to be afforded by the light microscope. Armed with these techniques and a knowledge of neurobiological principles, one can define different patterns of neurotoxic response to chemical agents and explain how these result in human diseases that vary in expression and prognosis. With the exception of certain developmental disorders associated with chemical overexposure, there is usually an excellent correlation between the neurotoxic disorders of mammals and human beings. Through studies in various animal models, structural and functional changes induced by numerous chemical substances have been characterized. These studies have also enabled investigators to pinpoint the initial site of neurotoxic damage or dysfunction—information that provides a sensitive method to assess no-effect and threshold levels for the substance of interest.

With few exceptions, biochemical mechanisms underlying the neurotoxic action of environmental chemicals are poorly understood. The best examples are the actions of certain organophosphate esters on acetylcholinesterase, which promptly induce cholinergic toxicity, and on neuropathy target esterase (formerly called neurotoxic esterase), which is associated with the development of polyneuropathy (Davis and Richardson, 1980). From measurements of enzyme levels in tissue and blood, one can estimate the degree of enzyme inhibition and associated neurotoxic illness. Such measurements provide a sensitive means of assessing the neurotoxic activities of agents with these properties. Biochemical studies of other disorders will most likely yield information on mechanisms that will, in turn, provide comparably sensitive biological markers to determine the degree of neurotoxic impairment produced by chemical agents. Data on biochemical changes underlying neurotoxicity also are critical for the prevention and treatment of such disorders.

Information correlating metabolism and pharmacodynamics with neurotoxic properties is also sparse. The usefulness of this approach, however, was elegantly demonstrated in studies of *n*-hexane and methyl *n*-butyl ketone in rats. O'Donoghue and colleagues (1982) found that these chemically distinct solvents appeared to produce qualitatively identical patterns of neurotoxic disease. The neurotoxic potency of each of the agents was directly proportional to the amount of γ-diketone generated by metabolism. Such studies in rats and subsequently in humans (DiVincenzo et al., 1978; Perbellini et al., 1979, 1980) demonstrated that these agents were meta

bolically related and each could be converted to the proximal metabolite 2,5-hexanedione. Moreover, the onset of characteristic neurological dysfunction (e.g., hindlimb weakness) was linearly related to the concentration of the proximal metabolite in serum (Krasavage et al., 1980). How many other neurotoxic compounds show a linear relationship between dose and effect is unknown.

INTERSPECIES EXTRAPOLATION

There are often major differences between the degree of neurotoxic response observed in animals and that found in humans. For example, the rat is relatively refractory to such agents as organophosphates, arsenic, thallium, MPTP, and thalidomide, all of which readily produce neurodegenerative disorders in humans. Other substances that cause neuropathy in humans, such as acrylamide (see Chapter 9), *n*-hexane (Spencer et al., 1980a), and carbon disulfide (Seppäläinen and Haltia, 1980), also produce neuropathy in rodents. Understanding in this area is so limited that there is no reliable prescription for choosing the appropriate animal for studies intended to shed light on human susceptibility. One important exception is the choice of fowl for the study of organophosphate neuropathy (Davis and Richardson, 1980).

Once a suitable test animal has been identified and a neurotoxic dose established, then the disorder of interest will usually appear in all adult animals after a similar dose and test period have elapsed. The pattern of dysfunction and the underlying structural and functional changes are also uniform across test animals within a single species. These principles may allow the experimentalist to test much smaller numbers of animals than those used, for example, in assays of carcinogenesis. Caution is appropriate, however, in studies focused on the establishment of threshold or low-risk levels of neurotoxic response (as is generally the case in risk assessment), where one is operating in a portion of the dose-response curve that is likely to be less steep than the region in which most experimental studies have been performed. These rules do not apply to neurotoxic disorders of the developing animal, in which response rate and type of teratogenicity are often variable.

RISK ASSESSMENT

Little effort has been directed toward assessing risk from exposure to chemical neurotoxicants. Because of the large number of potential health end points associated with neurotoxicants, attempts at risk assessment are substantially more complex than they are for chemical carcinogens. For example, the same substance may produce markedly different neurotoxic

effects, depending on age at time of exposure. Although the susceptibility of the nervous system to toxicity is well recognized, no bioassay has been developed for identifying and regulating environmental neurotoxicants.

Assessment of dose-response relationships is plagued by the usual uncertainties. Epidemiological dose-response information is extremely limited, and that which does exist may not be applicable to the general population. Although some animal models are particularly accurate for assessing neurological disorders in humans, the large number of potential toxic end points greatly complicates assessment since there may not be a single threshold dose for all end points.

Exposure assessment requires knowledge concerning chemical type and concentration, effect and its severity, route and duration of exposure, and identification of high-risk groups. Safety factors used in such assessments should reflect uncertainty due to variability within the population and variability between the population of interest and the sampled population. Until an accurate probabilistic model for risk assessment is developed, safety factors will have to be used in place of confidence limits. A more detailed discussion of risk assessment for neurotoxicants is found in Chapter 8.

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5

Mechanisms of Carcinogenesis

The public health effects resulting from reductions in exposures to various drinking water contaminants can be predicted with greater accuracy as the mechanisms underlying those effects become better understood. Among the possible chronic effects of concern to regulators faced with the task of estimating risk of such exposures, cancer ranks foremost. Carcinogenesis is a complex, multistep process that has been extensively reviewed by Becker (1981), Farber (1982), Farber and Cameron (1980), Slaga et al. (1980b), and Weinstein et al. (1984). This chapter provides an overview of principles that should be considered when assessing risk of exposure to drinking water. It is not intended to be comprehensive.

THE MULTISTAGE THEORY OF CARCINOGENESIS

Cancer is the product of a process involving complex interactions between environmental and endogenous factors. It is usually manifested by the uncontrolled proliferation of cells that have sustained heritable alterations. The discovery that many carcinogens interact with DNA and thus alter the genotype, i.e., specific DNA sequencing of encoded information, is important to the development of the current theories of carcinogenesis. It has also been learned that the inheritance of a single mutation (i.e., a gene with altered DNA) may not be sufficient to produce cancer (Farber, 1982; Weinstein et al., 1984). In the human body, many millions of cells are at risk, and many of them can be shown to have DNA lesions; however, few cells give rise to malignant tumors. When DNA is damaged, the body responds with the cellular mechanisms of repair or eliminates the aberrant

cell through immune-surveillance mechanisms. This process provides protection against both exogenous and endogenous mutagens and carcinogens. Cellular mutation is often an early stage in a multistage process.

Carcinogenesis has been experimentally demonstrated to be a multistage process in the cells of certain animal tissues, including skin, lung, liver, and bladder. This process is believed to occur in much of human tumorigenesis as well. According to current theories, at least three such stages (initiation, promotion, and progression) are evident in many experimentally induced cancers (Farber, 1984a,b; Slaga, 1983; Weinstein et al., 1984). These stages are phenomenological, and their mechanisms of action are not well understood. The distinction between the stages has been defined experimentally. Each stage appears to be influenced by several exogenous and endogenous factors, such as age, sex, diet, metabolic activity, and the dose and type of xenobiotic substance to which the organism is exposed.

The early work of Rous and Kidd (1941), Berenblum (1941), and Mottram (1944) demonstrated that cancer could be induced in experimental animals in two steps: initiation and promotion. Subsequent studies in animals showed that sequential induction of cancer can occur in a number of tissues or organs. Epidemiological evidence on epithelial cancers collected by Armitage and Doll (1954) also suggested a multistage process in carcinogenesis. This was later supported by the toxicological studies of Boutwell (1964), Slaga et al. (1980b), and Van Duuren et al. (1973). Consequently, the initiation-promotion model has been generally accepted as being representative of tumor induction.

Initiation

Initiators are mutagens that act either directly or indirectly by forming electrophilic species that interact with and modify DNA structure, or otherwise damage the DNA sequence, but do not by themselves induce tumor formation. Initiation is believed to cause a lesion that persists over a long period, as demonstrated by Van Duuren et al. (1975), who showed that mouse skin initiated more than 1 year before treatment with phorbol esters is still very susceptible to tumor induction. Thus, the initiation step is considered to be irreversible. In addition to demonstrating this, Boutwell (1964) showed that repeated doses of an initiator were additive in the number of tumors produced.

Promotion

A promoter is a substance that usually does not induce a carcinogenic response by itself but that results in a carcinogenic response when applied

in multiple doses following a single, subcarcinogenic dose of an initiator. This temporal sequence of administration is only demonstrable in the laboratory; such distinctions are difficult to demonstrate in humans, who receive simultaneous environmental exposures to many types of chemicals. Some promoters can also show weak initiating activity at high doses. Unlike initiators, promoters do not form electrophilic species that interact with DNA. Some evidence indicates that promotion itself involves several stages and that it may be possible to characterize a promoter as a complete or a first-or second-stage promoter (Fürstenberger et al., 1983; Slaga et al., 1980a). The effects of a first-stage promoter are thought to be reversible; i.e., if promoter administration is terminated, a carcinogenic response is not produced. Administration of a second-stage promoter produces irreversible effects.

A compound capable of acting as both an initiator and a promoter in the same tissue is defined as a complete (whole) carcinogen. Most chemicals that seem to have performed as initiators appear to be complete carcinogens.

Progression

The period during which ill-defined stages lead from benign tumor to malignant tumor is called progression. The transformation of neoplastic cells to a malignant tumor during this stage may involve several steps, such as oncogene activation (Weinberg, 1985), chromosome aberration (Weinstein et al., 1984), interaction between tumor cells and host defenses (Kripke and Morison, 1985), and various selection processes (OSTP, 1985). Progression can be considered a dynamic process, since tumors may continue to increase in their degree of malignancy and heterogeneity (Weinstein et al., 1984).

Cocarcinogenesis

Cocarcinogenesis is the process by which two or more compounds, when administered concurrently, increase the risk of tumor development. In some cases, a cocarcinogen is not carcinogenic by itself, but enhances the carcinogenic potency of an initiator. In other cases, both compounds are carcinogenic by themselves, but together elicit a response that is greater than that expected on the basis of simple additivity. Cocarcinogens differ from promoters by definition because promoters are administered after initiators and are not usually carcinogenic alone. Some compounds may be both cocarcinogens and promoters. However, not all tumor promoters are cocarcinogens and not all cocarcinogens are tumor promoters, sug

gesting that promotion and cocarcinogenesis proceed by different mechanisms.

ONCOGENE ACTIVITY

Oncogenes are naturally occurring genes that code for factors that regulate, among other things, cellular growth. They have been identified in several human tumors as well as in spontaneous and xenobiotically induced tumors in animals. The most recent studies on the mechanisms of cell interactions in cancer induction have attempted to elucidate the role of oncogenes in carcinogenesis (Weinberg, 1985). In the last 4 years, such research has led to several key discoveries that strongly support the multistage theory of carcinogenesis (Land et al., 1983; Slamon et al., 1984).

Approximately 40 oncogenes have been discovered (Weinberg, 1985). Most of these have been operationally classified as immortalizing genes (*myc* type) and transforming genes (*ras* type). Recent studies have shown that *ras*-type oncogenes can be activated by chemical carcinogens and by ultraviolet light (Sukumar et al., 1983). Immortalization of cells in culture has been carried out using chemical carcinogens, and these cells have been transformed and promoted by oncogene products and xenobiotic substances. Studies have shown that the DNA from chemically induced tumors contains active oncogenes, and recent research has demonstrated the potential importance of chromosome translocations in oncogene activation (Bishop, 1982; Land et al., 1983; Leder et al., 1983). Immortalization (presence of a *myc*-type oncogene) and transformation (presence of a *ras*-type oncogene) may be regarded as the biological counterparts of initiation and promotion. Recent findings have identified a third type of cancer gene (not yet classified) that may cause a cancer cell to metastasize (Bernstein and Weinberg, 1985). This finding of a unique oncogene in a metastatic tumor strongly supports the multistage model of carcinogenesis, since it indicates that different types of oncogenes may correspond to different stages of carcinogenesis.

The literature on the specificity of oncogenes and their activation, their location in spontaneous and induced tumors, and the ability of transformed cells in culture (activated *ras* gene) to induce tumors *in vivo* further indicates that tumor induction is indeed a multistage event. To reflect these experimental findings, risk modeling must consider at least three, if not more, stages in order to be consistent with the experimental and human evidence.

In particular, the observation that certain promoting agents can induce chromosome aberrations (including translocations) and aneuploidy is of interest in view of the fact that both translocations and aneuploidy have been observed in certain animal and human neoplasms, such as Burkitt's

lymphoma, retinoblastoma, and Wilms' tumor (Barrett et al., 1983; Cairns, 1981; Leder et al., 1983; Tsutsui et al., 1983; Yunis, 1983). Increased risk of tumor development has also been observed in patients with congenital aneuploidy, such as Down's syndrome (Windham et al., 1985). Recent research has illustrated the potential importance of chromosome translocations in oncogene activation (Bishop, 1982; Land et al., 1983; Leder et al., 1983).

MODEL SYSTEMS

Mouse Skin

The mouse has served as an especially useful test animal, since tumor induction in its dorsal skin is relatively rapid and can easily be observed and quantitated without sacrificing the animal. For 30 years, up to the late 1960s, the mouse skin model was used almost exclusively for initiation-promotion studies in chemical carcinogenesis.

For nearly 40 years, croton oil (an oil obtained from the seeds of *Croton tiglium* L.) was used as a promoting substance in mouse skin studies. The active component was identified in the late 1960s as phorbol myristate acetate (PMA) (Hecker, 1971; Van Duuren, 1969). Since that time, several PMA analogs have been synthesized. Although these PMA analogs have less promoting activity, they are important in studies designed to determine the mechanism of action of phorbol ester promoters (Boutwell, 1974).

In addition to the phorbol ester tumor promoters, there are other classes of compounds with promoting activity in the mouse skin. Dihydroteleocidin B, a derivative of a natural product isolated from streptomyces, has strong promoting activity in this model (Fujiki et al., 1981), as do certain natural products other than the phorbol esters extracted from plants (Mufson et al., 1979). Phenol and certain phenol derivatives were shown by Boutwell and Bosch (1959) to be weak promoters in skin, as are certain fatty acids and fatty acid methyl esters and long, straight-chain alkanes such as decane (C-10) and tetradecane (C-14) (Arffmann and Glavind, 1971; Van Duuren and Goldschmidt, 1976). Anthralin (1,8-dihydroxy-9-anthrone) was shown by Segal et al. (1971) to be a strong promoter in mouse skin, whereas detergents such as sodium lauryl sulfate and Tween 60 are weak promoters (Boutwell, 1964; Setälä, 1960). A class of tumor promoters discovered recently are peroxides such as benzoyl peroxide, which is a moderately strong promoter in mouse skin (Slaga et al., 1981). Direct-acting alkylating agents such as iodoacetic acid and 1-fluoro-2,4-dinitrobenzene are weak to moderate promoters (Bock et al., 1969; Gwynn and Salamon, 1953). Benzo(e)pyrene, which is not a whole carcinogen in skin, is a moderate promoter in that tissue (Slaga et al., 1979), whereas

7-bromomethyl benz(*a*)anthracene is a complete carcinogen and also a strong promoter (Scribner and Scribner, 1980). Suganuma et al. (1984) have recently identified a new class of tumor promoters, aplasiatoxins, which are structurally unrelated to PMA.

A number of chemicals that are tumor promoters in mouse skin are also cocarcinogens in mouse skin. The existence of cocarcinogenic substances was inferred from studies of carcinogenesis resulting from exposures to cigarette smoke condensate (CSC). Since the concentration of benzo(*a*)pyrene [B(*a*)P] in CSC and the dose delivered in CSC skin-painting experiments could not account for the observed tumor yield, it was surmised that other substances present in CSC enhanced the response to B(*a*)P (Hecht et al., 1981). Van Duuren and Goldschmidt (1976) showed that catechol (1,2-dihydroxybenzene), which is an abundant phenol in CSC, is a potent cocarcinogen in B(*a*)P-induced skin carcinogenesis. Thus when low carcinogenic doses of B(*a*)P were applied to the skin repeatedly over a long period in the presence of catechol, the number of skin tumors increased significantly compared with the number induced by treatment with B(a)P without catechol. Treatment with catechol in the absence of B(*a*)P did not cause tumor induction. Van Duuren and Goldschmidt (1976) also showed that PMA is a cocarcinogen as well as a promoter.

In addition to serving as a model system for identifying whole chemical carcinogens, initiators, and promoters, the mouse skin has been extremely useful for studying the mechanism of action of initiators and promoters since the early 1970s. At the tissue level, phorbol ester promoters cause hyperplasia and inflammation (Boutwell, 1964). Cellular responses observed within 24 hours after exposure include increased synthesis of DNA, RNA, and protein (Baird et al., 1971), increased phospholipid turnover (Rohrschneider and Boutwell, 1973), changes in cyclic nucleotide metabolism (Mufson et al., 1979), increases in protease activity (Troll et al., 1978), induction of ornithine decarboxylase—a key enzyme in polyamine metabolism (O'Brien et al., 1975), and decreases in the activities of the antioxidant defense enzymes superoxide dismutase and catalase (Solanki et al., 1981). Largely as a result of these studies, early investigations on the inhibition of promotion focused on substances that might reverse the tissue and biochemical responses caused by promoters. Thus antiinflammatory compounds such as cortisol, dexamethasone, and fluocinolone acetonide were found to be effective promotion inhibitors (Belman and Troll, 1972; Schwartz et al., 1977), as were protease inhibitors (Troll et al., 1978). Vitamin A derivatives (Verma et al., 1979) and free-radical scavengers such as dimethyl sulfoxide (DMSO) (Loewengart and Van Duuren, 1977) were also effective promotion inhibitors.

In the mouse skin, the two-stage initiation-promotion protocol results in the formation of a large number of papillomas, many of which progress

to squamous cell carcinomas. Burns et al. (1976) identified two classes of papillomas—one class that regressed after treatment with PMA was discontinued and another class that showed a decreased tendency to regress, i.e., a class of papillomas with autonomous growth. The greatest tendency to undergo malignant transformation occurred in the autonomous group. Klein-Szanto et al. (1983) have shown that the acquisition of γ-glutamyltranspeptidase (GGT) and the loss of high-molecular-weight keratins appear to be good indicators of progression from benign to malignant tumor in mouse skin.

Slaga et al. (1980a) demonstrated that the promotion phase in mouse skin can be subdivided into two stages. In the first stage, the initiated dorsal skin of mice is treated with a first-stage promoter such as PMA for 2 weeks. This treatment is followed by repeated treatments (18 weeks or longer) with a second-stage promoter (e.g., mezerein) (Slaga et al., 1980a,b). Treatment of initiated mouse skin with a first-stage promoter for 2 weeks without subsequent treatment with a second-stage promoter does not result in tumor induction, nor does treatment for a long time with a second-stage promoter without prior treatment with a first-stage promoter (Slaga et al., 1980a). In the two-stage promotion model, the complete promoter PMA is also a potent first-stage promoter. The weak promoter mezerein is a strong second-stage promoter, but the PMA analog 4-*O*-methyl-PMA, which is not a promoter in the two-stage carcinogenesis model, is a weak first-stage promoter (Slaga et al., 1980b). Fürstenberger et al. (1983) have shown that the first stage of promotion was not reversible for at least 2 months when PMA was given as a first-stage promoter and was followed by 12-retinylphorbol-13-acetate (RPA). They also demonstrated that partial inversion can occur between initiation and the first stage of promotion. In their model, initiation with a subthreshold dose of dimethylbenzanthracene (DMBA) could occur successfully up to 6 weeks after treatment with PMA.

Rat and Mouse Liver

This model system has in the past involved an invasive procedure (partial hepatectomy), followed by administration of the initiator [usually 2-acetylaminofluorene (2-AAF), diethylnitrosamine (DEN), or DMBA] and then by phenobarbital as the promoter. The interaction of phenobarbital with 2-AAF was first reported by Peraino et al. (1971), who established the promotional effect of phenobarbital in the liver. Recent results indicate that the rat and mouse liver models of carcinogenesis do not require use of the invasive technique and that dietary restriction of choline for less than 2 months after pretreatment with a cytochrome P450 modifier is sufficient to induce altered foci (Mylecraine, 1984).

Recent evidence suggests that intraperitoneal pretreatment of Fischer 344 adult male rats with β-naphthoflavone, a 2,3,7,8-tetrachlorodibenzo*p*-dioxin (TCDD)-type of P450 cytochrome inducer, enhances the production of altered foci by DEN (Mylecraine, 1984). A small percentage of these altered foci have been shown to progress to cancer (Williams and Weisburger, 1983). Mylecraine (1984) has shown that altered foci in rodent livers are enhanced when phenobarbital is injected intraperitoneally to the animals before initiators are administered. Thus, apart from increasing the production of reactive derivatives, some aspect of the proliferation induced by compounds known to induce one or more forms of cytochrome P450 may have an effect on the ability of the liver to respond to a carcinogen.

Induction of uncontrolled cell replication is believed to be an important contributing factor in the process of cocarcinogenesis, but the role of proliferation in promotion is not entirely clear. Investigations of several organ systems indicate that cells in the process of normal replication are more susceptible to mutagenesis and carcinogenesis than are cells that are relatively dormant and replicate more slowly. For example, synchronized rat liver epithelial cells were shown to be most sensitive to mutation at the phosphoribosyltransferase locus by methyl methane sulfonate or by the gastric carcinogen *N*-methyl*N*′-nitro-*N*-nitrosoguanidine (MNNG) during DNA synthesis (Tong et al., 1980). Following partial hepatectomy in the $B6C3F_1$ mouse, Newberne et al. (1982) observed a small increase in spontaneous liver tumors. Rats given a single injection of dimethylnitrosamine 24 hours after undergoing partial hepatectomy (the peak time of DNA synthesis in the regenerating liver) developed hepatocellular carcinomas, whereas nonhepatectomized rats did not (Craddock, 1971). However, the lack of proportionality between the rate of cell division and induction of tumors in carcinogen-exposed rat livers indicates that there are modulating factors beyond the rate of cell division (Becker, 1979).

J. M. Ward et al. (1984) have demonstrated that continuous, long-term exposure is not necessary for tumor promotion in the mouse liver. For example, di(2-ethylhexyl) phthalate administered after initiation with DEN resulted in the same significant increase in altered hepatic foci after 24 or 84 days of exposure. See next section, entitled Other Animal Systems, for further examples.

Studies in which 2-AAF is administered serve as excellent examples of the complexity of multistage (and multiorgan) carcinogenicity. This compound behaves as a promoter in the mouse bladder but as an initiator in the liver of the same animal (Hughes et al., 1983). Further evidence for tissue specificity has been provided by studies demonstrating that liver tumors in rodents are not promoted by PMA or by other phorbol esters but, rather, by polyaromatic hydrocarbons (PAHs), polychlorinated bi

phenyls (PCBs), TCDD, and phenobarbital (Peraino et al., 1980; Pitot et al., 1980). This finding is supported by evidence in humans for tissue-specific promotion by estrogens, cigarette smoking, and asbestos, the latter serving as a promoter of lung cancer development and as an initiator of mesothelioma (NRC, 1984, pp. 165-199).

Many inducers of hepatic cytochrome P450, e.g., phenobarbital, PCBs, dichlorodiphenyltrichloroethane (DDT), and TCDD, have been implicated as promoters in the rodent liver model. These agents may act as promoters through the generation of active oxygen molecules such as superoxide, hydroxyl radical, or peroxide, which may cause genetic effects, direct effects on the cell membrane through alteration of transport mechanisms, and immunotoxicity (see Kensler and Trush, 1984, for a review). Indeed, Dean et al. (1983a) have shown that 12-*O*-tetradecanoylphorbol-13-acetate (TPA) depresses cell-mediated immunity, T-cell function, and *in vivo* tumor resistance. These responses are consistent with findings using other suspected promoters, e.g., diethylstilbestrol (DES), TCDD, and dimethylvinylchloride (DMVC) (Dean et al., 1983a).

Other Animal Systems

The discovery of experimental tumor promotion in other tissues, such as the bladder (Hicks, 1983; Verma et al., 1983), breast (Rogers, 1983; Wotiz et al., 1984), colon (Reddy and Maeura, 1984; Rogers, 1983), lung (Witschi, 1983; Witschi et al., 1977), pancreas (Ohyama, 1985), and respiratory tract (Mossman et al., 1985), is relatively recent. Research in these areas has produced a great deal of information on possible mechanisms of carcinogenicity, including tissue specificity and a memory effect of promoters.

Multistage carcinogenesis in the liver and skin has been more clearly defined (J. M. Ward et al., 1984) than in the lung, colon, brain, kidney, and thyroid. Estrogen-sensitive tissues (i.e., tissues with high levels of estrogen receptors) such as the endometrium, prostate, and breast appear to respond to estrogens and nonhormonal compounds that exhibit estrogen-like activity in a manner similar to the initiator-promoter (multistage) model (Baxter and Funder, 1979; Lippman and Allegra, 1978; McGuire et al., 1978; Wein and Murphy, 1973).

Some promoters are active at only one site. However, others, such as 2-AAF, can act as an initiator in one tissue and a promoter in another (Hughes et al., 1983). Some agents can act as an initiator at one site, a promoter at another, and an inhibitor at a third. TCDD is an example of this phenomenon: in the mouse, it is a weak initiator in skin (DiGiovanni et al., 1977) and possibly in liver (Kociba et al., 1978); a promoter in liver (Pitot et al., 1980); and an inhibitor in skin (DiGiovanni et al., 1983)

and possibly in the pituitary, uterus, mammary glands, pancreas, and adrenal gland (Kociba et al., 1978). The effect of a promoter may vary, depending on the sequence of administration with the initiator. For example, when given to mice before an injection of urethan, the antioxidant butylated hydroxytoluene (BHT) will decrease the numbers of lung adenomas induced. When administered after urethan, BHT increases tumor yield (Malkinson and Beer, 1984). The sensitivities of different species and strains to promoter activity vary in many cases; mice are the most sensitive, forming multiple skin papillomas in response to an initiation-promotion regimen, as compared to less frequent basal cell carcinomas in rats and melanomas in hamsters (Slaga and Fischer, 1983). In light of these observations, it may be desirable to test potential promoters and inhibitors of promotion *in vivo* in several species and strains and in both sexes, to examine several organs for the response, and to administer the compounds in different sequences.

Studies in Humans

Evidence for multiple stages in human tumor promotion results from analysis of epidemiological data on several cancers. For example, epidemiological data on cigarette smoking and lung cancer have been used to develop a simple multistage model in which the incidence rate is proportional to time^k, where k is the slope of the log-log age-incidence curve and is believed to approximate the number of stages necessary for tumor development (Peto, 1977). Cessation of cigarette smoking reduces the risk of lung cancer, although not to the level of risk for nonsmokers (Doll, 1978), and is thus believed to affect the penultimate stage of lung cancer development (Peto, 1977). Cigarette smoke contains compounds capable of both initiating and promoting lung carcinogenesis. In another example, liver cancer in Africa appears to be linked to both aflatoxin B_1 exposure and hepatitis B virus infection (Linsell and Peers, 1977), each of which may affect different stages in liver cancer development. The risk of lung cancer for asbestos workers who smoke is 50 times greater than that of nonsmokers (NRC, 1984). Asbestos appears to be acting both as a promoter in the lung and as an initiator of mesothelioma (NRC, 1984, pp. 165-199). Tumors of the endocrine organs are linked to hormone availability; the hormones apparently exert a promoting effect (Day and Brown, 1980; Sivak, 1979).

GENETIC TOXICITY

Several contaminants in drinking water react chemically with DNA. A few of these are reviewed in Chapter 9. This genetic toxicity is of great

concern since the information that controls the structure, function, and reproduction of cells is encoded in the DNA. Moreover, both theoretical considerations and experimental evidence indicate that alteration of the DNA is involved in at least one step in the complex process of carcinogenesis.

Genotoxicity and, preferably, genetic toxicity are the general terms used to describe the mode of activity of an agent that itself or by way of its metabolites can interact with genetic material to induce heritable alterations in DNA sequence or chromosome number or structure in either somatic or germ cells. Mutagens are agents that can alter the primary base sequence of the DNA. Clastogenicity and aneuploidy refer to chromosome breakage or change in chromosome number, respectively. A distinction is sometimes made between genetic toxicants that act in different ways. In primary genetic toxicity, either the parent compound or its metabolite directly alters or binds to genetic material. For example, both methyl methane sulfonate (MMS) and B(a)P are primary genetic toxicants. MMS, however, is called a direct-acting agent because it interacts directly with DNA without the need for metabolic activation. In contrast, B(*a*)P is an example of a primary genetic toxicant that requires metabolic activation to exhibit genetic toxicity. Secondary genetic toxicity refers to activity in which genotoxicity is a secondary result of the primary action of the agent. Examples of this type of toxicity are effects on the DNA polymerase, inhibition of DNA repair, induction of a physiological state that results in genetic toxicity, or forced cell proliferation, resulting in an increase in the frequency of spontaneous mutations.

Some carcinogens are believed to act without affecting DNA. One example is DES, which can lead to a rare form of vaginal cancer in adolescent women who had been exposed transplacentally (Herbst et al., 1977). DES may affect the differentiation of endocrine organs, leading to cancer later in life due to either hormonal imbalances or altered hormone receptor response (Weisburger and Williams, 1982). Another example is asbestos, whose fibers do not seem to damage DNA directly (Fornace, 1982) or act as mutagens (Chamberlain and Tarmy, 1977) but which may transport PAHs (known initiators) into target cells because they adhere to asbestos fibers (Eastman et al., 1983).

THE ROLE OF THE IMMUNE SYSTEM IN CARCINOGENESIS

The immune system provides a major defense against invading microorganisms and altered cells. It functions in overall host resistance to infections, in the maintenance of homeostasis, and in surveillance against uncontrolled cell proliferation (Fidler, 1985). The immune system provides a natural defense against cancer. This conclusion is supported by

reports of increased incidences of some rare types of cancer among people with an impaired immune system, such as patients with acquired immunodeficiency syndrome (AIDS) (Biggar et al., 1985; Cairns, 1985). Furthermore, malignancies occur with enhanced frequency in patients on immunosuppressive therapy (McKhann, 1969; Penn, 1978). Although a variety of lymphoid cell types and macrophages are involved in immune response, evidence to date has indicated that the most effective host responses leading to tumor cell elimination are those of the T cells (Schatten et al., 1984; Zöller, 1985).

Neoplasms are frequently associated with the presence of a mononuclear cell infiltrate containing numerous macrophages that, in collaboration with B and T lymphocytes, can destroy neoplastic cells. Adams and Snyderman (1979) have suggested that tissue macrophages may recognize small colonies of newly formed tumor cells, cluster about them, and destroy the nascent neoplasm. Recently, Fidler and Kleinerman (1984) have shown that human blood monocytes (phagocytic blood cells that become macrophages in tissue) destroy tumor cells but not normal cells when they are cultured together. This effect is related to immunosurveillance.

The possibility that the immune system developed as a useful and effective mechanism for the early sensing and elimination of neoplastic cells was originally proposed by Thomas in 1959 and was later given the name immunosurveillance by Burnet (1970). Burnet's concept of immunosurveillance was based on two assumptions: (1) that cancer cells might be arising in small clones, or specialized subpopulations, in one organ or another, all the time and (2) that something foreign might always be displayed at their surfaces indicating their alien nature. Both T cells and macrophages have been shown to participate in host immunosurveillance.

Studies in laboratory animals have demonstrated that the immune system is particularly sensitive to the toxic effects of xenobiotic substances. The cellular components of the immune system are derived from bone marrow stem cells, a population of very rapidly proliferating cells that constantly replenish the immunocytes in the peripheral blood and tissues. Rapidly dividing cells are believed to be more sensitive to mutations than those that divide more slowly or not at all.

Both the cell-mediated and the humoral immune systems are affected by toxic chemicals. Suppression of immune responsiveness is typically manifested by a decreased resistance to viruses, parasites, bacteria, and tumor cell grafts (for a review, see Dean et al., 1984; NRC, 1977). Immune suppression has also been observed in people accidentally exposed to polybrominated biphenyls and PCBs (Bekesi et al., 1979; Chang et al., 1980; Shigematsu et al., 1978). Xenobiotic compounds may also induce

immunologic enhancement, resulting in autoimmune-type disorders and possibly hypersensitivity reactions.

A variety of carcinogens and cocarcinogens have been shown to have profound effects on the immune system. Immune suppression has been reported following exposure of animals to B(*a*)P, ultraviolet irradiation, and DMBA (Dean et al., 1983b; Kripke and Morison, 1985; E. C. Ward et al., 1984). These effects were manifested by decreased resistance to infections and tumor cell grafts and by decreased spleen weight and marrow cellularity.

Tumor-promoting agents such as TPA, DES, TCDD, DMVC, and urethan have been shown to decrease both the cell-mediated immune response and overall resistance to infections (Dean et al., 1983a; Luster et al., 1980a,b, 1982). However, phorbol ester tumor promoters have been reported to activate macrophages and to stimulate reactive oxygen release from macrophages (Laskin and Pilaro, 1985; Laskin et al., 1981; Pilaro and Laskin, 1984). In this case, stimulation of the immune response by tumor promoters may also lead to enhanced development of neoplasia.

Thus both suppression and enhancement of the immune system by xenobiotic compounds and carcinogens may lead to increased cancer incidence. These effects may be manifested at any stage during carcinogenesis.

CARCINOGEN EVALUATION

Animal Bioassays

Positive results in properly conducted animal bioassays are considered to be predictors of qualitative response in humans (IARC, 1980; NRC, 1977, 1983; NTP, 1984; OSTP, 1985; OTA, 1981). The scientific rationale for this approach is simply that animals are the closest models to the human for cancer studies. In addition, many carcinogens produce cancer in several species, and all known human carcinogens have been shown to produce tumors in at least one animal model (NTP, 1984). Benzene and arsenic trioxide, the two former holdouts from this general rule, have now been shown to be carcinogenic in animals (Goldstein et al., 1982; Maltoni and Scarnato, 1979; Pershagen et al., 1984). For some chemicals (e.g., aflatoxin B_1, DES, vinyl chloride, mustard gas, melphalan, and 4-aminobiphenyl), the positive results in experimental animals preceded the epidemiological evidence. The overall patterns of chemical metabolism are generally similar in humans and laboratory animals (Rall, 1979), although the rates of metabolism and the type and site of cancer may

differ (IRLG, 1979; OTA, 1981). For example, the metabolism of B(*a*)P is qualitatively the same in all species and systems studied (Sims, 1976).

At present, the most widely used data for carcinogen evaluation and regulation are those derived from animal bioassays. Alternative approaches to identifying carcinogens, such as short-term tests and the study of structure-activity relationships, are generally viewed as supplemental to but not as substitutes for long-term bioassays (NTP, 1984; OSTP, 1985). These two approaches are discussed in later sections of this chapter.

The standards for experimental design and conduct as well as for the analysis and reporting of data from animal bioassays have been well described (IARC, 1980; see OSTP, 1985, for a review). Subjects of extensive discussion have been the necessity of using high doses or the maximum tolerated dose (MTD) to maximize the sensitivity of the bioassay and the validity of this approach (Food Safety Council, 1980; NRC, 1977; NTP, 1984; OSTP, 1985; OTA, 1981). The MTD has been operationally defined as the dose that will neither reduce longevity nor inhibit the maximum growth or weight gain of the experimental animals by more than 10%. For some materials that produce important deleterious effects without causing serious weight loss, however, the MTD must be related to the other toxic responses and normal longevity of the animal (NTP, 1984). Recently, two expert panels have reaffirmed the use of MTD as a valid and necessary procedure (NTP, 1984; OSTP, 1985).

There are several important limitations to animal bioassays. For example, the test animals are usually homogenous, healthy animals exposed to a single agent, whereas the human population is genetically heterogenous with widely varying susceptibilities to disease and multiple exposures to carcinogenic substances. Moreover, the assays do not necessarily predict the target site of action in humans. For example, 2-naphthylamine induces bladder cancer in humans, monkeys, dogs, and hamsters, but hepatic cancer in rats (IARC, 1977). These problems are discussed further in Chapter 8 in the context of quantitative risk assessment based on animal bioassay data. In addition, animal bioassays are time-consuming, difficult to conduct, and expensive. For example, a well-conducted inhalation study usually requires 3 to 4 years from the planning stage to completion of the final report and can cost in excess of $500,000. Despite their limitations, however, bioassays are currently the most acceptable way to predict effects in humans when, as is usually the case, there are no adequate epidemiological data on humans (OSTP, 1985).

Short-Term Tests

Short-term tests can be completed in only a fraction of the time it takes to complete traditional 2 year bioassays in rodents. They can be used to

determine the mutagenicity of chemicals in *in vitro* systems of bacteria and mutagenicity or transformation in mammalian cells in culture. They can also be used to study very specific end points, such as DNA binding or sister chromatid exchange, in *in vivo* systems following limited exposure to a chemical. Short-term assays often can provide information regarding the putative carcinogenicity of a chemical; that is, a positive response in a short-term test can indicate the likelihood of a positive response in a chronic carcinogenesis bioassay.

Numerous assays can be conducted to obtain a measure of the genetic toxicity and potential carcinogenicity of a chemical (Bridges et al., 1982; Butterworth, 1979; Hollstein et al., 1979). Various combinations of these assays are currently used in batteries or tiers by industry and government agencies to characterize chemicals regarding potential carcinogenicity and to assist in regulatory decision making (EEC, 1979; EPA, 1984; OECD, 1981; OTA, 1981). The theories supporting the use of such short-term tests have been reviewed in detail (Ashby, 1983; de Serres and Ashby, 1981; IARC, 1980, 1982; OSTP, 1985).

In the widely accepted multistage theory of carcinogenesis, genetic toxicity occurs during the early or initiating phase of neoplasia. Such end points are of considerable value in detecting initiating and complete carcinogens, which are believed to exert their effect, at least in part, by interacting with genetic material. However, these assays are limited in their ability to detect events such as promotion, which may also be of considerable importance in the process of carcinogenesis. As more is learned about the molecular biology of cancer, new assays that measure other events in the process of carcinogenesis are becoming available. For example, cell transformation assays measure the ability of chemicals to convert a normal cell to a preneoplastic state or to convert a preneoplastic cell to a tumorigenic state (Barrett et al., 1984; Heidelberger et al., 1983).

Chemicals that participate in carcinogenesis by means other than altering genetic material have been termed nongenetic, or epigenetic, carcinogens. These chemicals can include promoters, which accelerate the multistage process of carcinogenesis. Some cell culture assays, such as the cell transformation assay, may be useful in detecting promoting activity (Abernethy et al., 1984; Frazelle et al., 1983), but there is still a need for short-term tests for promotion (IARC, 1983; NTP, 1984; OSTP, 1985).

Short-term tests are highly complex, sometimes technically demanding procedures for measuring the molecular effects of chemicals on cells. Skilled personnel are required to perform them properly and to interpret the results (Butterworth, 1981). For example, cytotoxicity can have a profound effect on the end points measured. Thus, experiments must be designed so that results are not artifacts of excessive cytotoxicity. In many cases, a large amount of data is generated, especially for chemicals of

interest to the general public (see, for example, EMIC, 1986). Occasionally, there is inconsistency between the results of different tests. The reasons for varying responses must be examined and reconciled. When testing a known carcinogen, investigators may look for and give weight to any positive response at the expense of a more balanced view of the data.

Because chemicals may act by different mechanisms, a battery of tests is required for an appropriate evaluation of the genetic toxicity of a chemical. Assays vary in complexity—from tests on bacteria that are exquisitely sensitive to mutagens (McCann et al., 1975) to measurements of genetic toxicity in the treated animal or cells from exposed individuals (Bridges et al., 1982). Results from bacterial assays alone are rarely sufficient to classify a chemical as genetically toxic or not and should generally be confirmed in mammalian cells. For example, vitamin C is positive in the *Salmonella* mutation assay under certain conditions (Norkus et al., 1983), but TCDD, a potent carcinogen, is not (Geiger and Neal, 1981). Thus, knowledge of the principal mechanism of action of a chemical can be critical in determining which short-term tests will have meaningful predictive value. This consideration could be important for assessing the safety of a substance, identifying noncarcinogenic analogs of a compound for use in new products, or choosing alternatives to currently used carcinogens. For example, data from promotion assays would be far more valuable than mutagenicity data in searching for a noncarcinogenic analog of TCDD. In reality, however, the mechanism of action of chemicals is generally not known.

Both false-negative and false-positive results may occur when using short-term tests, because they do not reflect the complexity of interactions in the whole animal. In the absence of an appropriate metabolic activating system, false negatives can occur. Measurements in the whole animal are particularly useful because the results are influenced by such inherent factors as metabolism, distribution, excretion, and repair (Ashby, 1983). One must be concerned that the events measured *in vitro* truly reflect the critical events that occur in the animal. The importance of this concern is illustrated by the effects of nitroaromatic carcinogens. The potent hepatocarcinogen technical-grade dinitrotoluene (DNT), as well as the individual DNT isomers, are weakly mutagenic in the *Salmonella* mutation assay without the need for an added metabolic activation system (Couch et al., 1981). No activity is seen, however, in cell culture assays, including those of metabolically competent primary hepatocytes (Bermudez et al., 1979). The weak activity in bacteria bears no resemblance to the complex pattern of activation in the whole animal, which involves the enterohepatic circulation and sequential steps of metabolism by the liver, gut flora, and the liver once again (Rickert et al., 1984). Only when genetic toxicity,

forced cell proliferation, and promotion are measured in the whole animal do the results correlate with the striking differences in carcinogenic potency of the various isomers and the sex-specific susceptibility to the carcinogenic action of DNT (Rickert et al., 1984). When conducting short-term tests to determine the potential carcinogenicity of nitroaromatic compounds, it is mandatory to examine the effects in the whole animal, because the presence of gut flora is obligatory for the metabolic activation of this class of chemicals (Doolittle et al., 1983; Mirsalis et al., 1982).

Germ Cell Mutagenesis

Genetic toxicants must be regarded as potential germ cell mutagens and, thus, as threatening irreversible damage to the human gene pool. Although there is as yet no documented case of chemically induced heritable genetic disease in humans (Mohrenweiser and Neel, 1982), epidemiological and animal studies suggest that such an association may exist (Strobino et al., 1978). This potential association is of particular concern when chemical exposure occurs through contaminated drinking water, since exposure may be chronic throughout the reproductive years.

Systems for evaluating human germ cell mutagens are evolving. Cigarette smoke is a potent human carcinogen and is the most important factor in more than 120,000 lung cancer deaths annually in the United States (Cairns, 1975; Mommsen and Aagaard, 1983; Silverberg, 1984). It contains so many mutagens that smokers have mutagenic urine (DeMarini, 1983; Yamasaki and Ames, 1977). There is also an increase in morphologically abnormal sperm in smokers, and smoking during pregnancy increases the incidence of spontaneous abortion (Evans, 1982; Kline et al., 1977). Thus, there is reason to suspect that cigarette smoke may be a germ cell mutagen. Nonetheless, there is as yet no evidence directly linking the induction of heritable mutations in humans to cigarette smoking (Bridges et al., 1979). Whether these negative results reflect the inadequacy of current germ cell mutagenicity assays or the relative mutagenic potency of cigarette smoke in germ cells is not clear.

One of the few tests that can be conducted in humans is the sperm morphology assay, which at least provides an indication that the parent compound or its metabolites reach the testes (Wyrobek et al., 1982). The value of this assay is limited because induction of abnormal sperm morphology may not be related to genetic damage in the exposed male. Biochemical assays for human gene mutation (Mohrenweiser and Neel, 1982) show promise as mutational screening techniques, but they also require precise assessment of the background germinal mutation rate. Although these biochemical assays are of little use in measuring mutational events in germ cells of exposed individuals, they do provide information

on mutation rates in exposed populations. There is no effective assay for measuring chemically induced mutations or even chemically induced DNA damage in germ cells of human females.

STRUCTURE-ACTIVITY RELATIONSHIPS

An important consideration in the evaluation of a chemical for potential carcinogenicity is that of structure-activity relationships with other chemicals in the same class. Carcinogenic potency and target organ specificity can sometimes be predicted from such relationships.

A number of methods have been developed to relate structure with activity; one of the best known is the Hansch-Taft relationship. Quantitative Hansch-Taft relationships have been established for several classes of chemicals, based on water-hexane partition coefficients, electronic factors (Taft α^* values), which are believed to provide an indication of the electron-donating or-withdrawing properties of substituents present on a methylene group bound to a reactive center (Gould, 1959; Hansch, 1969), and dose-response data for chemicals within the class for which such data exist. These relationships can be used to predict the carcinogenicity of other chemicals within the class. For example, a Hansch-Taft relationship has been established for the carcinogenicity of nitrosamine (Wishnok et al., 1978). This was based on data for 60 nitrosamines that had been tested in rats (Druckrey et al., 1967). The relationship for nitrosamines was extended to include target organ specificity, revealing that specificity is directly associated with molecular structure and a complex interplay between the parent molecule and its metabolites with the exposed organism (Edelman et al., 1980). Structure-activity relationships have also been established for groups of promoters such as anthralin derivatives (Van Duuren et al., 1978) and phenolic compounds (Boutwell and Bosch, 1959).

RISK ASSESSMENT

Risk assessments for carcinogenesis reflect assumptions about the underlying mechanisms of the disease. Considerable data from studies in animals and humans support the multistage concept of chemical carcinogenesis. Classic noncancer toxicology assumes that there is a threshold dose for toxic effects, and that below the threshold dose no ill effects will or can occur. Sound biological arguments support this assumption. However, cancer is a disease originating at the molecular level, involving unrepaired or ill-repaired damage to cellular genetic material (DNA) (Hattis and Ashford, 1982). Consequently, the insult by an initiating xenobiotic compound is believed to add to an already on-going natural process, so

that a threshold for cancer induction by a xenobiotic compound does not exist.

Most laboratory evidence indicates that promoters need to be administered continuously, or in large doses, implying that promoters should have discoverable thresholds (Hoel et al. 1983). A difficulty arises when the same chemical appears to behave both as an initiator with respect to one tissue and a promoter with respect to another tissue, raising questions of the importance not of the chemical but of the chemical-tissue interaction. Examples of this chemical-tissue interaction are asbestos, which is probably a promoter with respect to lung cancer and an initiator with respect to mesothelioma in humans (NRC, 1984), and 2-AAF, which has promoting activity in both bladder and liver carcinogenesis in laboratory animals (Hughes et al., 1983).

In assessing cancer risk, projections from high animal doses to low-dose exposures of humans are driven more by the choice of the extrapolation model than by the available data (Whittemore, 1980). Thus, further research, development, and validation of more sophisticated models are required. For example, models are needed to distinguish between responses to lifetime exposures at relatively uniform increments of dose and those resulting from more erratic accumulation of doses that range from high to no dose for varying periods of time at irregular intervals. Studies of cohorts exposed to radiation or asbestos indicate that dose rate needs to be included in future dose-response models.

When assessing the risk of exposure to carcinogens in drinking water, one should consider information on metabolism and pharmacokinetics. This information may be used to ascertain the validity of a linear dose-response assumption, which can misstate the risk because of inadequate information on metabolism. Determination of the biologically effective dose delivered to the target molecule (such as DNA) will allow a more realistic evaluation of risk.

Because of the multistage nature of carcinogenesis, the concept of threshold dose, or no-observed-effect level, is not applicable as it is for other toxic effects. Instead, the multistage, no-threshold, low-dose extrapolation model described in Chapter 8 can be used to predict a finite excess risk of cancer at any low carcinogen dose. However, no model can unambiguously predict low-dose effects from data obtained at high doses. A major reason for this ambiguity is that at high doses, the behavior of an enzyme system responsible for the activation or detoxication of a carcinogen may often be governed by a set of parameters different from those that apply at low doses.

Chapter 8 contains a more detailed discussion of risk assessment for carcinogens.

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6

Dose-Route Extrapolations: Using Inhalation Toxicity Data to Set Drinking Water Limits

This chapter presents a pharmacokinetic model for the disposition of volatile organic compounds (VOCs) and their metabolites in biological systems. It is intended to allow extrapolation from the inhalation dose route in animals to the ingestion route in humans and may play a useful role in the overall risk assessment for such compounds.

VOCs are present in many drinking water supplies throughout the United States (Brass et al., 1977; Symons et al., 1975). Chloroform and certain other trihalomethanes are believed to form during chlorination processes, in which chlorine reacts with humic acids and other organic materials in water supplies. Other VOCs are emitted into drinking water during manufacturing and other activities involving the use of chemicals. Waterborne VOC concentrations typically vary from several micrograms per liter (ppb) to a few milligrams per liter (ppm). Higher concentrations are found in river water downstream from chemical spills and in well water near pollutant point sources such as hazardous-waste disposal sites.

Drinking water standards have been established to protect people from potentially adverse health effects associated with ingestion of contaminated waters. Such effects must often be determined by conducting toxicity studies in laboratory animals and in some way extrapolating these results to predict toxic effects in exposed humans. The best toxicity data base from animal studies for predicting risk to humans would be experiments in which VOCs were provided to laboratory animals in their drinking water. Two constraints make this difficult, however. First, achievable concentrations are small because the water solubility of most VOCs is

limited (i.e., their water:air partition coefficients are small), and second, rats consume only about 30 ml of water daily.

TABLE 6-1 Physiological Constants[a] Used in Kinetic Modeling of Rats and Humans

Parameter		Rat	Human
(bw)	Body weight (kg)	0.3	70
(Q_p)	Alveolar ventilation (liters/hr)[b]	5.74	325
(Q_c)	Cardiac output (liters/hr)[b]	5.74	325
(k_a)	Absorption rate constant[c]	5.0	5.0
(V_w)	Water intake (liters/day)	0.030	2.0
Blood flow rates (portion of total)			
(Q_l)	Liver	0.25	0.25
(Q_f)	Fat	0.09	0.09
(Q_m)	Muscle	0.19	0.19
(Q_r)	Richly perfused tissue (viscera)	0.47	0.47
Tissue group volumes (portion of body weight)			
(V_l)	Liver	0.041	0.025
(V_f)	Fat	0.090	0.200
(V_m)	Muscle	0.720	0.610
(V_r)	Richly perfused tissue (viscera)	0.059	0.075

[a] Values similar to those used by Ramsey and Andersen (1984), with minor changes in alveolar ventilation (increased from 4.5 to 5.7 liters/hr) and percentage of cardiac output that perfuses the liver (decreased from 37% to 25%).

[b] Calculated from an allometric relationship: $y = 14(bw)^{0.74}$.

[c] Absorption rate was assumed to be invariant with body weight. Uptake can be modeled as arising from a blood perfusion rate into a given tissue volume, in which case the human rate constant would be:

$$ka_2 = ka_1\left(\frac{bw_2}{bw_1}\right)^{1/3}$$

In industry and commerce, humans are exposed to VOCs usually by inhalation and to a lesser extent by skin contact. Over the years, the toxicity of many VOCs has been examined through subchronic or chronic inhalation studies in laboratory animals. These are often conducted at high vapor concentrations and can generally be designed to ensure overt toxicity in animals exposed at the highest concentration. In addition to high airborne vapor concentrations, the inhalation dose rate is much higher than that achieved in a drinking water study, because the daily alveolar ventilation (approximately 140,000 ml; see Table 6-1) is much greater than daily water consumption. For many VOCs, inhalation studies provide the only data from which drinking water standards can be derived. Using such results to predict the risks associated with human consumption of contam

inated drinking water therefore requires both a dose-route and interspecies extrapolation of toxicity data.

Even in a prospective sense, inhalation may be a very good surrogate route, and perhaps the only one available for estimating expected toxicity of contaminants in drinking water. Inhalation studies, in which a chemical is absorbed at a fairly uniform rate over a specified exposure period, are not very different from drinking water studies, in which a chemical is absorbed at a variable, moderate rate throughout a day. Well-designed, properly conducted inhalation toxicity studies may, in fact, provide an excellent experimental model for deriving drinking water standards for a variety of volatile chemicals. The differences between these two exposure routes are not insignificant, however, as discussed in this chapter and further developed in the two appendixes to this chapter.

BACKGROUND

There has been no consensus on how or even whether inhalation studies could be used to establish drinking water standards. Various groups responsible for assessing chemical hazards in drinking water have handled the problem differently. Some have declined to use inhalation data, reasoning that the target organ, disposition, and ensuing toxic effects of inhaled chemicals may differ markedly from that which occurs when the agents are ingested. Others have argued that whereas results of inhalation studies may be of value from a qualitative standpoint, inhalation data are likely to be of limited utility quantitatively in predicting consequences of the ingestion of chemicals.

Stokinger and Woodward (1958) advocated use of threshold limit values (TLVs) to set water standards. They proposed a direct conversion from uptake at the TLV concentration to an acceptable waterborne concentration, assuming that humans ingest 2 liters of drinking water per day. Their approach also attempted to take into account the proportion of chemical absorbed by either route of administration. The general calculation for a water standard based on a TLV would be:

$$\frac{\text{TLV (in mg/m}^3) \times 10\ \text{m}^3\text{/day} \times \text{proportion absorbed in inhalation}}{\text{proportion absorbed orally} \times 2\ \text{liters/day} \times \text{safety factor}} = \text{mg/liter}, \quad (1)$$

where 10 m^3/day represents an estimate of a moderately active employee's total ventilation during an 8-hour work shift. Since TLVs apply to human occupational exposure, no additional safety factor was proposed for general use. Stokinger and Woodward (1958) did provide estimates for both inhalation and ingestion absorption factors.

A variation of this general approach has been used in most attempts to establish drinking water standards for humans from inhalation data based

on animals. When toxicity data from animal studies are used, the TLV is replaced by the highest no-adverse-effect concentration in the inhalation experiments in animals. The safety factor, varying from 10 to 1,000, depends on the nature of the inhalation study, the severity of the response, and the presence or absence of a history of human exposure to the test chemical. This approach has recently been used to derive adjusted acceptable daily intake (AADI) values for trichloroethylene, perchloroethylene, and methylchloroform (EPA, 1984). These calculations represent an attempt to conduct a direct route-to-route extrapolation based on delivered dose, given the most fundamental assumption that the relationship between delivered dose (milligrams absorbed per day) and the dose received by target organs is independent of the route of exposure and the species. There is no obvious toxicological or pharmacokinetic basis for such an assumption.

Routes of Exposure

The route of exposure significantly influences the quantity of a chemical that reaches a particular target tissue, the length of time it takes to get there, and the degree and duration of effect. Volatile organic compounds are readily absorbed by the lung because of its large surface area, intimate alveolar-capillary interfaces, and high rate of blood perfusion. VOCs are small, uncharged, lipophilic molecules that are quickly absorbed from the alveolus into the systemic circulation (Åstrand, 1975). Although the uptake of inhaled volatile organics varies with the exposure concentration and the chemical, in humans it typically ranges from 25% to 75% (Åstrand, 1975). The uptake in the first few breaths is related to blood solubility and ventilation: perfusion ratios. The proportion retained was derived by Haggard (1924a,b,c) and, in the terminology used in this chapter (see Appendix B), is:

$$\text{Fraction retained} = \frac{Q_c P_b}{Q_c P_b + Q_p} \times \frac{Q_t - Q_{ds}}{Q_t} . \qquad (2)$$

This relationship also applies throughout exposures to soluble chemicals (i.e., P_b of about 5 or larger) that are well metabolized at the exposure concentrations used. Compounds absorbed into the pulmonary circulation are transported via the arterial blood directly to potential target organs.

The gastrointestinal (GI) tract is also well suited for the absorption of volatile organic compounds, although its total surface area is less than that of the lung and it receives only about 4% of the cardiac output that perfuses the lung. The presence of food in the GI tract delays absorption and reduces the availability of orally administered halocarbons (Counts et

al., 1982; D'Souza et al., 1985). Compounds absorbed from the GI tract into the bloodstream are also subject to first-pass elimination by the liver and lungs (i.e., reduction of blood concentrations before the chemical reaches the systemic circulation). Although Andersen (1981a) and his coworkers (Andersen et al., 1980) evaluated hepatic metabolism of inhaled halocarbons and estimated the metabolism of inhaled styrene during a single pass through the systemic circulation (Andersen et al., 1984), no one has yet directly measured first-pass hepatic elimination of orally administered volatile organics. Nevertheless, there is no reason why the liver should not metabolize ingested VOCs as efficiently as it does those that are inhaled (i.e., the liver removes virtually all the VOC presented to it by the blood when inhaled concentrations are not high enough to saturate metabolism). For most VOCs encountered in the environment, a substantial proportion of a low oral dose will be removed by the liver before it reaches the arterial circulation. Andersen (1981 a) compiled a list of VOCs that would be expected to demonstrate substantial first-pass extraction by the liver when present in portal blood at concentrations below saturating levels for enzymes. For organics that do undergo extensive first-pass hepatic extraction, the liver will receive a higher dose and may therefore be injured more by an oral dose than it would by a comparable inhalation exposure. This process is expected with well-metabolized halocarbons that are metabolically activated to cytotoxicants.

Ingested VOCs will also be subject to a first-pass pulmonary exhalation. The extent of this effect is determined by the blood:gas partition coefficient of the volatile chemical. It is relatively easy to use an analysis similar to that of Haggard (1924 a,b,c) or Andersen (1981a) to derive a relationship for the proportion of circulating volatile compound that will be eliminated in a single pass through the lung when inhaled air contains none of the test chemical:

$$\text{Fraction exhaled} = \frac{Q_p}{Q_c P_b + Q_p}, \qquad (3)$$

which is about $1/(1 + P_b)$, since cardiac output (Q_c) and alveolar ventilation (Q_p) are approximately equal. Acting sequentially, presystemic elimination by the liver and lungs could significantly diminish the amount of VOC that reaches the bloodstream after low-dose oral ingestion.

Dose-Response Curves

For all routes of administration, VOCs are absorbed by complex processes involving passage from an exterior compartment through a series of cells, tissues, and organs until some portion of the original dose reaches

the systemic arterial circulation. The toxic chemical can then be distributed to various organs remote from the site of entry. Frequently, toxicologists know what organs are affected but lack detailed knowledge of the molecular mechanism(s) of toxicity. Despite the inadequacy of the data base on such mechanisms, toxicologists and regulators must make prudent, well-documented decisions about expected risks to humans based on data derived largely from studies in animals.

The linchpin of any risk assessment is determination of a dose-(or concentration-) response curve under specified experimental conditions. The dose-response curve can either assess a virtual no-effect or minimal-effect level (for nongenotoxic or noncarcinogenic effects) or support extrapolation to expected low-level incidence (for genotoxic or carcinogenic effects). These curves should, of course, be based on an estimate of target-tissue dose, which, however, may not be related in any simple manner to applied dose (Andersen, 1981b; Gehring et al., 1978). Applied dose for inhalation or drinking water studies is normally expressed as ppm or mg/m^3 (in air) or as mg/liter (in drinking water). On the other hand, target-tissue dose, also called internal dose (O'Flaherty, 1985), might be the area under the blood or tissue concentration-time curve, the peak tissue concentration, the total amount metabolized, the area under a tissue metabolite concentration curve, or some other appropriate measure of target-tissue exposure.

The proper measure of target-tissue dose must be selected carefully. Consideration must be given to whether the toxic chemical is presumed to be the parent compound or a metabolite (i.e., whether the dose-response curve is consistent with parent chemical toxicity or more complex), and to the relative reactivity of the toxic metabolites (i.e., whether they are stable or very short-lived). The pharmacokinetic models developed for risk assessment extrapolations must have sufficient biological and biochemical detail to describe these various measures of target-tissue dose. In addition, the toxicologist and regulator should be aware of the critical distinction between target-tissue dose and applied dose and should understand how a proper measure of the former can influence risk assessment computations.

Measurement of Tissue Dose

Selection of the appropriate measure of target-tissue dose depends on the nature of the chemical component that is associated with the toxic effects. Toxic chemicals can interfere with normal physiological and cellular function through a variety of biochemical mechanisms. For the VOCs of interest, toxic effects are most frequently associated with stable metabolites or with reactive metabolites that bind covalently and essentially

irreversibly with important cellular macromolecules. Their toxicity is not usually simply associated with concentrations of a parent chemical but, rather, with amounts or concentrations of the toxic metabolites. Some toxicants, such as curare, reversibly bind to endogenous receptors. Their toxicity depends on their tissue concentration, receptor binding affinity, and receptor concentration. This is more of a classical pharmacological interaction. Among environmentally important chemicals, the prime example of a pharmacologically acting toxicant that has very marked interspecies differences in toxic potency is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Murphy, 1980, p. 389).

For chemicals with pharmacological activity, the proper measure of target-tissue dose would be the tissue concentration divided by some measure of the receptor binding constant for the toxic chemical. High receptor binding affinity arises from strong noncovalent interactions between specific portions of the protein binding surface and the chemical structure of the toxicant. Interspecies differences arise when portions of the binding site are not conserved from species to species, in which case both affinity and toxicity can become markedly species-dependent. Fortunately, a pharmacological mechanism of action, with its potential for significant species differences, is not frequently observed with the chemicals usually found in drinking water.

Much more commonly, toxicity is caused by the intrinsic chemical reactivity of foreign compounds or their metabolites. In these cases, there is a direct reaction between critical cellular constituents and reactive chemicals, and the reactions leading to toxicity are expected to proceed according to second-order rate equations. Extreme differences in toxicity among species are not expected under these conditions, since tissues and organs are very similar in content and function among species, and the reactivity of their cellular constituents should be similar when exposed to any particularly reactive toxic chemical. The second-order rate equation for loss of a critical cellular component, C_{crit}, as it reacts with a toxic chemical, T, is:

$$\frac{dC_{crit}}{dt} = -kC_{crit}T. \qquad (4)$$

In a simplistic way, the rate equation can be rewritten to express the dependence of the reduction in cellular components on the time integral of the toxic chemical, i.e., the area under the tissue-concentration curve (AUTC):

$$\int \frac{dC_{crit}}{C_{crit}} = -k\int T dt = -k(\text{AUTC}); \text{ thus,} \qquad (5)$$

$$\ln \frac{(C_{crit})_t}{(C_{crit})_0} = -k(\text{AUTC}). \qquad (6)$$

In this case, with a directly reactive species, the loss of critical sites is related to integrated tissue dose.

Frequently, metabolites are responsible for toxicity. Metabolite formation may occur through first-order processes or capacity-limited, enzyme-catalyzed ones; the latter are more usual. When the reactive toxic chemical is short-lived, the appropriate measure of tissue dose is the ratio of the amount of toxicant produced divided by the volume of tissue in which the reaction takes place (V_r). For an enzyme-mediated formation of reactive metabolite, *TM**,

$$\frac{d(TM^*)}{dt} = \left(\frac{V_m \times T}{K_m + T}\right)\frac{1}{V_r}. \qquad (7)$$

By analogy with Equation 5, the burden of metabolite per unit volume of tissue becomes:

$$\int d(TM^*) = \frac{1}{V_r}\int \frac{V_m \times T}{K_m + T}\,dt = \frac{\text{AURMC}}{V_r} = \text{AMEFF}, \qquad (8)$$

where the AURMC (area under the rate-of-metabolism curve) could also have been developed for first-order production of metabolites. This equation does not describe a true concentration; it describes an effective concentration (AMEFF; see Appendix B of this chapter) that would have been achieved by the production of a specified amount of reactive metabolite, *TM**, in a particular volume, V_r. The concept of correlating toxicity with the area under the rate-of-metabolism curve was previously developed by Andersen (1981a). Further elaboration of the concept to include the effective volume of reaction is necessary to extend its use to interspecies comparisons (see the section entitled Interspecies Extrapolation later in this chapter).

The toxicity of reactive intermediates is related to the portion of the intermediate that ultimately reacts with the critical cellular targets for both carcinogenic and noncarcinogenic end points. In chemical terms, this is illustrated by a scheme in which there are several pathways for consumption of the reactive metabolite. Each pathway has a first-order rate constant, the sum of which is Σk_i. There is also a pathway that leads to reaction with critical cellular components; its rate constant is k_r. Therefore, the relative concentration of the reactive intermediate, *TM**, involved in toxic interactions is:

$$C_{TM^*} = \frac{k_r}{\Sigma k_i}(\text{AMEFF}). \qquad (9)$$

This relationship is more important for interspecies extrapolation, i.e., when $k_r/\Sigma k_i$ might vary between species, than for a dose-route extrapolation in a given species, where it is assumed that the ratio does not change very much with concentration of the parent VOC.

In addition to situations in which the parent chemical or short-lived metabolites are the reactive toxic chemical, other, more complex patterns may exist when stable metabolites are the reactive chemicals associated with toxicity. An example is the neurotoxicity associated with inhalation of *n*-hexane or methyl *n*-butyl ketone, which is due ultimately to circulating concentrations of the metabolite 2,5-hexanedione (DiVincenzo et al., 1978). Although the relationship between concentration of precursor and formation of metabolite may be complicated (Clewell et al., 1984), the appropriate target-tissue dose on which risk assessment would be based would be similar to that expressed in Equation 7, except that AUTC would be replaced by area under the target-tissue metabolite-concentration curve (i.e., AUTMC).

In a simple system involving a stable toxic metabolite *TM* formed from a precursor *T* at a concentration C_T, in which a constant fraction of the metabolic pathway is converted to the metabolite of interest, the metabolite is retained in a specified volume of distribution V_{TM} and eliminated by a first-order process with rate constant k_{TM}. The mass-balance equation for the metabolite in its volume of distribution is:

$$\frac{V_{TM}dC_{TM}}{dt} = \frac{k_r}{\Sigma k_i}\left(\frac{V_m \times C_T}{K_m + C_T}\right) - k_{TM}V_{TM}C_{TM}. \qquad (10)$$

The AUTMC then is related to the metabolism rate of the parent chemical, but the dependence cannot be expressed as a simple analytical expression. The expected steady-state concentration of the metabolite can be written in a simple form:

$$(C_{TM})_{s\text{-}s} = \frac{k_r/\Sigma k_i\left(\frac{V_m \times C_T}{K_m + C_T}\right)}{k_{TM} \times V_{TM}}. \qquad (11)$$

The denominator in Equation 11 is identical to the compartmental clearance Cl_{TM} of the stable metabolite.

PHARMACOKINETIC MODELS

An attractive and potentially economical approach to risk-assessment extrapolations is the development of predictive physiologically based pharmacokinetic (PB-PK) models for the disposition of volatile organic compounds and their metabolites in biological systems. These models are based

on the known physiology of the experimental animals and on known or easily measurable physical, chemical, and biochemical properties of the VOCs. PB-PK models predict general behavior of toxicants, and models successful with one chemical are usually easily adapted to describe the kinetic behavior of many others.

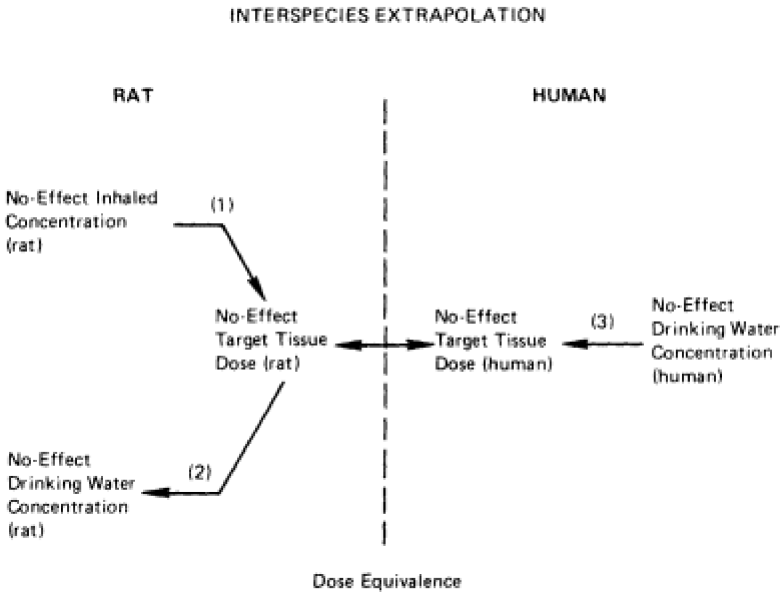

Figure 6-1
Physiologically based pharmacokinetic extrapolations involved when results of inhalation studies in laboratory animals are used to establish drinking water standards for humans.

This chapter demonstrates a possible PB-PK approach for conducting dose-route and interspecies extrapolation. The underlying premise in a pharmacokinetic risk assessment is that there is a coherent dose-effect curve relating the incidence or severity of response to an appropriate measure of target-tissue dose. Pharmacokinetic risk-assessment extrapolations are conducted by assuming that a particular target-tissue dose achieved by one route of exposure in a particular species will have the same biological effect as an equivalent target-tissue dose achieved by another route of exposure or in some other species. The dose-route extrapolation between inhalation and oral ingestion is based on an understanding of the physiological differences in the processes of chemical absorption by these two exposure routes (see Appendix A of this chapter). Interspecies extrapolations are conducted by determining the drinking water concentration that would lead to the same target-tissue dose in humans achieved at the no-effect (Figure 6-1) or minimal-effect concentration in

the inhalation studies in animals. In Step 1 of Figure 6-1, the target-tissue dose is estimated in a test species based on the no-effect inhalation exposure concentration. Step 2 determines the drinking water concentration associated with an equivalent target-tissue dose in the test species. Step 3 determines the drinking water concentration that produces a target-tissue dose in humans equal to the no-effect target-tissue dose from the inhalation study in the test species. In this chapter, the two extrapolation processes are handled separately, but in practice they would be combined and the dose-route extrapolation for test animals would not be conducted as an independent step.

To be useful in risk-assessment extrapolations, the pharmacokinetic models used to estimate tissue doses must have sufficient biological detail to describe the differences in absorption by the two exposure routes and to account for structural and physiological differences between various mammalian species. Some PB-PK models do contain the anatomical and biochemical detail for such extrapolations (see, for example, Andersen, 198 la; Fiserova-Bergerova, 1976; Gerlowski and Jain, 1983; Himmelstein and Lutz, 1979; Ramsey and Andersen, 1984). These models, which are particularly easy to develop for a variety of VOCs, are nothing more than a series of mass-balance differential equations describing the movement of a chemical through a number of tissue compartments within the body.

In the past, solving such equations was difficult and time-consuming, and there was substantial reluctance to use these descriptions unless the system of equations had a formal, analytical solution (Gibaldi and Perrier, 1975; Teorell, 1937a,b). With modern digital computers and improved techniques for numerical integration, however, the solution of even relatively large sets of differential equations is easy. Furthermore, many physiologically based models that are useful for risk assessment with VOCs require only 4 to 10 ordinary differential equations and can be solved rapidly with microcomputer-based procedures.

Data-Based Inhalation Models

A typical data-based compartmental model attempts to relate the blood or tissue concentration profile of a parent chemical or metabolite to the administered dose of parent chemical using a set of mathematical equations. The parameters for these equations are determined from experiments that follow the time course of the chemical in body fluids, usually blood, and occasionally in specific tissues. A simple data-based inhalation model (Figure 6-2) was developed to examine the pharmacokinetics of inhaled styrene (Ramsey and Young, 1978). This model was used by Young et al. (1979) to describe kinetic behavior in rats and by Ramsey et al. (1980) to describe kinetic behavior in humans. It consisted of a central com

partment in equilibrium with the blood and a peripheral compartment with a concentration related to the central compartment by rate constants in each direction. The time course of a chemical in each compartment is described by a single, mass-balance differential equation. In applying these data-based compartmental models, time-course concentration curves are first determined experimentally. Then compartment volumes and rate constants are adjusted to give curves that fit the experimental data. These models can be used for interpolation and limited extrapolation in the same animal species.

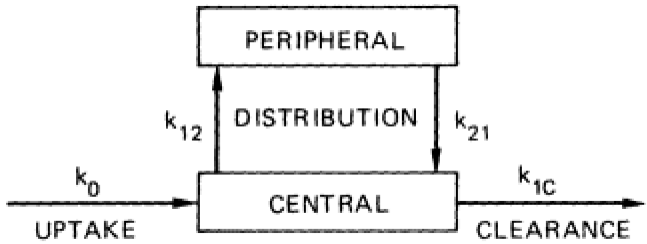

Figure 6-2
A conventional, data-based compartmental model used for describing inhalation of styrene in rats and humans.

The two equations for the rate at which the amount of chemical in each compartment changes are:

$$\frac{dAmt_1}{dt} = \frac{V_1 dC_1}{dt} = k_0 - k_{1C}V_1C_1 - k_{12}V_1C_1 + k_{21}V_2C_2, \text{ and} \quad (12)$$

$$\frac{dAmt_2}{dt} = \frac{V_2 dC_2}{dt} = k_{12}V_1C_1 - k_{21}V_2C_2. \quad (13)$$

In these mass-balance equations, $k_{12}$and $k_{21}$are intercompartmental transfer rate constants, $V_1$and V_2 are volumes of the two compartments, k_0 is a zero-order input rate, and k_{1c} is a first-order elimination rate constant. Reitz et al. (1982) used a similar model to describe ethylene dichloride inhalation kinetics but replaced k1c with a term for saturable metabolic clearance when extending this model to examine the kinetic behavior of oral doses. Historically, these data-based descriptions have been very popular because their simple form permits their exact solution as a sum of exponential terms. The existence of a direct solution avoids using more recently developed numerical integration techniques.

Physiologically Based Models

PB-PK models differ from conventional data-based compartmental approaches in that they are based largely on the actual physiology of the

test species. Instead of compartments whose characteristics are defined by experimental time-course data, these models are based on compartments representing organs and tissue groups with realistic weights and blood flows derived from the literature (Bischoff and Brown, 1966; Himmelstein and Lutz, 1979). Because an accurate anatomical description is used along with measured tissue solubilities, the intercompartmental rate constants are now defined by blood flows, tissue partition coefficients, and tissue volumes. A model derived from this approach can predict the qualitative behavior of the experimental time course without being based directly on the time-course data themselves. Refinement of physiological models to incorporate additional insights gained from direct comparison with experimental data yields validated physiological descriptions that can be used for quantitative extrapolation well beyond the range of experimental conditions.

In a physiologically based model, each tissue (or tissue group) is still described by a mass-balance differential equation, but the individual rate constants are related to blood flow, tissue solubility, and volumes of the particular organs. The physiological model used by Ramsey and Andersen (1984) to examine styrene kinetics is shown in Figure 6-3. In this physiological description, the mass-balance equation for the liver was:

$$\frac{dAmt_l}{dt} = \frac{V_l dC_l}{dt} = Q_l C_a - Q_l C_{v,l} - \frac{V_{max} C_{v,l}}{K_m + C_{v,l}} . \qquad (14)$$

This mathematical shorthand indicates that the amount of chemical in the liver is increased by a chemical entering in the arterial blood ($Q_l C_a$) and diminished by that leaving via the liver venous blood ($Q_l C_{v,l}$) and by metabolism $(V_{max} C_{v,l})/(K_m + C_{v,l})$.

The physiological model used to examine styrene was based on the assumption that diffusion of the chemical from blood into tissues is much faster than tissue blood flow and therefore was called flow-limited. In addition, in the flow-limited situation, the concentration in effluent tissue blood is in equilibrium with the tissue concentration as dictated by the tissue:blood partition coefficient P_i. The mass balance differential equation for the liver (l) then becomes:

$$\frac{dAmt_l}{dt} = \frac{V_l dC_l}{dt} = Q_l C_a - Q_l \frac{C_l}{P_l} - \frac{V_{max}(C_l/P_l)}{K_m + C_l/P_l} . \qquad (15)$$

The rate constants for tissue uptake and elimination are now defined by physiological parameters and partition coefficients. These are Q_l / V_l and $Q_l / (V_l \times P_l)$, respectively. Kinetic constants of the metabolizing enzyme are V_{max} (mg/hr) and K_m (mg/liter). In this model, metabolism only occurs in the liver. Mass-balance equations for other tissue groups only have the

equivalent of the first two terms in Equation 15. Diffusion-limited models are used when chemical diffusion from blood into tissue is slower than tissue blood flow (Lutz et al., 1980). Diffusion-limited models have been used with Kepone (Bungay et al., 1981), polybrominated biphenyls (Tuey and Matthews, 1980), and tetrachlorodibenzofuran (King et al., 1983), but they are unnecessary for describing the biological behavior of small lipophilic chemicals that readily diffuse through biological membranes.

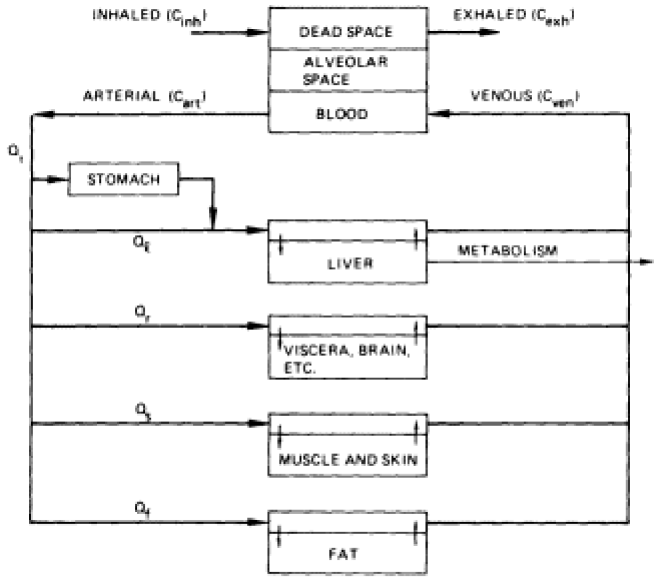

Figure 6-3
Schematic diagram of a physiologically based pharmacokinetic model for VOC inhalation, used to describe styrene kinetics. Organs or groups of organs are defined with respect to their blood flows (see Appendix B of this chapter for variables). Adapted from Ramsey and Andersen, 1984.

The chief advantage of a physiologically based model is its much greater predictive power than that of conventional data-based models. Because fundamental metabolic parameters are used in this approach, dose extrapolation over a known range of doses, including those where saturation of metabolism occurs, is possible. And because the physiological description of the test species is used in the simulation, the behavior of the chemical can be predicted in a different species simply by replacing the appropriate physiological constants (Dedrick, 1973). Similarly, the behavior for a different route of administration can be determined by adding equations that describe the nature of the new input function. Use of the kinetic model for extrapolation from one exposure scenario to another is

relatively easy and only requires a little ingenuity in writing the equations for this dosing regimen. Finally, because measured physical-chemical and biochemical parameters are used, the behavior of a different chemical can quickly be estimated by the same physiological model after determining the appropriate biochemical constants.

The trade-off is the need for an increased number of parameters and equations and for a numerical technique to solve the resulting equations. Values for many parameters, especially for physiological constants, are provided in the literature, however, and several techniques have been developed for the rapid determination of such compound-specific parameters as tissue solubilities and kinetic constants for metabolism. The development of physiological models may even be based entirely on data obtained from *in vitro* or very simple *in vivo* studies. For many VOCs, tissue partition coefficients can be determined by a simple *in vitro* technique called vial equilibration. The chemical is added to a vial containing a liquid, and then the concentration of the chemical in the head space above the liquid is determined and used to calculate the partition coefficient of the chemical (Sato and Nakajima, 1979a). A variation of this method has also been used to estimate metabolic coefficients (Sato and Nakajima, 1979b). Alternatively, there are rapid *in vivo* approaches for determining metabolic constants based either on steady-state conditions (Andersen et al., 1984) or on gas uptake experiments (Andersen et al., 1980; Filser and Bolt, 1979; Gargas et al., 1986). These experimentally accessible constants for tissue solubility and VOC metabolism, together with the general physiological parameters, provide a model of parent chemical behavior and rate of metabolism that can predict parent chemical kinetic behavior at various concentrations, for various dose routes, in a variety of species, and with any number of exposure scenarios.

An Inhalation Model

The absorption, distribution, and elimination of gases and vapors have been extensively studied to understand the time course of action of volatile anesthetics. In the early part of this century, Haggard (1924a,b,c) described the role that blood flow, pulmonary ventilation, and solubility coefficients play in determining the kinetics of the uptake of diethyl ether. Over the intervening years, there have been other important contributions, which have provided general models for the physiological pharmacokinetics of volatile anesthetics (Fiserova-Bergerova et al., 1980; Kety, 1951; Mapleson, 1963; Riggs, 1963). More recently, the importance of understanding the role of metabolism in order to elucidate the mechanism of a chemical's toxicity has become more widely recognized. Physiological models for inhaled agents can now describe both the formation and con

sumption of the metabolites formed during and after exposure to volatile chemicals (Fiserova-Bergerova, 1984; Gargas and Andersen, 1985; Ramsey and Andersen, 1984).

Several basic assumptions are made in PB-PK models. For example, in the physiological models for pulmonary uptake, end alveolar air and arterial blood are assumed to be in equilibrium and arterial blood concentrations are equal to the end alveolar air concentrations times the blood:air partition coefficient (P_b). The total uptake of an inhaled chemical depends in a complex way on the blood:air partition coefficient, pulmonary alveolar ventilation (Q_p), cardiac output (Q_c), and the difference between arterial and venous blood concentrations (Andersen, 1981a). This difference in concentrations is related to the extent that the vapor undergoes systemic metabolism. The equation used to calculate the arterial blood concentration (C_{art}) is:

$$C_{art} = \frac{P_b(Q_p C_{inh} + Q_c C_{ven})}{P_b Q_c + Q_p}. \qquad (16)$$

This equation represents the steady-state solution of a mass-balance equation of the total lung compartment. Mixed venous blood concentration (C_{ven}) is the weighted average of the concentration from all the tissue groups:

$$C_{ven} = (\Sigma Q_i C_{v,i})/Q_c. \qquad (17)$$

These equations and four simultaneous, mass-balance differential equations for the four tissue groups constitute the basic physiological model for inhalation (Figure 6-3). The use of this inhalation model to analyze the pharmacokinetics of inhaled styrene in rats at concentrations from 80 to 1,200 ppm (344 to 5,160 mg/m^3) has been described in detail (Ramsey and Andersen, 1984). For styrene, the use of realistic metabolic constants for V_{max} and K_m (Andersen et al., 1984) enabled investigators to make both accurate predictions of kinetic nonlinearities in this concentration range and determinations of the general physiological factors that govern kinetic behavior of lipophilic, well-metabolized vapors.

An Oral Model

The physiological kinetic model used to study inhaled styrene was modified to examine styrene absorption after intubation of a single bolus dose in a saline vehicle. Withey (1976) studied the intragastric kinetics of styrene. Ramsey and Andersen (1984) fit these data with the inhalation model by varying a first-order uptake rate constant until the best representation of the data was obtained (Figure 6-4). In this description, the chemical was absorbed by a first-order process directly into the liver. The

best-fit absorption rate constant was 5.5/hr. The uptake of other chemicals—methylene chloride, trichloroethylene, 1,2-dichloroethane, 1,1-dichloroethylene, and vinyl chloride monomer (Withey, 1976; Withey et al., 1983)—also occurs very rapidly when they are instilled in the stomach in aqueous solution. When the published curves for the first four chemicals were analyzed with the styrene model, the best-fit uptake rate constant was found to be about 7/hr (Gargas and Andersen, Air Force Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio, personal communication, 1985).

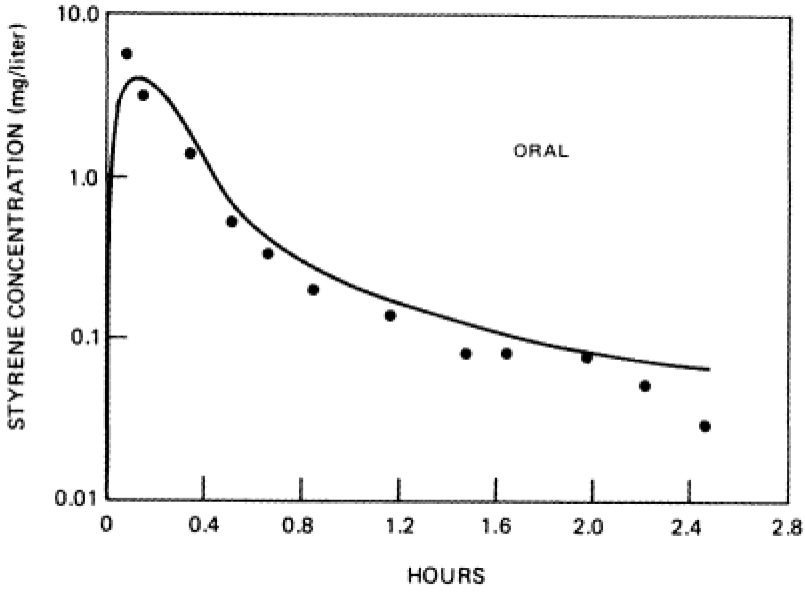

Figure 6-4
Styrene concentrations over time in mixed venous blood from a rat after a 9.3-mg/kg oral dose of styrene in aqueous solution, as determined by Withey (1976). Solid line is the model prediction with a first-order rate constant for absorption of 5.5/hr. Reproduced with permission from Ramsey and Andersen (1984).

To describe drinking water exposures accurately, the temporal patterns of water ingestion in the test species must be approximated by the input equations. Figure 6-5 shows the results of an analysis based on a 12-hour water intake by four different drinking patterns in the rat—a series of sips (equal amounts of water every 10 minutes, shown in the insert for the first 4 hours of the dosing period), two episodic consumption patterns

(equal amounts every 1 or 2 hours), and bolus intubation of the entire daily intake. The curves show the amount in the stomach compartment during a single 24-hour period. The VOC concentration in ingested water was 800 mg/liter, and the total water consumption was assumed to be 30 ml/day for a 300-g rat, as compared with 2 liters/day for a 70-kg human (see Table 6-1). This is equivalent to an allometric relationship of the following form:

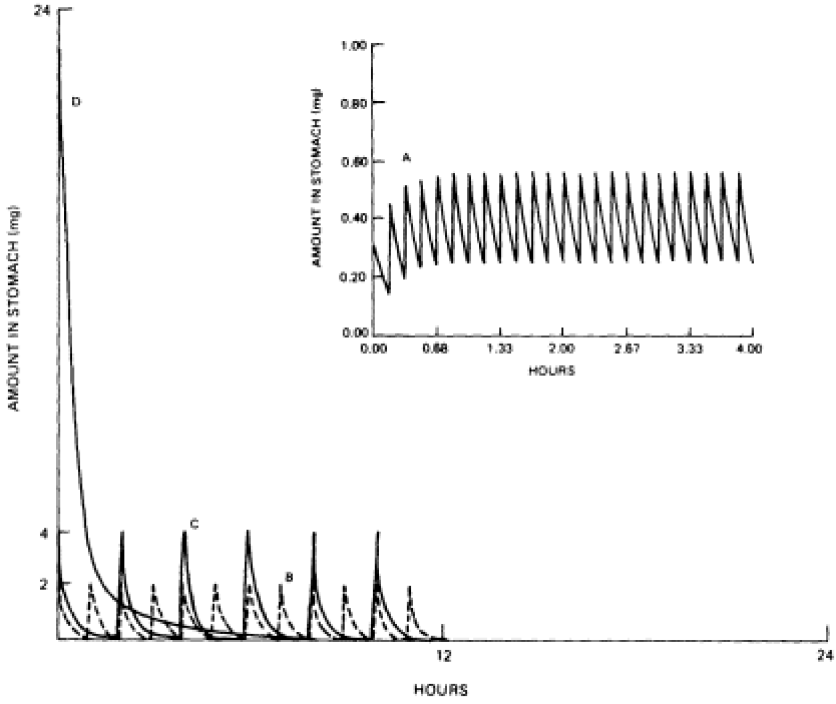

Figure 6-5
Multiple dosing. Computer-generated drawing. VOC presence in the stomach of every 10 minutes (sipping); B = Equal amounts every hour; C = Equal amounts every 2 hours; rats based on four drinking water consumption patterns: A = Approximately equal amounts D = Single bolus intubation.

$$y\ (\text{ml/day}) = 76(bw)^{0.77}. \qquad (18)$$

In this equation, bw represents body weight in kilograms and y represents intake per day.

Adolph (1949) published a similar equation for water intake based on an adult intake of 4.4 liters per day. When expressed in the same units, his equation is:

$$y\ (\text{ml/day}) = 104.8(bw)^{0.88}. \qquad (19)$$

TABLE 6-2 Partition Coefficients[a] and Metabolic Constants for Trichloroethylene (TCE) and Benzene (BEN) in Rats

Parameter	TCE	BEN
Saline:air partition	1.3	2.8
Blood:air partition	22.0	18.0
Fat:blood partition	26.0	28.0
Liver:blood partition	1.3	1.0
Muscle:blood partition	0.5	0.6
V_{maxc}[b] (mg/kg)	12.0	3.3
K_m (mg/liter)	0.25	0.25
Intrinsic clearance (liter/hr)	48.0	13.0
Human blood:air partition	9.5	8.0

[a] Determined by vial-equilibration (Sato and Nakajima, 1979b).
[b] $V_{max} = V_{maxc}(bw)^{0.7}$.

Equation 18 was a recalculation of Equation 19 based on a daily water intake of 2 liters by a 70-kg human, an assumption used in most risk-assessment procedures, rather than the original 4.4 liters. In the model developed here for drinking water consumption, a small dose of chemical appears in the stomach at equally spaced intervals. The chemical is completely absorbed into the liver compartment with a first-order absorption rate constant of 5/hr. The same rate constant was used with both rats and humans (see Table 6-1).

TWO EXAMPLES OF DOSE-ROUTE EXTRAPOLATIONS

Two extensively studied chemicals—trichloroethylene and benzene—are used in this section to illustrate how the results of inhalation studies can be applied to estimate the equivalent dose in a drinking water study. These two chemicals were selected because subchronic inhalation toxicity studies have defined no-effect or minimal-effect levels for the noncarcinogenic responses to these VOCs in rats and because the target organs for toxicity are different. Trichloroethylene causes hepatic toxicity, and benzene is toxic to the blood-forming tissues in bone marrow. For both chemicals, the kinetic constants for oxidative metabolism, determined by gas uptake experiments (Table 6-2), indicate that the parent chemical has a high affinity for the metabolizing enzymes (i.e., low K_m) and that first-pass effects in the liver (i.e., the ability of metabolism to reduce blood concentrations before the chemical reaches the systemic circulation) will be significant at low oral doses.

The PB-PK extrapolations developed here focus on determining the equivalent no-effect levels for inhalation and drinking water exposures,

but not on risk assessments of the carcinogenic activity of these chemicals. Yet this approach could also be applied to assessing carcinogenic risk, a process that is essentially a high-dose to low-dose extrapolation. Regardless of whether the effect investigated is chronic toxicity or carcinogenicity, risk assessment should still be based on the amount of toxic chemical at the appropriate target tissue. The development of PB-PK models for trichloroethylene and benzene requires knowledge of their partition coefficients, metabolic constants, and physiological indices in the rat.

Trichloroethylene

In a subchronic inhalation study, Kimmerle and Eben (1973) found increased liver weights in rats exposed to 56-ppm (302.4-mg/m^3) concentrations of trichloroethylene (the lowest-observed-effect concentration) for 8 hours/day, 5 days/week for 98 days. Essentially continuous exposure (23.5 hours/day) to 35 ppm (189 mg/m^3) for 90 days caused no untoward effects in exposed rats (Prendergast et al., 1967). Considered together, these two studies suggest that 56 ppm (302.4 mg/m^3) is the minimal effect concentration and that the liver should be used as the target tissue in the pharmacokinetic tissue-dose extrapolation.

The toxicity of trichloroethylene appears to be associated with its metabolites—not with the compound itself. Trichloroethylene is metabolized by microsomal oxidation to an unstable, reactive epoxide intermediate, the majority of which is rearranged to yield trichloroacetaldehyde. The aldehyde is oxidized to trichloroacetic acid or reduced to trichloroethanol, which is then conjugated with glucuronic acid and excreted in the urine. The choice of the measure of target-tissue dose depends on the presumed mechanism of toxicity. If toxicity is due to the concentration profile of one of these stable metabolites, the model would have to include information on the disposition and elimination of these metabolites (see section on PB-PK Models in Risk Assessment toward the end of this chapter). If toxicity results from the presence of the short-lived reactive epoxide, the target-tissue dose can be estimated with Equation 8 and should be related to the total amount metabolized per unit liver volume. This latter relationship was chosen as the measure of target-tissue dose for the trichloroethylene extrapolations. Buben and O'Flaherty (1985) have shown that liver enlargement in mice orally dosed with trichloroethylene dissolved in corn oil correlates well with the amount metabolized.

The next task is determination of the target-tissue dose in the rat following an 8-hour inhalation exposure to 56 ppm (302.4 mg/m^3) (Figure 6-6A). This amount was the lowest-observed-effect concentration reported by Kimmerle and Eben (1973), which is used in place of the no-effect tissue dose indicated in Figure 6-1. To do this, the inhalation PB-PK

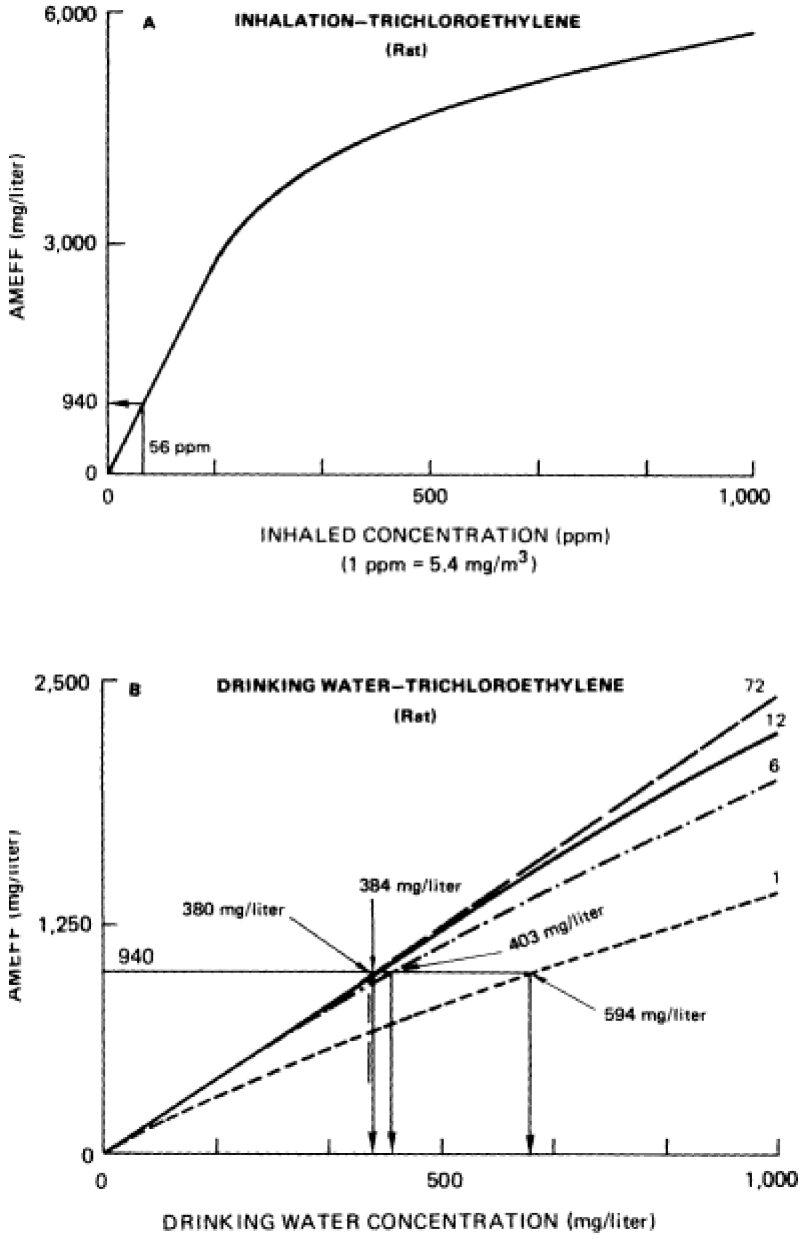

Figure 6-6
Dose-route extrapolation for inhalation and drinking water exposures of rats to trichloroethylene. AMEFF is the effective concentration of reactive metabolite formed in a compartment of specified volume. The numbers at the ends of the drinking water curves indicate the number of equal doses given to the test animal.

model was run at a variety of exposure concentrations to calculate the effective concentration of metabolite formed in the liver (the AMEFF, in mg/ liter). This integrated measure of metabolite dose was taken over a 24-hour period, which included the 8 hours of exposure and the 16 hours before the next exposure. At each inhaled concentration, the computer program calculated the total amount of metabolite produced in the liver for the entire 24-hour period, i.e., the AURMC. The curve at the high inhaled concentrations shows the nonlinear behavior introduced by metabolic saturation. All values of AMEFF from the multiple simulations were then plotted to yield a smooth curve. The value of AMEFF for rats following an 8-hour exposure to 56 ppm (302.4 mg/ m^3) was calculated to be 940 mg/liter.

The physiological model was next used by the committee to estimate the drinking water concentration that would produce a dose of toxic metabolite in rat liver equivalent to that produced when rats were exposed to 56 ppm (302.4 mg/m^3) by inhalation (Figure 6-6B). The model was applied at a variety of drinking water concentrations to estimate target-tissue dose for the reactive trichloroethylene metabolite. With this model, tissue dose was determined for the 24 hours beginning with ingestion of the first aliquot of water. Curves are depicted for water ingestion for four different exposure patterns: repetitive sipping, two episodic drinking patterns, and bolus ingestion. The goal of these simulations was to determine the drinking water concentration that produced a target-tissue dose of 940 mg/liter in the liver. The concentrations were 380, 384, 403, and 594 mg/ liter for the models in which the dose was divided into 72, 12, or 6 equal parts, or given as 1 dose only, respectively. For this particular measure of target-tissue dose, the three (i.e., more realistic divided dose) scenarios provided very similar estimates of the minimal-effect drinking water concentration in rats.

Benzene

Deichmann et al. (1963) exposed Sprague-Dawley rats to inhalation concentrations of benzene ranging from 15 to 831 ppm (45 to 2,493 mg/ m^3). The animals were exposed 7 hours/day, 4 days/week for periods ranging from 4 weeks to 8 months. Leukopenia was observed in rats exposed to 47 ppm (141 mg/m^3) during 180 days within an 8-month period and in those exposed to 44 ppm (132 mg/m^3) for 45 to 54 days within a 3- to 4-month period, whereas no toxicity was observed at exposure concentrations of 31, 29, or 15 ppm (93, 87, or 45 mg/m^3). The exposures to 29 ppm (87 mg/m^3) were administered for 62 days within an 88-day period. For the physiologically based extrapolation with benzene, the no-observed-effect concentration of 31 ppm (93 mg/m^3) given 7 hours/day

for 90 days within a 126-day period represented the benchmark exposure. The target-tissue dose of benzene must in some way be related to the length of time that the benzene remains in the blood-forming tissues. Rickert et al. (1979) found that benzene concentrations in bone marrow closely follow blood benzene concentrations, indicating that the marrow responds kinetically as if it were part of the well-perfused visceral tissue compartment. Although the myelotoxicity of benzene is believed to be due to further metabolism of some unidentified benzene metabolites in the marrow tissue itself, there are no estimates of the kinetic constants for benzene metabolism in marrow tissue. In the absence of more complete information, the best measure of target-tissue dose for benzene will be estimates of the integrated visceral tissue dose of some unidentified stable metabolite (see Equation 10). The model parameters for benzene do not include estimates of either $k_T/\Sigma k_i$ or of Cl_{TM}. Since these terms are linear, an estimate of each was used in the present calculations. It was assumed that $k_T/\Sigma k_i$ was 1.0 and Cl_{TM} for the rat was 250 ml/hr, which is equivalent to clearance of a chemical with a 1/2-hour half-life from the body water of rat. These values were used to derive a surrogate for the AUTMC in the simulations of the benzene model. A second measure of tissue dose, AUBC (area under the blood concentration-time curve)—Equation 4—was also taken.

The procedure for setting the no-effect benzene tissue dose is the same as that used for trichloroethylene, except that the units of surrogate tissue metabolite dose are mg/liter x hours instead of mg/liter. The no-effect tissue metabolite dose in the 7-hour, 31-ppm (93-mg/m^3) exposure with 17 hours between the end of exposure and the next exposure period is calculated to be 10.13 mg/liter x hours. The overall behavior of the AUTMC for exposures to a variety of concentrations of benzene again is nonlinear at high concentrations (Figure 6-7A). The benzene drinking water model predicted equivalent no-effect target-tissue doses for drinking water concentrations of 109, 111, 118, and 183 mg/liter for the sipping, 1 hour, and 2 hour episodic scenarios and for the bolus dosing regimen of water consumption, respectively (Figure 6-7B).

For visceral tissue doses of benzene itself, the 7-hour, 31-ppm (93-mg/ m^3) exposure produced an AUBC of 2.87 mg/liter x hours, whereas the four drinking water regimens provided equivalent tissue doses of 310, 219, 167, and 89 mg/liter x hours (Figure 6-8). With benzene, the pattern of water ingestion by the rat has a marked effect on tissue dose. This physiologically based approach could obviously be refined by including a more realistic pattern of water intake. In the drinking water model, ingestion was divided into equal increments at evenly spaced intervals over 12 hours. More complicated patterns of water consumption

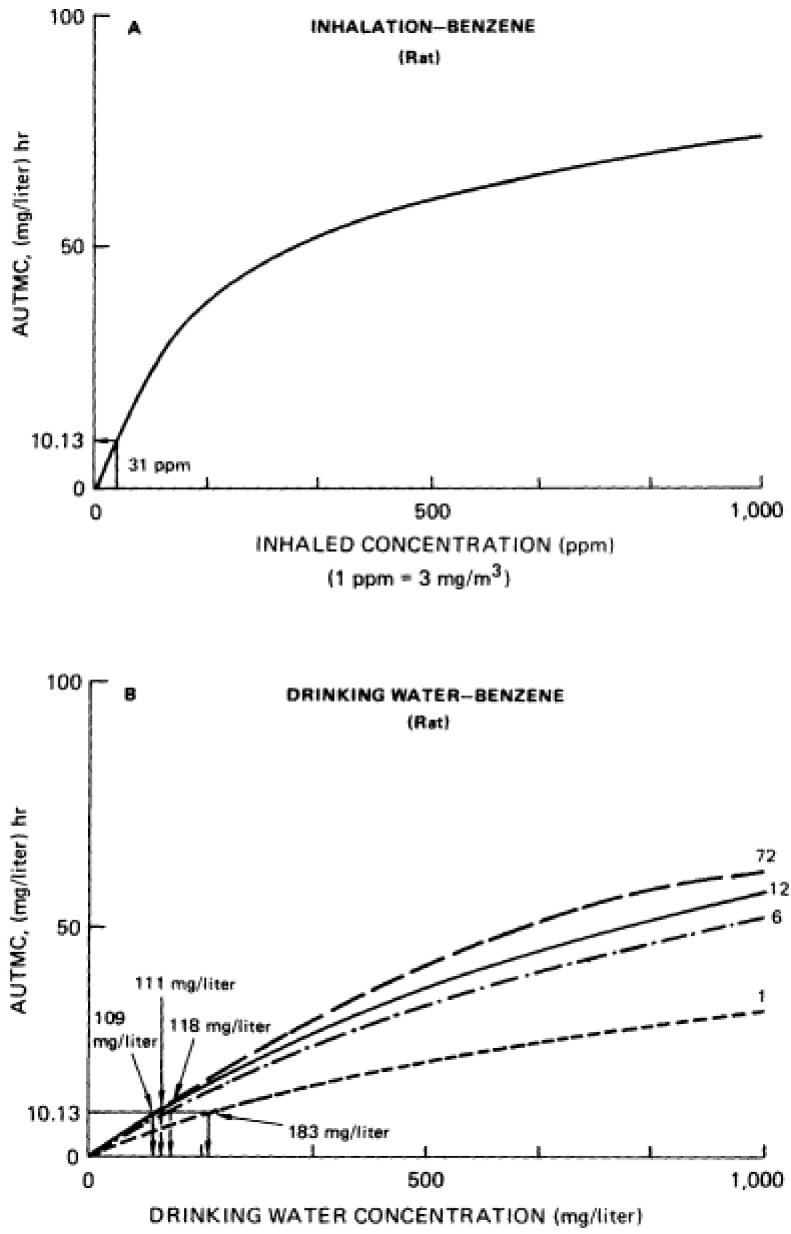

Figure 6-7
Dose-route extrapolation for inhalation and drinking water exposures of rats to benzene. AUTMC is area under the target-tissue metaboliteconcentration curve. The numbers at the ends of the drinking water curves indicate the number of equal doses given to the test animals.

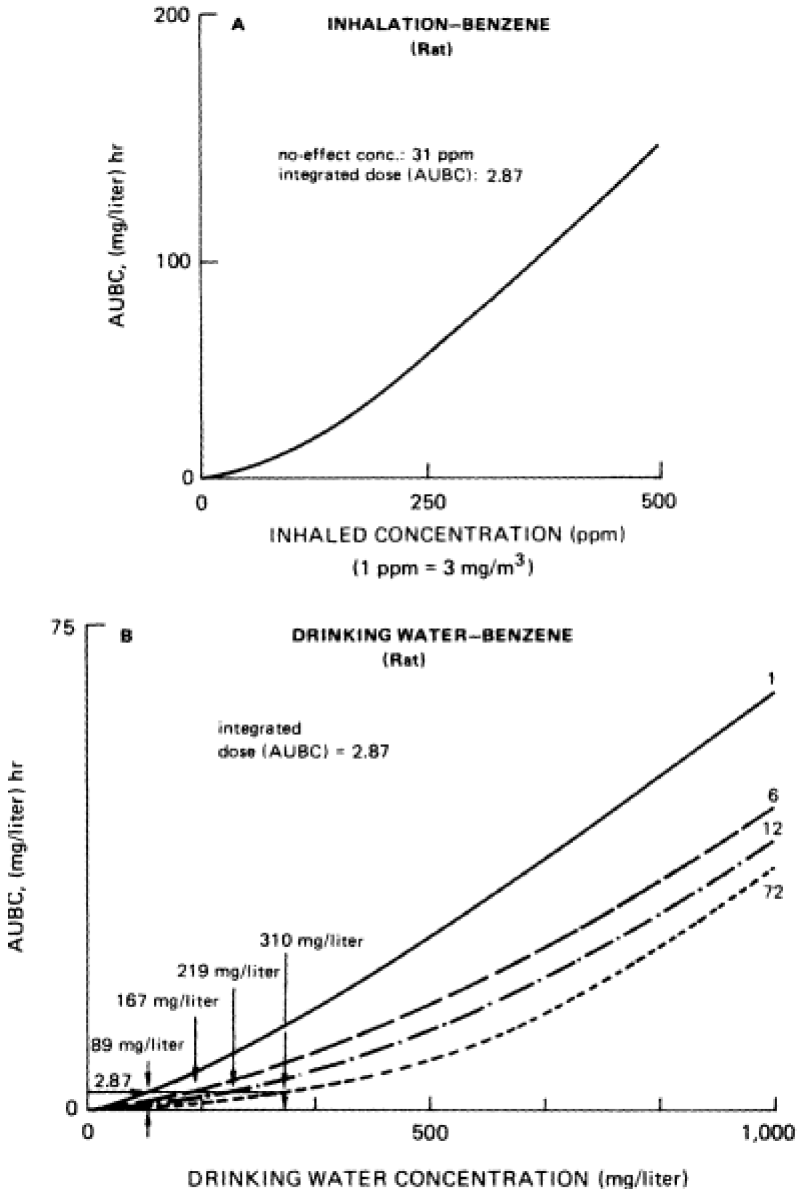

Figure 6-8
Dose-route extrapolation for inhalation and drinking water exposures of rats to benzene, based on area under the blood benzene concentration curve. The numbers at the ends of the drinking water curves indicate the number of equal doses given to the test animals. Panel A: Same as in Figure 6-7 except AUBC was evaluated instead of AUTMC. Panel B: Same as in Figure 6-7, but the extrapolation corresponds to a no-effect AUBC value of 2.87 (mg/liter x hours).

could be readily modeled if there were more detailed data on the daily water consumption patterns of rats.

INTERSPECIES EXTRAPOLATION

Allometric Relationships

In applying Stokinger and Woodward's (1958) approach to the calculation of acceptable water concentrations from TLVs, an exact correspondence is assumed between an acceptable absorbed dose from inhalation and the acceptable absorbed dose from oral ingestion. When this type of calculation also includes interspecies scaling, some assumptions are then made to estimate the relative sensitivity of humans compared with the test species. Presently, the no-effect level in humans is estimated from the rodent no-effect level by applying a surface area correction, i.e.,

$$\text{Human dose (mg)} = \text{rat dose (mg)} \left(\frac{\text{human } bw}{\text{rat } bw}\right)^{2/3}, \text{ and} \quad (20)$$

$$\text{Human dose (mg/kg)} = \text{rat dose (mg/kg)} \left(\frac{\text{rat } bw}{\text{human } bw}\right)^{1/3}. \quad (21)$$

This adjustment uniformly reduces the acceptable weight-adjusted dosage for humans compared with that determined in smaller laboratory test animals. This correction factor is based largely on limited experience with chemotherapeutic drugs (Freireich et al., 1966) and appears applicable to some chemicals. However, it does not have either a toxicological or a pharmacokinetic basis and should not be applied to every chemical. Since a number of kinetic factors may be important in influencing tissue doses in various species, the appropriateness of a rigid approach to interspecies scaling for all chemicals must be more closely examined.

The cornerstone of a scientific approach to interspecies extrapolation of physiological pharmacokinetic behavior is the predictable relationship observed between the values of various physiological parameters and species body weight. The allometric relationship for water intake from Adolph (1949), given in Equation 19, describes the dependence of one particular physiological process on body weight. This allometric relation has the following form:

$$y = a(bw)^x. \quad (22)$$

Dependencies of this type have been developed for a variety of physiological processes (Adolph, 1949; Schmidt-Nielsen, 1970, 1984). As a general rule, organ volumes tend to increase in direct proportion to body weight ($x = 1.0$), whereas surface area, blood flows, ventilation rates,

and metabolic rates are related to a fractional power of body weight ($x < 1.0$) that is closer to 0.7. For instance, the exponents proposed in the equations for ventilation and surface area are 0.74 (Guyton, 1947) and 0.67 (Spiers and Candas, 1984), respectively. These general allometric relationships can be used as the basis of interspecies scaling strategies.

Tissue Dose Scale-Up

The basis for choosing an appropriate tissue dose scale-up strategy is the kinetic behavior of the particular chemical and its mechanisms of toxicity. In this first example, the chemical has a well-defined volume of distribution; it is eliminated by processes dependent on organ perfusion in the liver, kidney, or lung, and its toxicity is related to the area under its blood or tissue concentration curves. A dose of this chemical is distributed into a volume (V_d) and eliminated with a rate constant determined by its clearance (C_l) and Vd. Clearance is some proportion (P) of perfusion to the organs of elimination, i.e., $C_l = PQ_i$. By conventional compartmental kinetics, the AUBC in any species (i) is:

$$\text{AUBC}_i = \frac{\text{dose}}{V_d}\left(\frac{V_d}{Cl}\right) = \frac{\text{dose}}{PQ_i}. \qquad (23)$$

If P (the proportion of flow cleared) is species-invariant, the dose in a second species that has the same integrated target-tissue dose AUBC becomes:

$$(\text{mg of dose})_2 = (\text{mg of dose})_1\left(\frac{Q_2}{Q_1}\right). \qquad (24)$$

Since organ blood flows are proportional to $(\text{bw})^{0.7}$,

$$(\text{dose})_2 = (\text{dose})_1\left(\frac{bw_2}{bw_1}\right)^{0.7}. \qquad (25)$$

This equation is consistent with the correction factor used in Equation 20.

Species sensitivity could also be calculated as a direct function of the exposure concentration instead of absorbed dose. For example, in a simple compartmental model, consider a chemical metabolized to a stable metabolite that is eliminated by flow-dependent processes and whose toxicity is related to the AUBC of the metabolite. During metabolism of brominated hydrocarbons such as halothane or bromochloromethane, this kinetic behavior is expected to occur following the release of the bromide ion (Gargas and Andersen, 1982). Oxidative metabolism yields inorganic bromide that is eliminated by renal filtration. In the kidney, most of the bromide is resorbed by the same processes that conserve chloride; only small amounts

escape resorption. In the animal, therefore, bromide concentrations are increased by its metabolism and subsequent distribution into extracellular water. Metabolic rates are proportional to and limited to $(bw)^{0.7}$, as clearance. Steady-state concentrations ($C_{s\text{-}s}$) are reached after exposure certain airborne concentrations of a chemical:

$$C_{s\text{-}s} = \frac{\text{rate of formation}}{\text{clearance}} = \frac{k_1(bw_1)^{0.7}}{k_2(bw_1)^{0.7}} = \frac{k_1}{k_2}. \qquad (26)$$

The rate of approach to or recession from a steady state depends on the clearance and the volume of distribution. Thus, the rate constant for the approach will decrease as body weight increases (see Table 6-1). However the extra area under the uptake curve for the smaller species is exactly offset by the decreased area under the elimination curve, and the net area under the bromide curve, for inhalation of equivalent concentrations of the brominated hydrocarbons, should be invariant with body weight for the bromide produced during their metabolism.

The same analysis used for the bromide ion applies to inhalation of a VOC by various species. If metabolism is not extensive, the steady-state blood concentrations are determined by the blood:air partition coefficients. To the extent that blood solubility is a thermodynamic parameter (Dedrick, 1973), it is not expected to be widely variable from species to species, and the achieved blood concentrations should be quite similar regardless of species body weight. Rate constants for the approach to a steady state will decrease with increasing body weight, but the area under the blood concentration-time curve during inhalation of an equivalent VOC concentration will be nearly independent of the body weight of the species.

An examination of drinking water ingestion can also be based on the water concentration required to give a particular integrated tissue dose of parent chemical. Ingested dose (Equation 23) is the water concentration (C_w) times the volume of water consumed (V_w). The latter is determined by the allometric relationship shown in Equation 18. Thus, ingested dose is used in Equation 23 to give:

$$\text{AUBC} = \frac{C_w \times V_w}{P_i \times Q_i}, \text{ and} \qquad (27)$$

$$\text{AUBC} = \frac{C_w \times a_1(bw)^{0.7}}{P_i \times a_2(bw)^{0.7}} = \frac{C_w \times a_1}{P_i \times a_2}, \qquad (28)$$

where AUBC is independent of body weight and essentially only a function of water concentration.

An examination of the AUTMC relationship reveals that steady-state blood concentrations are proportional to the ratio of V_{max} to the clearance

of the metabolite from its volume of distribution (see Equation 11). Both V_{max} and Cl_{TM} should be proportional to about the same fractional power of body weight. $C_{s\text{-}s}$ and AUTMC should also be essentially independent of body weight when expressed as a function of drinking water concentration. Whether toxicity is correlated with integrated tissue dose of a parent or a stable metabolite, equivalent water or inhaled concentrations are expected to produce similar tissue doses *regardless* of body weight. Even with a conservative approach to standard setting, therefore, no-effect concentrations in test animals should provide a good estimate for the expected no-effect concentrations in humans. This very useful rule of thumb was derived from an examination of the pharmacokinetic bases of interspecies extrapolation strategies.

Short-Lived Reactive Metabolites

Some toxicity is mediated by chemically reactive intermediates that are not sufficiently stable to be isolated. For these chemicals, the rate of metabolism is assumed to be proportional to $(bw)^{0.7}$ and the intermediate is formed in a target tissue whose volume is directly related to body weight. The tissue load of reactive metabolite becomes:

$$\text{dose}_{TM^*} = \frac{(\text{AURMC})_1}{V_{(\text{tissue})_1}} = \frac{a_1(bw)^{0.7}}{a_2(bw)^1} = \frac{a_1/a_2}{(bw)^{0.3}}. \qquad (29)$$

The relationship that expresses the ratio of maximum target-tissue dose of an unstable reactive metabolite in one species to that in another species with different body weight is:

$$\frac{(\text{dose})_2}{(\text{dose})_1} = \frac{a_1/a_2}{(bw_2)^{0.3}} \times \frac{(bw_1)^{0.3}}{(a_1/a_2)} = \left(\frac{bw_1}{bw_2}\right)^{0.3}. \qquad (30)$$

The maximum effective tissue dose in the smaller species will be significantly larger than the maximum effective dose in the larger species. An argument very similar to this was proposed by Gehring et al. (1978) for assessing risk to humans from vinyl chloride inhalation on the basis of data from toxicity and metabolism studies in rats.

The appropriate measure of target-tissue dose is likely to be a subject of lively controversy for many chemicals. Yet, decisions on how to conduct interspecies scaling must be based on some presumed measure of the toxic moiety. The equations for interspecies scaling are not intended as hard-and-fast rules. Rather, they point out the variety of behaviors that can be expected and emphasize that it would be shortsighted to depend on a standard equation for conducting interspecies extrapolation. A defensible interspecies extrapolation strategy can only be developed once an

argument has been made for a particular measure of target-tissue dose and once a realistic description of the exposure scenario has been developed for use in an appropriate PB-PK description.

Interspecies PB-PK Model Simulations

Ramsey and Andersen (1984) successfully modeled human styrene kinetics by scaling all volumes in direct proportion to body weight and all flows by body weight raised to the 0.7 power. The same partition coefficients were used in each species, except that the blood:air partition coefficient was independently determined for the blood of rats and humans. The values were 40.2 ± 3.7 (Ramsey and Andersen, 1984) and 51.9 ± 2.0 (Sato and Nakajima, 1979a), respectively. Following the line of reasoning developed by Gehring et al. (1978) for vinyl chloride metabolism, Vmax was scaled as $(bw)^{0.7}$ and the same K_m was used for both humans and rats. As pointed out earlier in this chapter for dioxin toxicity, binding constants for molecules much more complex in structure than styrene may vary unpredictably among animal species. With these simple VOCs, there are no intricate functional groups to dictate highly selective binding that could give rise to significant species variability in metabolism. More work is clearly needed to evaluate species dependencies of xenobiotic affinity constants for protein binding in general and for binding to metabolizing enzymes in particular. Even with the most successful descriptions of pharmacokinetic behavior in a test species, there is still some element of uncertainty and art involved in restructuring the model to describe kinetics in humans. Part of this uncertainty would quickly evaporate with conscientious efforts to validate some of the assumptions, such as the scaling of V_{max} and the body weight independence of K_m, presently made when scaling up animal kinetic models to predict human kinetic behavior.

In the interspecies extrapolation strategy based on a PB-PK model, the measure of target-tissue dose in the human is the same as that used in the rodent model. A drinking water model for humans is then used to determine which concentration leads to the same value for target-tissue dose as that associated with the no-effect (or minimal-effect) level in rodents (Figure 6-1). Again, the model for humans requires a description of the pattern of water consumption by humans and an estimate of the total volume consumed per day. For this report, water intake was calculated from Equation 18 and calculations were based on all four scenarios used in the drinking water model for rats. Another change in scaling the physiological model for rats was an adjustment for differing amounts of fat in adult humans and in young adult rats. The human fat compartment volume was set at 20% of body weight versus 9% in a 300-g rat (Table 6-2). Blood:air partition coefficients for humans were determined from fresh samples of

blood taken from volunteers at Wright-Patterson Air Force Base; they were found to be significantly lower than those for rats (M. L. Gargas, Air Force Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio, personal communication, 1985). The blood:air partition coefficients for humans are in close agreement with those published by Sato and Nakajima (1979a,b).

The PB-PK drinking water models for humans were run at a variety of concentrations to predict target-tissue dose for various ingested concentrations and the four drinking patterns for both trichloroethylene and benzene (Figure 6-9). The no-effect or minimal-effect drinking water concentrations are determined as follows: the no-effect target-tissue dose from Figure 6-6A or Figure 6-8A is located on the *y*-axis, and a line parallel to the *x*-axis is drawn through this point. To estimate the human no-effect drinking water concentration associated with each pattern of water consumption, lines are dropped down perpendicular to the *x*-axis from the intersection points on the curves. For trichloroethylene, the target-tissue, minimal-effect 940-mg/liter dose of reactive metabolite is expected at a drinking water concentration of 1,528 mg/liter, assuming ingestion of 2 liter/day in six equal portions. In agreement with the expectations from Equation 30, the scaling for trichloroethylene predicts that humans can be exposed to a much higher drinking water concentration before attaining a similar target-tissue dose of metabolite (1,528 mg/liter, compared with 403 mg/liter for rats). For benzene, the target-tissue, no-effect dose for the area under the surrogate tissue metabolite curve (10.13 mg/ liter x hours) is expected at a drinking water concentration of 98 mg/ liter (not shown). As expected from Equation 28, similar drinking water concentrations (118 compared with 98 mg/liter) yield equivalent tissue doses, expressed as the area under the benzene tissue metabolite curve, regardless of species. Alternatively, because AUBC and AUTMC are expected to have similar interspecies relationships, the extrapolation for benzene could be based on AUBC. In this case, the target-tissue, no-effect dose for AUBC in the rat (2.87 mg/liter x hours) is expected in humans at a drinking water concentration of 183 mg/liter (Figure 6-9). The equivalent value for the rat was 167 mg/liter (Figure 6-8). Both these measures of tissue dose of benzene are surrogate estimates: AUBC because it examines parent compound behavior; AUTMC because it has to assume the fraction metabolized to active metabolite and clearance of this unknown metabolite.

These estimates of no-effect or minimal-effect drinking water concentrations are based on strict equivalence of target-tissue dose, as determined from a PB-PK model representative of a standard, healthy adult male. Safety factors have not been incorporated in the models for either rats or humans to account for any uncertainties in the data base. Nonetheless,

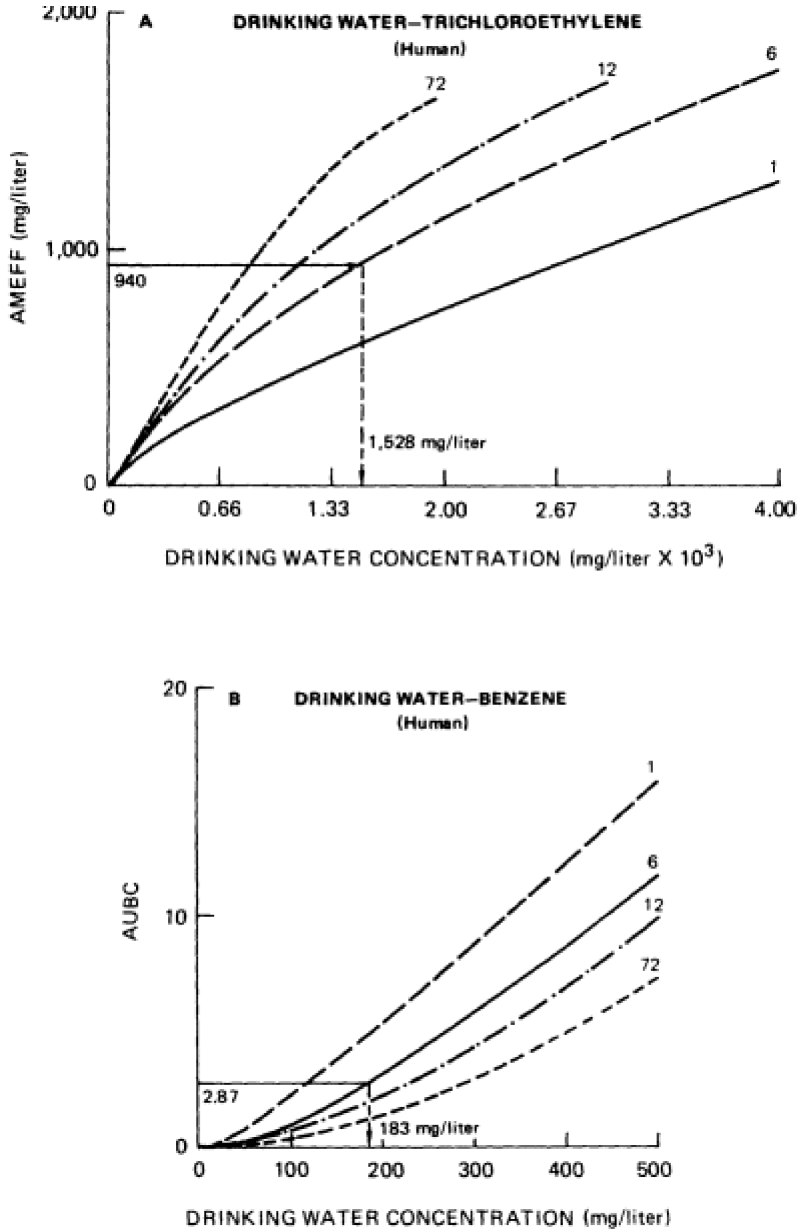

Figure 6-9
Interspecies extrapolations for trichloroethylene and benzene, based on PB-PK estimates of equivalent target-tissue doses. The numbers at the ends of the drinking water curves indicate the number of equal doses given to the test animals.

there is a need to recognize the potential uncertainties in any PB-PK description when determining the chemical nature of the toxic component and when extrapolating from a homogeneous rodent population to the heterogeneous human population. These uncertainties could be addressed by using the PB-PK models for changes in various physiological or biochemical parameters to predict, for example, the kinetics in young children drinking large amounts of water or in elderly persons with impaired metabolism. Another approach would be to assume a particular range of sensitivities in the human population and to base the lower acceptable exposure limit on the tissue concentration associated with the lower limit of susceptibility in the population.

Incorporating Time-Dependent Physiological and Metabolic Changes

In rats, chronic exposure and aging can alter kinetic behavior. Repeated exposure might induce xenobiotic metabolism, as it does with chlorobenzene (Sullivan et al., 1983) or styrene (Andersen et al., 1984). Stable metabolites might accumulate and either inhibit or induce metabolism of the parent chemical, as observed with metabolites of hexane (Clewell et al., 1984). Basal enzyme activities will change with age. Volumes of distribution may also change as rats mature and gain a larger amount of body fat (Yang et al., 1984). In much older rats, there is degeneration of physiological function, which can influence kinetic behavior.

The potential for such factors to confound kinetic modeling becomes more serious in long-term studies. The value of long-term chronic studies could be enhanced considerably if small groups of test animals were maintained exclusively for evaluation of important biochemical and physiological parameters toward the end of the test animals' life span. The results of such studies could form the basis for introducing time-dependent physiological changes into the general PB-PK model developed for risk assessment.

Not only does the modeling use a description of a standard rat, the scale-up approach simulates a standard, healthy, young adult male weighing 70 kg. Given the extensive heterogeneity in the human population, it is vital to either evaluate or simulate the effect of altered metabolism or altered physiological states on kinetic behavior. Physiological models can be readily used for this, since metabolic constants and physiological parameters are explicitly defined for organs of elimination and for target tissues. One possibility for predicting the variability in expected target-tissue dose is to simulate kinetic behavior for different conditions of organ function and metabolic capacity and for both extremely young and very old members of an exposed population. This would indicate the variability

expected in target-tissue doses for extremes of physiological status, age, and patterns of water consumption.

Although more research is needed to demonstrate the general utility and limits of PB-PK approaches in assessing risk based on extrapolation of data on other classes of environmental chemicals, techniques already exist for collecting the necessary data to develop PB-PK models for many VOCs. Implementation of this general approach could improve the scientific basis of risk assessment extrapolation procedures and could usefully focus toxicologists' attention on acquiring experimental data more relevant for quantitative physiological models that describe the kinetic behavior of important toxicants and their metabolites.

PB-PK MODELS IN RISK ASSESSMENT

A commonly voiced concern about introducing pharmacokinetic considerations in general risk-assessment decision making is that it is too difficult to determine the time-course curves necessary to model parent chemicals and to undertake the analytical studies needed to quantify and identify the metabolites of the test chemical. Although this is true, PB-PK models can still be useful without complete pharmacokinetic data.

The kinetic models for trichloroethylene and benzene described in this chapter are not complete models. They can be used to keep track of the parent chemical and the total amount metabolized, but they do not identify the metabolites that are formed, the concentrations these metabolites reach, or the routes by which they are eliminated. Yet, even these simple PB-PK parent chemical models account for metabolic nonlinearities, exhalation, and first-pass liver and pulmonary effects. And they are known to provide a good approximation of kinetic behavior in the rat (Murphy et al., 1984). Furthermore, such PB-PK models do not require an extensive data base on blood or tissue concentrations as related to time at various dose levels.

Time-course studies are essential to the development of data-based kinetic models, but they are only secondary in the design of PB-PK models, where the data serve more for model validation and fine tuning than for model articulation. For many volatile chemicals, the necessary biochemical, physiological, and solubility constants needed for a PB-PK model can easily be obtained in short-term experiments requiring a limited number of animals.

Extending Basic Models to Accommodate Reality

These basic PB-PK descriptions of VOC disposition can play a role in risk assessment, especially in providing a framework for analyzing the

effect of various factors on target-tissue dose. The simulations presented here show the complex influence of drinking water patterns on various measures of target-tissue dose in rats and humans. Not every physiological or biochemical factor that can alter kinetic behavior is accounted for by these simple descriptions. For instance, does repeated exposure to trichloroethylene induce metabolism of the compound in the same way that styrene induces its own metabolism (Andersen et al., 1984)? These factors are not included initially, because their importance is unclear until they have been demonstrated by laboratory experimentation. However, the absence of such information in these basic models reflects less the accuracy or utility of the model than its completeness.

Other important factors can be incorporated as further information and new insights are gathered during toxicity testing or through epidemiological investigations. In this respect, PB-PK descriptions are much like toxicity data bases. They contain the best available data, yet they are never truly complete and may require restructuring or rethinking based on new observations. Nonetheless, even provisional data bases or kinetic models can be useful. In fact, refinements may not substantially alter the conclusions reached during their original use in risk assessment. For instance, enzyme induction may occur at high concentrations but may be of no practical importance at environmental concentrations. The goal of developing these rudimentary PB-PK models is to explore the possibility of introducing a quantitative, scientifically sound pharmacokinetic strategy into risk-assessment extrapolations.

When more elaborate models are required, formulation of the kinetic model at an early stage of chemical toxicity evaluation can guide experimental strategies for collecting the biochemical and kinetic constants necessary for successful model development. For example, a metabolite of trichloroethylene, trichloroacetic acid (TCA), may cause peroxisomal proliferation in rats and mice, and this proliferative response may be involved in the carcinogenicity of trichloroethylene in gavage bioassay experiments (Green and Prout, 1985; Prout et al., 1985). To assess relative risks from an integrated tissue dose of this metabolite, the kinetic model would have to include TCA production and its elimination and predict liver concentrations of TCA at different times. A model then would be articulated to include major pathways for this metabolite (Figure 6-10).

Experiments or literature searches should focus on obtaining kinetic constants and describing localization of the enzymatic steps shown in Figure 6-10 for aldehyde reduction (3), aldehyde oxidation (1), and alcohol glucuronidation (4). Other required information would include plasma protein-binding characteristics of TCA itself (5), urinary excretion rates for the glucuronide (7) and the acid (6), and the equilibrium constant in biological media for the formation of the hemiacetal from the aldehyde

(2). Experiments should be designed to provide quantitative values for the biochemical factors as well as a physiological basis for tissue localization, volumes of distribution, and filtration rates. This coordinated approach to data acquisition would facilitate ultimate interspecies extrapolation with the kinetic model, a process that should be a primary goal of any pharmacokinetic model intended for risk-assessment extrapolations. Similarly, a more extensive kinetic model for benzene might include biochemical constants for metabolism in marrow, identification of the stable toxic metabolite, and determination of its clearance mechanisms. The model then could examine AUTMC for visceral tissues like marrow, the concentration dependence of metabolism in these tissues, and the target-tissue dose of metabolite either as total amount metabolized or total amount metabolized per target-tissue volume.

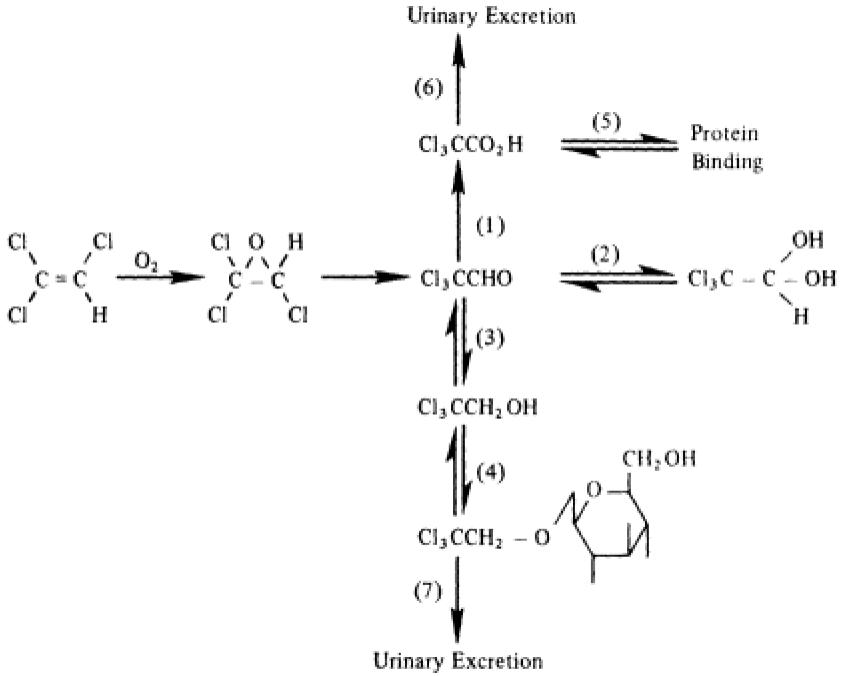

Figure 6-10
Major metabolic pathways for trichloroacetic acid.

Other factors concerning xenobiotic metabolism might also be incorporated into more complex PB-PK models. It appears paradoxical that short-lived toxic metabolites are often presumed to be responsible for toxicity, although they do not need to be explicitly identified in the kinetic models. In general, this approach is justified by assuming that the amount

of active toxic chemical formed will be linearly related to the total amount metabolized at all doses or exposure concentrations. This may not be true for chemicals when cofactor depletion by reactive metabolite shifts the distribution of chemical reactions by various pathways. The toxicity of a reactive metabolite is partially determined by the ratio of the amount reacting with target sites divided by the total amount reacting by all pathways, i.e., the $k_r/\Sigma k_i$ term in Equation 9. The interspecies extrapolations assume that this distribution of reactive intermediate would be the same in humans as in rats. If evidence indicated a species variation in this ratio, it should be incorporated into the calculations of AMEFF and in the risk-assessment extrapolations.

For chemicals with large intrinsic hepatic clearances, first-pass hepatic elimination is important at low concentrations or low dose rates in decreasing the amounts of chemical reaching the systemic circulation. This behavior is readily apparent when examining the AUBC for benzene administered in drinking water (Figure 6-8). Other tissues could be involved to a lesser extent in presystemic elimination, including the gut and intestinal microflora (for oral ingestion) and the nasal mucosa and upper airway pulmonary tissues (for inhalation). If these tissues are not themselves targets for toxicity, their metabolic capacity would reduce delivery of VOCs to blood at low concentrations. Thus, even the PB-PK risk assessment procedure could overestimate risk at very low administered concentrations. Much work must be done to understand the metabolic capabilities of nonhepatic tissues and their role in presystemic elimination of these VOCs.

Appendix A:

Metabolic Processes in the Respiratory Tract, the Gastrointestinal Tract, and the Liver

To understand the principles described in the body of Chapter 6, the committee prepared this appendix, which describes in greater detail the metabolic processes that occur in the respiratory tract, the gastrointestinal tract, and the liver. All these processes must be considered when assessing risk from exposure to chemicals.

THE RESPIRATORY TRACT: ABSORPTION, METABOLISM, AND PRESYSTEMIC ELIMINATION

Pulmonary Structure and Cell Types

One of the striking features of the lung is the diversity of cell types within it. At least 40 have been identified, each with a characteristic location and function (Gil, 1982). Lung cells provide an epithelial lining, an immunological defense, an endocrine function, and xenobiotic metabolism. Cell types differ widely in the various regions of the lung. The conducting airways are lined with ciliated cells that move mucous secretions and goblet cells that secrete mucus. At least five other cell types are present in the airways; the exact number depends on the species (Kuhn, 1976). The bronchioles contain Clara cells and are frequent targets of injury by toxicants (Boyd, 1977; Reznik-Schüller and Lijinsky, 1979). As distance from the trachea increases, the goblet cell number decreases and the number of Clara cells increases.

The gas-exchanging regions of the lung, the alveolar sacs, also have many cell types. The alveoli have continuous linings by two cell types—the Type I cell, which is squamous and covers 95% of the alveolar surface, and the Type II cell, which is cuboidal and is purported to be the progenitor of the Type I cell (Gil, 1982). Type II cells are responsible for the production of pulmonary surfactant. Macrophages, also present in the alveolar spaces, are important because of their immunological role (Brain et al., 1977).

The metabolic activities of the cells differ widely (Gil, 1982). Ciliated cells, basal cells, epithelial serous cells, and many other cells in the upper airways have little capacity for xenobiotic metabolism. Clara cells have considerable cytochrome P450 activity, because they have large amounts of smooth endoplasmic reticulum. Alveolar Type I cells, although they generally have few organelles, have relatively large amounts of smooth

endoplasmic reticulum, which seems to proliferate under the effects of some toxic compounds. Type II cells generally have a large supply of organelles, including smooth endoplasmic reticulum, and therefore are often used in pharmacokinetic studies for examining mechanisms of action (Sahebjami et al., 1978; Young and Silbajoris, 1985). Thus, in addition to its function in gas exchange, the lung has varying regional capacities for the metabolism of xenobiotic compounds.

Metabolic Properties of Lung Cells

Clara Cells

Clara cells are nonciliated bronchiolar cells that increase in number as distance from the bronchioles increases. Although their physiological function is unknown, Clara cells differentiate to replenish injured ciliated bronchiolar cells.

The potential for the metabolism of xenobiotic compounds by Clara cells was studied extensively by Boyd (1977, 1980). Clara cells contain mixed-function oxidase activity capable of metabolizing xenobiotic compounds by several pathways. Boyd used 4-ipomeanol to demonstrate selective damage to Clara cells after intraperitoneal and oral administration. Toxicity was apparently related to a reactive metabolite formed by cytochrome P450. Liver microsomes also metabolized 4-ipomeanol, but the K_m for lung microsomal metabolism was more than 10 times lower than that of hepatic microsomes. The selective toxicity for the lung may be explained by these kinetic factors.

Carbon tetrachloride is also a Clara-cell toxicant (Boyd, 1980; Longo et al., 1978). Severe damage, including lipid accumulation, dilation of endoplasmic reticulum, and frank necrosis, was observed in rats and mice given oral doses of the compound. These changes were selective within the bronchiolar region for Clara cells.

Clara cells are also a target for reactive intermediates formed during the metabolism of other toxicants, e.g., 3-methylfuran, other furans, and bromobenzene (Boyd, 1980). Reznik-Schüller and Lijinsky (1979) suggested that Clara cells might be responsible for pulmonary tumors induced by some nitrosamines. Paraquat, although primarily a toxicant to alveolar pneumocytes, has been shown to damage bronchioles in mice (Etherton and Gresham, 1979). Studies by Devereux et al. (1981) on rabbit Clara cells showed the presence of two major forms of cytochrome P450 with molecular weights of 52,000 and 58,000 daltons.

Alveolar Type II Cells

Type II pneumocytes are present along the alveolar wall and are probably the progenitors of the Type I cells. The capability of Type II cells for metabolic activation of toxicants is considerable. This is consistent with the fact that they share a common embryologic origin with Clara cells (O'Hare and Sheridan, 1970). Studies by Devereux et al. (1981) supported this conjecture by showing that the cytochrome P450 isozymes present in rabbit Type II cells were identical to Clara cell P450 isozymes, both immunologically and with respect to molecular weight.

Carbon tetrachloride produced alterations in Type II cells in guinea pigs after a single intraperitoneal injection and in rats after oral administration or inhalation (Chen et al., 1977; Gould and Smuckler, 1971; Valdivia and Sonnad, 1966). The damage was similar to that (i.e., fatty changes) produced by carbon tetrachloride in the liver. These data are consistent with metabolic reduction of carbon tetrachloride by cytochrome P450 in the Type II cells.

Other Pulmonary Cells

There are few data on the capabilities of other pulmonary cell types to metabolize xenobiotic compounds. Alveolar macrophages have been investigated by Devereux et al. (1981) and Hook et al. (1972), who concluded that their xenobiotic-compound metabolizing activity is very low or nonexistent. The vascular endothelium is exposed to large quantities of xenobiotic compounds in the bloodstream, but there have been no reports that reactive intermediates have been formed at this site as the result of the metabolic processes. Ciliated bronchiolar cells bind 4-ipomeanol to a much lower extent than do Clara cells, suggesting that their metabolic activity is rather low (Boyd, 1980).

There is evidence that butylated hydroxytoluene (BHT) damages alveolar Type I cells in mice (Boyd, 1980). BHT is metabolized to a reactive intermediate in liver, but identification of this metabolite and its site of formation in the lung is incomplete.

The lack of xenobiotic compound-metabolizing enzymes in the tracheobronchial region presents an apparent paradox, since this region is a common site of tumor formation in humans. This apparent inconsistency may be resolved by the development of deposition and metabolism models that account for true regional deposition. Xenobiotic compounds may be metabolized in Clara cells, and the products of that metabolism may be translocated to the tracheobronchial region via mucociliary transport. Some reactive intermediates, such as bromobenzene-3,4-oxide, readily trans

locate from intracellular metabolic sites into the extracellular milieu (Monks et al., 1984).

Elimination of Compounds by the Lung

In addition to its functions as an organ of absorption and metabolism, the lung serves as a site of elimination of volatile xenobiotic compounds. The most extensively studied compounds eliminated by the lung are the volatile anesthetics. In general, these compounds have a low capacity for metabolism (i.e., small *Vmax*), and at the concentrations generally used in humans, their elimination rate is controlled by the same factors that are important to uptake: pulmonary ventilation, blood flow, and solubility in blood and tissues (Cowles et al., 1968).

The respiratory loss of xenobiotic compounds may be an important means of lowering toxicant concentrations in the blood. Volatile compounds, such as the halogenated hydrocarbons, are eliminated in part by diffusion across the pulmonary epithelium into the air spaces of the lung. The epithelial lining of the alveolus is only 5- to 10-µm thick in some regions covered by Type I cells, thus forming an efficient surface for gas exchange (Gil, 1982). Compounds with low blood:air solubilities (P_b) diffuse quickly out of the blood if the air in the lung is relatively free of that compound. Since the entire cardiac output perfuses the lung, this concentration gradient could allow rapid elimination by gas exchange.

There have been several published examples of the elimination of halogenated organic compounds via the lung. Elimination of carbon tetrachloride has been investigated in humans, monkeys, and rats. Humans given 80-ppm (480-mg/m^3) concentrations of carbon tetrachloride in a single breath expired 33% of the dose unchanged within 1 hour (Morgan et al., 1970). Monkeys expired 51% of a dose unchanged 1,800 hours after inhalation exposure (McCollister et al., 1951), and within 18 hours, rats expired 75% of an intraduodenally administered dose (Paul and Rubinstein, 1963). A small amount of chloroform formed as a metabolite of carbon tetrachloride was also exhaled by the rats, but this accounted for less than 1% of the total metabolism (Paul and Rubinstein, 1963).

Chloroform was excreted unchanged in the breath of several test species given chloroform orally (Brown et al., 1974). The expired levels ranged from 6% of the dose in mice to 78% in squirrel monkeys after 48 hours. Similar results were found by Paul and Rubinstein (1963). Humans given chloroform by ingestion exhaled 68.3% of the dose unchanged (Fry et al., 1972). In this study, obese subjects were found to exhale less chloroform than did subjects of normal weight. Brown et al. (1974) and Fry

et al. (1972) noted that most of the carbon in the chloroform that was not eliminated unchanged was exhaled as carbon dioxide.

The chloroethylenes and chloroethanes are also eliminated unchanged by the lung. In mice, 10% to 42% of an intraperitoneally administered dose of 1,2-dichloroethane was expired unchanged (Yllner, 1971). Hake et al. (1960) showed that 98.7% of an intraperitoneal dose of 1,1,1-trichloroethane was exhaled unchanged by rats within 25 hours. Rapid pulmonary excretion of 1,1,1-trichloroethane by humans has been reported after a single inhalation exposure (Morgan et al., 1970). In 1 hour, 44% of the dose was exhaled unchanged.

Reichert and Werner (1978) reported that an oral dose of vinylidene chloride given to rats was eliminated unchanged by the lung in a dose-dependent manner. At 0.5 mg/kg *bw*, 0.9% was expired unchanged. At 50 mg/kg *bw*, 20% of the dose was expired unchanged. Jones and Hathway (1978) reported that 28% of a vinylidene chloride dose was exhaled unchanged by rats after oral administration of a 50-mg/kg *bw* dose. Tetrachloroethylene was eliminated by the lung to an even greater extent. Rats given the compound orally expired 97.9% unchanged within 48 hours (Daniel, 1963).

The amount of benzene in the breath of both occupationally exposed and nonexposed volunteers has been measured (EPA, 1979). At work, the breath of filling station attendants exposed to airborne benzene concentrations ranging from 190 to 260 μg/m^3 contained levels of 17 to 432 μg/m^3. Only nonsmoking, nonoccupationally exposed subjects had lower levels while at work. The breath of residents of Love Canal in Niagara Falls, New York, contained benzene levels ranging from 0.7 to 6.9 μg/ m^3, the highest levels being exhaled by smokers. Carbon tetrachloride, chloroform, 1,1,1-trichloroethane, tetrachloroethylene, dichlorobenzene, chlorotoluene, and tetrachlorobenzene were also measured in the breath of subjects not occupationally exposed.

At first glance there seems to be unexplained variation in the percentage of chemical eliminated by exhalation when different dose routes, dosages, and species are compared for any particular VOC. This is not at all unexpected, however, and is nothing more than an expression of the differences in kinetic character of metabolic clearance and clearance by exhalation. The former is capacity-limited and displays zero-order kinetics at high blood concentrations, whereas the latter displays first-order kinetics at all concentrations.

For most volatile, metabolized substrates, total clearance (Cl_T) is the sum of hepatic, metabolic, and pulmonary clearance, i.e.,

$$Cl_T = Cl_{\text{metabolism}} + Cl_{\text{exhalation}}.$$

The two organ-specific clearance terms written more explicitly become:

$$Cl_T = \frac{Q_l V_m/(K_m + C_l)}{Q_l + V_m/(K_m + C_l)} + \frac{Q_p Q_p}{Q_p + Q_c P_b}.$$

The hepatic term (see Pang and Gillette, 1978) is saturable, since it has the liver concentration (C_l) in the denominator. The pulmonary term was developed earlier in Equation 3. The percentage exhaled is the ratio of $Cl_{exhalation}$ to Cl_T. As circulating concentrations become lower ($C_l << K_m$), the proportion exhaled decreases. Then as concentrations increase, hepatic clearance diminishes and the percent exhaled becomes greater. This behavior is readily explainable kinetically but greatly complicates the interpretation of limited data on the relative amounts of VOCs exhaled after exposure by various routes at different dosages. These differences in elimination would be much more easily interpreted if investigators estimated such basic constants as the maximum rate of metabolism (V_{max}), the substrate binding affinity (K_m), and the blood:air partition coefficients (P_b) of the test substances.

THE GASTROINTESTINAL TRACT: ABSORPTION, METABOLISM, AND PRESYSTEMIC ELIMINATION

The Absorption Process

The major function of the GI tract is the ingestion, digestion, and absorption of nutrients as well as the excretion of nonabsorbable material and certain waste products. Anatomically, the GI tract may be regarded as layers of muscle cells supporting a single layer of mucosal cells. This apparent simplicity is deceiving, however, in that the various regions of the tract are in fact highly complex and specialized for their different functions. For this brief review, focus is placed on the aspects that are important in the absorption of toxicants.

The pioneering studies of Brodie and colleagues in the 1950s clearly established that the major mechanism by which nonionizable toxicants are absorbed from the GI tract is their passive diffusion through the lipoidal membranes of the mucosal cells (Hogben, 1971; Hogben et al., 1957, 1959; Schanker et al., 1957, 1958). The solubility of toxicants in the aqueous milieu of the lumen of the tract is also of obvious importance in that to be absorbed, a molecule must be kinetically free to reach the absorbing surface. Thus, particulate matter or toxicants strongly absorbed onto macromolecules at the absorbing surface may completely pass through the gastrointestinal tract without significant absorption.

The area of the absorbing surface is also a major factor in the uptake of xenobiotic compounds. The extent of involution of the mucosal surface varies widely in different regions of the tract and is greatest in the small intestine. As discussed here, the small intestine is the major site at which toxicants are absorbed into the body.

Anatomical Regions of the Gastrointestinal Tract

Buccal Cavity and Esophagus

The buccal cavity and the esophagus are the sites of the initial processing of food and liquids. These tissues represent about 12% of the length of the tract in humans and have a relatively high blood supply. As a general rule, absorption of toxicants from the buccal cavity is considered to be minimal, largely because of the short time most materials remain in this initial section of the tract (Beckett and Hossie, 1971). One important feature of this site, however, is that the venous blood supply drains from this top portion of the GI tract directly into the systemic venous system. That is, it does not enter the portal vein and hence does not first pass through the liver before reaching the general circulation.

Stomach

This region, which accounts for approximately 7% of the total length of the GI tract in humans, is specialized for the processing and digestion of food (Davenport, 1982). The inner surface of the stomach is involuted, thereby increasing the absorbing surface. However, the lumen is large relative to other areas of the tract; the overall ratio of lumen volume to mucosal surface area is lower than in the small intestine.

The length of time material stays in the stomach is highly variable. The gastric emptying time (between ingestion and elimination from the stomach) depends on the food content, the nature of the food, and the general muscular tone of the stomach. With a few exceptions, the most notable being ethanol, the absorption of toxicants from the stomach is usually minimal. However, it is likely that appreciable amounts of VOCs would be absorbed from the stomach when administered by intubation in an aqueous solution. Factors influencing gastric absorption include residence time, which may be short if the stomach is empty; the diluting effect of food; the availability of macromolecules for absorption and lipid as a solvent if the xenobiotic compound is ingested with or immediately after a meal; and, as noted above, the relatively low ratio of mucosal surface to lumen volume (Schanker, 1971).

Small Intestine

The small intestine has three distinct areas: the duodenum, the jejunum, and the ileum, which together account for approximately 30% of the length of the GI tract in humans. Anatomically, these areas are characterized by their highly involuted surfaces. These involutions (termed villi) provide the highest ratio of absorbing-surface-to-lumen volume found in the GI tract. The pH of the lumen contents is significantly higher (about 7.5) than that of the gastric juice (Davenport, 1982, p. 119). Of particular importance is the entry of bile into the small intestine, where it emulsifies and otherwise promotes the dissolution of lipoidal toxicants. Collectively these factors—the high surface-area-to-lumen-volume ratio, the moderate pH, and the presence of bile—act together to provide the most favorable environment for absorption.

Blood draining the stomach, small intestine, and colon passes through the hepatic portal vein. As noted below, absorbed nutrients and toxicants in the portal vein must traverse the liver before reaching the systemic circulation.

Colon and Rectum

Although the colon and rectum together are almost half the length of the gastrointestinal tract in humans, they are generally considered to contribute only minimally to the absorption of toxicants. Factors minimizing absorption in this region include a less favorable surface-area-to-lumen-volume ratio and the decrease in fluidity of the lumen contents as water is withdrawn.

Part of the blood supply draining the lower rectum and the anal canal passes directly into the systemic venous circulation and hence bypasses the portal circulation.

Presystemic Elimination

The amount of an orally ingested VOC that eventually reaches systemic target tissues depends largely on the physiochemical characteristics of the compound, which determine its rate of absorption across the membranes of stomach and small intestine, and on the extent to which it is degraded within the GI tract, the liver, and the lung on its way to the systemic arterial circulation. Elimination before reaching the systemic arterial circulation is referred to as presystemic elimination.

Metabolism Within the Intestinal Lumen

Within the gut lumen, metabolic elimination may be catalyzed by the digestive enzymes of the host, by the enzyme content of crypt cells that have been shed from the villi, or by the enzymatic activities of the intestinal flora. Except for a few toxicants that structurally resemble normal nutrients such as peptides, lipids, and carbohydrates, the host digestive enzymes are generally considered to be unimportant in presystemic elimination. Mucosal cells, shed in humans at an estimated rate of 250 g/day (Davenport, 1982), are known to have significant xenobiotic-metabolizing activity (see below) and probably contribute to metabolism of xenobiotic compounds in the lumen. Little attention has been paid to this source of lumen activity, however, and its quantitative importance is largely unknown.

A greater role is played by the enzymes produced by the flora of the gut. Microorganisms within the lumen of the intestine represent a dynamic and diverse population, the nature of which varies widely among host species and within a species, depending on its location within the GI tract. For example, the stomach and small intestine of the rat support relatively high populations of enterobacteria, lactobacteria, enterococci, and bifidobacteria, whereas these regions of the fasted healthy human intestine may approach sterility. Generally speaking, bacterial counts increase with distance down the intestinal tract.

In contrast with the metabolic activity of the host tissues, the major enzyme activities of the flora are hydrolytic and reductive in nature. Oxidative reactions occur, but they tend to be minor in extent and are often restricted to specific types of compounds. It is these oxidative processes that initiate the metabolism of most VOCs.

The reductive enzymes catalyze the reduction of a variety of alcohols, aldehydes, ketones, azo compounds, hydroxylamines, and nitro derivatives. Reduction of nitro groups in this group of enzymes is of particular interest, since this activity appears to be absent from host tissue. For example, the conversion of the nitro-containing antibiotic chloramphenicol to its amine derivative appears to be entirely due to the action of enzymes of the intestinal flora.

Metabolism by Gastrointestinal Mucosa

The ability of the GI mucosa to metabolize a variety of toxicants is now well established. As with the flora of the lumen, however, the quantitative importance of this metabolism is less well defined.

The metabolic reactions in the intestinal mucosa are generally similar to those of the liver. The major differences are quantitative with respect to both the specific activities of the individual enzymes in the two tissues

and, of course, the weight of the two organs. Metabolic reactions found in the mucosal cells include typical Phase I activities, such as oxidation of aliphatic and aromatic carbons, *N*- and *O*-dealkylation, *S*-and *N*-oxidation, and desulfurization. Phase II reactions are equally well represented and include glucuronide, sulfate, glutathione, and glycine conjugation and *N*-acetylation. Epoxide hydrolase and a variety of hydrolytic activities are also present.

Cytochrome P450 and associated Phase I oxidative activities in the intestine are only approximately 2% of that found in the liver (Vainio and Hietanen, 1980). Phase II activities, such as uridine diphosphate (UDP)-glucuronyl transferase and 3'-phosphoadenosine-5'-phosphosulfate (PAPS)-sulfotransferase, may occur in relatively greater amounts (up to approximately 10% to 15% of liver levels), whereas others, such as reduced glutathione (GSH)-transferase and epoxide hydrolase, occur to a considerably lesser extent (0.5% of liver levels). However, these relative percentages may be misleading when comparing the contributions to xenobiotic clearance made by the two organs. During absorption, virtually all the toxicant must traverse the mucosal cell barrier and, hence, be exposed to the metabolic activity within these cells. In contrast, during passage through the liver, some part of the xenobiotic dose may remain associated with plasma proteins and be excluded from the parenchymal cells of the liver. Relatively speaking, the metabolic machinery of the mucosal cells may have greater access to the toxicant than the liver does, and clearance in the mucosal cells may be disproportionally greater. VOCs, however, do not seem to be restrictively bound to plasma proteins.

This concept should be extended further. Since the concentration of toxicant in the mucosal cells may transiently become very high during absorption, the enzymes of the mucosal cells may become transiently saturated or, and perhaps more likely, become capacity-limited due to the cell's inability to generate essential cosubstrates such as PAPS and acetyl coenzyme A at a sufficiently high rate. These two possibilities—a greater-than-expected organ clearance and a greater likelihood of capacity limitation—point to the complexity of assessing the role of intestinal mucosa in the metabolism and toxicity of xenobiotic compounds. Major change in the relative importance of these two factors may be expected as the dose of toxicant is increased. At low doses or concentrations, first-pass clearance by the mucosal cells may be a major component of body clearance, comparatively small amounts of toxicant penetrating to the systemic arterial circulation. At higher doses or concentrations, however, nonlinear behavior is to be expected, and much greater proportions of the toxicant could reach the general circulation and, hence, target tissues. These intestinal first-pass effects are very likely to have a substantial influence on the uptake of well-metabolized VOCs ingested at low concentrations.

METABOLISM IN THE LIVER

The liver constitutes approximately 2% of the body weight of the adult human and about 4% of the adult rodent. It is an unusual organ in that it receives both an arterial (hepatic artery) and venous (portal vein) blood supply. Of the total blood flow (approximately 1 ml/min/g liver), an estimated 70% to 80% arrives via the portal vein. It is particularly noteworthy that the portal vein drains more than 90% of the length of the GI tract, including the bulk of the absorbing surface of the intestine. Anatomically, most orally ingested VOCs must pass through the liver to reach the systemic arterial circulation.

Within the liver, blood from both arterial and venous supplies traverses the sinusoids (incompletely lined channels between the parenchymal cells of the liver) before exiting via the hepatic vein. This arrangement ensures that toxicants sufficiently lipophilic to have gained entry across the mucosal membrane will rapidly equilibrate into the parenchymal cells and become available to the xenobiotic chemical-metabolizing enzymes there. Metabolism of a toxicant during this first passage through the liver is a major component of the presystemic elimination of toxicants.

The metabolic capability of the liver for the elimination of toxicants is well known and has been described extensively elsewhere (for a general review, see Goldstein et al., 1974; Testa and Jenner, 1976). Briefly, two groups of reactions are recognized. The first, termed Phase I metabolism, is usually oxidative in nature and acts to insert or reveal a polar function in the toxicant. The second, or Phase II metabolism, acts to conjugate such polar groups with endogenous, highly water-soluble compounds, such as glucuronic acid and inorganic sulfate. In only two steps, Phase I and II metabolism usually converts highly lipophilic toxicants that cannot be excreted in the kidneys to highly hydrophilic derivatives that cannot be retained in the body.

Phase I reactions oxidize toxicants and are usually catalyzed by enzymes dependent on cytochrome P450. Phase II reactions include a wide range of activities. In addition to glucuronide and sulfate conjugation, activities in this category involve addition of the sulfur in glutathione and the oxygen in water to arene oxides, glutathione substitution reactions, conjugation of carboxylic groups with the nitrogen of glycine, and the acetylation of basic groups. Of particular importance, all these reactions require endogenous cosubstrates that, with the exception of water required for epoxide hydrolase activity, are capable of being depleted during the metabolism of xenobiotic compounds.

The fraction of a dose of VOC that penetrates to the systemic arterial circulation (F) may be expressed in terms of the fraction extracted by the liver during the first pass (E). Thus,

$F = 1 - E$.

Furthermore, since liver clearance (Cl_l) is the effective volume of blood from which the toxicant is completely removed per unit time, hepatic clearance (Cl_l) is given by the product of the blood flow to the liver per unit time (Q_l) and the fraction extracted (E). Thus,

$Cl_l = Q_l E$.

The fraction extracted (known as the extraction ratio) depends on several factors, including the ratio of unbound to bound (to plasma proteins) toxicant, mass transfer and permeability terms, and the intrinsic clearance of the toxicant in the liver (Cl_{int}). During the latter, clearance occurs under conditions that do not limit the rate at which the toxicant is delivered to the surface of the metabolizing enzymes, i.e., when the tissue substrate concentration is much below the apparent binding constant (see the second equation in this appendix). From a biochemical viewpoint, the free intrinsic clearance under first-order conditions may be estimated by dividing the maximal velocity of the enzyme reaction (V_{max}) by the apparent Michaelis-Menten-Henri binding constant (K_m). Thus, the intrinsic clearance is equivalent to the first-order rate constant for the enzymic reaction (see the second equation in this appendix) as Cl_l approaches zero.

Rearrangement and substitution of these and associated mathematical expressions lead to the following relationship:

$$F = \frac{Q}{Q + Cl_{int}} ; Cl_{int} = \frac{V_m}{K_m} .$$

Since intrinsic clearance can be related to the enzyme parameters V_{max} and K_m, this expression indicates that under first-order conditions, the fraction of the dose reaching the systemic arterial circulation depends on flow of blood to the liver and the metabolic capability of the liver to remove that drug.

Several important consequences follow from this relationship. First, the coadministration of an inhibitor or a competitive substrate for a major metabolic elimination pathway of a potentially toxic substance would lead to a decrease in the pathway's intrinsic clearance and a major increase in systemic availability. Thus, a dose of substance normally considered safe could, under appropriate circumstances, become highly toxic. Conversely, induction of the xenobiotic chemical-metabolizing enzyme (that is, an increase in V_{max}) would act to decrease systemic availability. Because both the constituent level and inductive capacity of hepatic metabolism are under genetic control in humans, there may be a great variation in intrinsic clearance, and the activity of specific pathways for specific toxicants in some subsets of the population may be very low. Thus, some persons

may have very low intrinsic clearance rates for specific xenobiotic compounds and may exhibit atypical systemic availability and toxic response.

When intrinsic clearance is very high relative to liver blood flow, the relationship reduces to:

$$F = Q/Cl_{\text{int}}.$$

Since the intrinsic clearance for a given toxicant and person may be regarded as constant, the systemic availability will depend largely on liver blood flow. Thus, drugs that alter blood flow may influence availability, whereas induction or even partial inhibition may have little effect. Conversely, for toxicants with intrinsic clearances that are low relative to blood flow, availability may be very sensitive to induction or inhibition effects and resistant to blood-flow alteration.

In recent years, it has become increasingly apparent that most VOCs are well metabolized by hepatic oxidation at appropriate concentration ranges. The term *well metabolized* refers to the condition where Cl_{int} is much greater than liver blood flow. Andersen (198 1a) has provided a list of VOCs that show this behavior. Although these compounds are well metabolized at low concentrations, the enzymes have only moderate maximum velocities and are readily saturated. The earlier idea that VOCs were poorly metabolized came from experiments in which high oral doses were administered and large amounts of VOC were eliminated unchanged. As noted earlier, this does not indicate lack of metabolism but, rather, saturation of metabolism and an increased relative clearance by exhalation (see Equation 3). Even many anesthetics are now known to be well metabolized. The V_{max} for the oxidative metabolism of halothane in a 250g rat is approximately 2.5 mg/hr. At low inhaled concentrations, its intrinsic clearance is greater than hepatic blood flow (Andersen, 1981a; Gargas and Andersen, 1982). However, its metabolism becomes saturated at an inhaled concentration of 100 ppm, which corresponds to an arterial blood concentration of only 2 mg/liter. Not surprisingly, when humans or animals were exposed to anesthetic concentrations, about 1,500 ppm, most absorbed halothane was eliminated in exhaled breath as the parent chemical. More recent, as-yet-unpublished studies by Gargas and associates show clearly that diethyl ether is also well metabolized by microsomal oxidation at inhaled concentrations below 200 ppm (M. E. Andersen, Air Force Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio, personal communication, 1985). In fact, most VOCs have large intrinsic clearances and will be subject to very significant first-pass elimination upon oral ingestion.

Appendix B :

Definitions of Symbols and Abbreviations

Symbol or Abbreviation	Units	Definition
AUBC	mg/liter x hours	Area under the blood concentration-time curve
AMEFF	mg/liter	The effective concentration of reactive metabolite formed in a compartment of specified volume
AURMC	mg	Area under the rate of metabolism curve
AUTC	mg/liter x hours	Area under the tissue concentration curve
AUTMC	mg/liter x hours	Area under the tissue metabolite time curve
bw	kg	Body weight
C	mg/liter or ppm	Concentration
Cl	liter/hr	Clearance
Cl_{int}	liter/hr	Intrinsic metabolic clearance
C_w	mg/liter	Water concentration of contaminant
E		Extraction ratio for an organ of elimination
F		Fraction of substance passing through an organ of elimination
K_m	mg/liter	Apparent Michaelis constant for substrate binding to metabolizing enzyme
k_r	hr^{-1}	Rate constant for pathway leading to reaction with critical cellular components

Symbol or Abbreviation	Units	Definition
P		Proportion of flow cleared by perfusion to organs of elimination
P_b	$liter_{air}/liter_{blood}$	Blood:air partition coefficient
Q_c	liter/hr	Cardiac output
Q_{ds}	liter/hr	Dead-space ventilation
Q_f	liter/hr	Blood flow in fat
Q_l	liter/hr	Liver blood flow
Q_p	liter/hr	Alveolar ventilation
Q_r	liter/hr	Blood flow in highly vascularized organs
Q_s	liter/hr	Blood flow in muscle and skin
Q_t	liter/hr	Total pulmonary ventilation
T	concentration	Toxic substance
TLV	concentration	Threshold limit value
TM	concentration	Toxic metabolite
V_d	liter	Volume of distribution
V_m	percent body weight	Muscle volume
V_{max}	mg/hr	Maximum rate of enzymatic reaction
V_w	liter	Water consumption in a given time period
y	ml/day	Intake

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7

Data on Humans: Clinical and Epidemiological Studies

The implications for human health posed by exposure to environmental contaminants, including those in drinking water, can be derived from studies in laboratory animals or in *in vitro* cell and tissue systems, from reports of clinical observations of isolated exposed individuals or experimental exposures and intervention studies in humans, or from direct observations of exposed human populations. To determine the effects on human health, the latter approaches are clearly preferable, but they are constrained by ethical considerations limiting experimentation to certain types of studies designed to prevent or treat disease. Thus, the data on humans generally fall into one of two categories: clinical data, which describe the effects of specific agents on certain individuals, and epidemiological data, which reveal patterns of disease or death in groups of humans exposed to single agents or to a variety of substances.

CLINICAL OBSERVATIONS: CASE REPORTS

Much of the knowledge concerning the human health effects of toxic materials comes from clinical information amassed after exposure to high doses, such as poisonings and industrial accidents. Such reports typically identify acute effects but can at times also describe chronic, delayed-onset diseases. For instance, Letz et al. (1984) reported two fatalities among workers following an acute inhalation exposure to ethylene dibromide that also produced liver and kidney damage. Such case reports are valuable for describing clinical and pathological effects in humans and suggesting underlying cause-and-effect relationships. When exposure levels can be

documented, they may help in defining dose-response relationships in humans. More commonly, broad comparisons are made, as in a study comparing elevated immunoglobulin levels in controls with levels in workers who had inhaled pentachlorophenol (Zober et al., 1981). However, it is usually not possible to link a specific exposure with specific illnesses, other than in illnesses with short latency periods (days or weeks), because the clinical features are not unique for many exposures. Thus the neurotoxicity of acrylamide, *n*-hexane, and methyl *n*-butyl ketone and the hepatotoxicity of 1,2-dichloropropane, pentachlorophenol, and ethylene dibromide (see Chapter 9) have been identified in humans largely because illness or adverse health effects, such as reduced reproductive capacity, occurred shortly after exposure.

In general, one can attribute delayed health effects to specific exposures only when there is a unique clinical manifestation. Toxic agents that might cause syndromes with clinical patterns that are not unique are unlikely to be associated with those syndromes. Occasionally, e.g., for Kepone-induced neuropathy (Cannon et al., 1978), a distinctive clinical pattern of illness as well as short latency reinforces a suspected exposure-response relationship. Other illnesses or health effects sufficiently unusual or distinctive to link disease and exposure include sterility in men (Whorton et al., 1977), a reduced proportion of male offspring following exposure to dibromochloropropane (DBCP) (Potashnik et al., 1984) (see Chapter 9), hepatic angiosarcoma following exposure to vinyl chloride monomer (Creech and Johnson, 1974), and the appearance of pleural mesotheliomas many years after exposure to asbestos (Wagner et al., 1960). Observations of similar illnesses in laboratory animals experimentally exposed to DBCP, 1,3-dichloropropene, bis(chloromethyl) ether, 4-aminobiphenyl, and vinyl chloride supplement the findings in humans. For several of these materials, the evidence for carcinogenicity was found first in test animals and later confirmed in humans (Davis and Mandula, 1985).

When there is both delayed onset of effects and a lack of distinctive clinical characteristics, risk assessments must be based on data from analytical epidemiological studies, clinical or experimental case reports, and extrapolation from laboratory experiments. Because of the long latency periods, difficulties encountered in making quantitative estimates of exposure, and problems in identifying and conducting follow-up studies of exposed populations, epidemiological data are unlikely to be available for most carcinogens and other chronic-disease agents found in drinking water until many years after first exposures. Clinical observations are thus important for suggesting potential exposure-disease relationships. They involve studying individual cases of illness or investigating cases with similar clinical features that appear to cluster in time and place or in relation to certain personal or exposure characteristics. A description of two cases

of lymphoma that occurred 6 years after accidental exposure to 1,3-dichloropropene (Markovitz and Crosby, 1984) illustrates the potential value of such hypothesis-generating clinical reports (see Chapter 9).

EPIDEMIOLOGICAL OBSERVATIONS

Most epidemiological studies conducted to date have focused on the evaluation of accidental or workplace exposures. There have been relatively few studies of humans exposed to contaminants in drinking water.

As an observational science, epidemiology attempts to assemble data answering the basic investigational questions: Who? What? Where? When? How? Who have been adversely affected by exposures? What did they do, or what were the substances to which they were exposed? Where did these events take place? When did exposure occur? How did the exposure come about? And how can the exposure be linked to the observed illness? For each of these questions, the student of human disease must consider whether the observed events could be explained in some way other than through exposure to the substance under consideration. In other words: Who else? What else? Where else? At what other times? In what other ways?

In laboratory studies in animals and in *in vitro* systems, strictly observed protocols can ensure that there are no confounding variables to cloud interpretation of the results of the specific exposure of interest. In contrast, many populations include people who smoke cigarettes, some people whose jobs involve exposure to some chemicals, some who live in rural areas, and others with different life-styles and, thus, exposure opportunities. The constraints people place on themselves (or that are placed on them) or the choices they make create a multitude of separate subpopulations that usually cannot be compared in simple ways.

Underlying many epidemiological studies is the assumption that exposure, however estimated, provides an adequate surrogate for the dose of a pollutant delivered in laboratory studies. Recent advances in molecular and biochemical epidemiology have clarified the definition of exposure along with the meanings of other terms used in the field of epidemiology. *Exposure* commonly refers to levels of pollutants measured externally in the physical environment. *Internal dose* indicates the quantity of the substance or its metabolites that reaches body tissues. *Biologically effective dose* designates the quantity of a pollutant or its metabolites that interacts with a particular target tissue or a surrogate for that target tissue (Perera and Weinstein, 1982). In this report, unless otherwise specified, the term *exposure* refers generically to all aspects of exposure, from levels in the surrounding environment to doses at a target tissue.

Early Studies

The beginning of modern epidemiology can be traced back to Snow's analysis of the patterns of cholera mortality in London during 1853 and 1854, which linked health risks to consumption of polluted water (Snow, 1855). Using available mortality data, Snow was able to pinpoint a source of the disease and recommend a means to neutralize it (removing the handle of the water pump) 28 years before Koch identified *Vibrio cholerae* as the cholera bacillus in 1883 (Black, 1980). Snow's study had two major parts: detailed observations concerning an explosive local outbreak attributed to infected water that appeared to come from a single neighborhood pump and a comparison of death rates in two intermingled populations that were distinguished only by their use of two different sources of drinking water—one relatively polluted and the other not. Snow's work underscores a basic feature of epidemiological evidence: although it depends upon observations of individuals, such data can only be interpreted epidemiologically in the context of the exposed population as a whole.

Descriptive Epidemiology

In looking at the occurrence of disease in a population, epidemiologists often begin by describing the specific disease and the conditions that previous knowledge suggests may be related to it. Attempts are made to discover if the disease incidence has increased over time and whether specific segments of a population are especially affected. By comparing increases in disease incidence with changes in possibly related exposures, hypotheses are generated for testing in more clearly focused studies. Geographical and demographic characteristics (age, sex, race) are also considered to sharpen the hypotheses to be examined. For example, some cancer mortality rates were higher in Louisiana parishes that obtained nearly all their drinking water from the lower Mississippi River basin in contrast to parishes that consumed little such water. This finding supported the need for further testing of an exposure-disease hypothesis (Page et al., 1976). Among the major contaminants found in the river water were the trihalomethanes. Reviewing 14 epidemiological studies relating cancer mortality to drinking water containing trihalomethanes, Williamson (1981) found some mixed evidence of increased risks of bladder, colon, and rectal cancer (see also NRC, 1980, pp. 6-7).

Epidemiological research on arsenic contamination of drinking water has also produced differing results. For example, in a geographic area of Taiwan with a stable population, many people have been exposed to drinking water with high arsenic levels for 40 years (Tseng, 1977; Tseng et al., 1968). A similar long-term exposure was documented in a popu

lation living in Antofagasta, Chile (Borgoño et al., 1977). People in both of these populations experienced endemic chronic arsenic poisoning, which was associated with elevations in skin cancer. After the installation of a water treatment plant in Chile, disease patterns changed, further strengthening the link between cause and effect. However, smaller arsenic-exposed populations observed for shorter periods in Alaska (Harrington et al., 1978) and Oregon (Morton et al., 1976) had no significantly elevated risk for skin cancer. These different findings may reflect differences in the respective study designs.

Two major limitations in descriptive studies are common to other study designs as well: the lack of specific information over periods of time on drinking water contamination and the inability to control for potential confounding variables, such as cigarette smoking, workplace or other indoor exposures, and nutrition. Hence, the multiple regression analyses used in descriptive studies are best suited to generating hypotheses for additional, controlled investigation. Evidence from such studies must be weighed against the presence of many other suspected or known causes of cancer (DeRouen and Diem, 1975) and against the criteria for consistency and plausibility elaborated below. In the meantime, the hypothesized relationship between exposure to carcinogens in drinking water and cancer patterns is undergoing serious examination.

Exposure assessment is a pivotal part of environmental epidemiology. For example, the National Human Monitoring Program of the U.S. Environmental Protection Agency assessed exposure of 21,000 subjects to selected pesticides through analysis of blood and urine specimens (Murphy and Harvey, 1985). These data were collected as part of the Second National Health and Nutrition Examination Survey (NHANES II)—a 4 year study conducted by the National Center for Health Statistics to collect dietary intake data as well as clinical, biochemical, and anthropometric data to determine the health and nutritional status of the U.S. population. Statistically weighted results of the blood serum analyses indicated that the general population is being exposed to some of these pesticides. For example, NHANES II data indicated that 31% of the 12- to 74-year-old subjects living in the Northeast, Midwest, and South had been exposed to *p,p′*-dichlorodiphenyltrichloroethane (DDT) (median level 3.3 ppb); 99% had been exposed to *p,p′*-dichlorodiphenyldichloroethylene (DDE), a metabolite of DDT (median level 11.8 ppb); and 13.9% had been exposed to β-benzene hexachloride (median level 1.7 ppb) (see Figures 7-1 through 7-3).

Analytical Epidemiology

Follow-up epidemiological studies are generally grouped under the heading *analytical epidemiology*, although all epidemiological studies include anal

ysis. Analytical epidemiology studies include case-comparison studies, cohort studies, some mixtures of the two, and experimental (or intervention) studies. Recognizing that descriptive epidemiological studies do not provide proof of cause-and-effect relationships, Hill (1953) proposed several criteria for examining the resultant data and strengthening the conclusions derived from them. Did the putative cause precede the observed effect in time? Was the observed effect pervasive, and did it occur in a large segment of the population? Was it seen at more than one time and in more than one place? After intervention, was a reduction in exposure

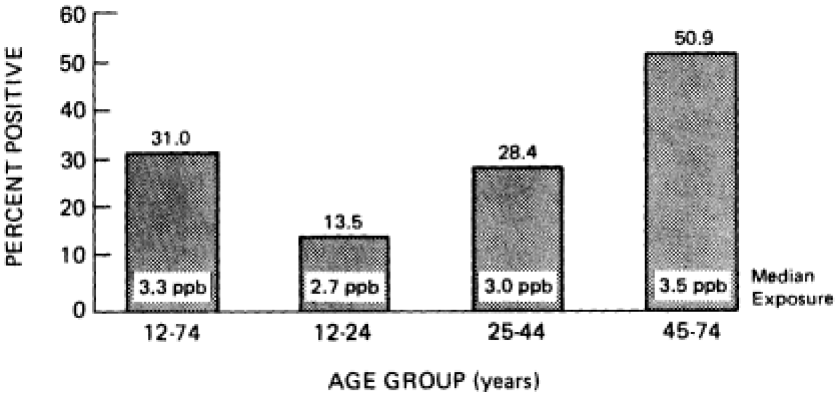

Figure 7-1
Percent positive and median *p,p'*-DDT levels in blood serum for positives by age. Limit of detectability, 2 ppb. Data for Northeast, Midwest, and South only. Data from Murphy and Harvey, 1985.

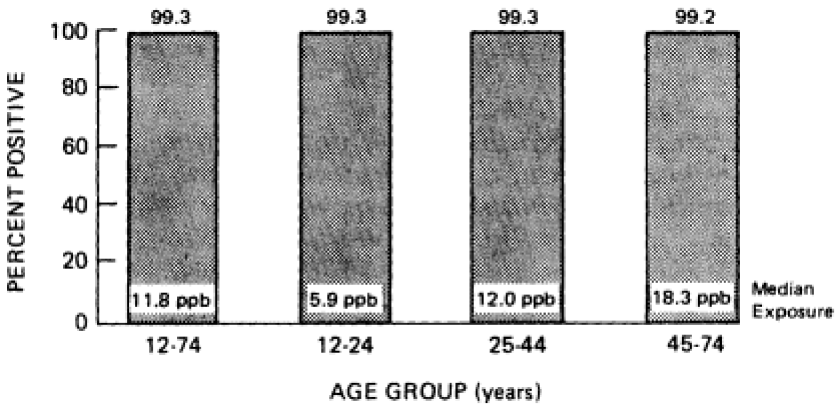

Figure 7-2
Percent positive and median *p,p'-DDE* levels in blood serum for positives by age. Limit of detectability, I ppb. Data for Northeast, Midwest, and South only. Data from Murphy and Harvey, 1985.

followed by a reduction in disease? Is there a reasonable biological basis for linking the presumed cause and effect? Is the effect consistent among studies? Is it not explainable by other known or suspected causes? Is the effect specific to this cause, and does this presumed cause lead only to this effect? These last considerations do not apply to diseases that may have multiple causes—as, for example, cancer—or to single exposures that can lead to several end results (e.g., cigarette smoking, which can lead to different kinds of cancer, low birth weight of infants, and heart and circulatory illnesses).

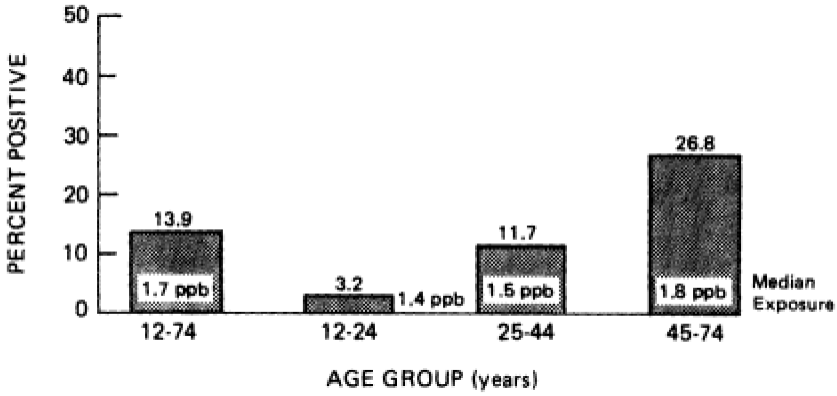

Figure 7-3
Percent positive and median b -benzene hexachloride levels in blood serum for positives by age. Limit of detectability, 1 ppb. Data for Northeast, Midwest, and South only. Data from Murphy and Harvey, 1985.

Case-Comparison and Cohort Studies

Epidemiological studies can attempt to discover the etiology of a disease by starting from either the exposure or the biological effect. Thus, tests of hypotheses suggested by descriptive studies can start with illnesses and evaluate different exposure histories of the sick and the well (case-comparison studies), or they can first observe the patterns of exposure in populations and then note whether the more extensively exposed develop more (or more serious) illnesses than the unexposed or less exposed (cohort studies). When the nature of exposure and disease permit, both kinds of observations may be made concurrently (cross-sectional studies), as with sperm counts measured in DBCP production workers (Whorton et al., 1979).

Case-comparison studies are useful in that they can be performed in small groups of subjects over relatively short periods. For instance, in a case-comparison study conducted in rural South Australia on 218 matched

case-control pairs, an association was found between maternal prenatal groundwater consumption and congenital malformations of the central nervous system and musculoskeletal system (Dorsch et al., 1984). Women who bore a malformed child were three times more likely to have consumed groundwater during pregnancy rather than rainwater. Further analysis showed an increased risk associated with nitrate concentrations in drinking water. Women who drank water containing 5 to 15 ppm nitrate had a nearly threefold increase in risk, and those who drank water containing concentrations higher than 15 ppm had a risk four times greater than that of controls.

Case-comparison studies deal only with a specific illness or cause of death. Thus, they are unable to determine whether the exposure under study is capable of increasing or decreasing the incidence of other illnesses or other causes of death. These studies also move backward in time; i.e., attempts are made to associate changes in morbidity or mortality rates with some previous exposure alteration.

In contrast, cohort studies move forward in time. However, such studies are more difficult to use when considering rare disorders such as some cancers, since very large sample sizes are often needed to detect even large excesses in risk. In some situations, morbidity or mortality data assembled in a cohort study can provide the basis for case-comparison studies within that study ("nested" case-comparison studies), thereby enabling investigators to make more detailed assessments of risk (Marsh, 1983).

Experimental or Intervention Studies

When a well-defined cause-and-effect relationship has apparently been demonstrated, it is sometimes possible to test the presumed relationship by eliminating or reducing the cause or causes. The elimination or reduction can be part of a well-designed randomized trial—as in the MRFIT (Multiple Risk Factor Intervention Trial) studies of the National Heart, Lung, and Blood Institute (Tillotson and Hulley, 1985) or in studies of the effect of fluoride-treated drinking water on health. A variety of epidemiological studies have documented a number of health effects of fluoridation. Low levels of fluoridation prevent tooth decay (Arnold et al., 1953; Carlos et al., 1962; Dean et al., 1950) and osteoporosis (Bernstein et al., 1966) while increasing bone density (Leone et al., 1955, 1960). However, excessive levels of fluoride can produce fluorosis (Geever et al., 1958a,b; Leone et al., 1954).

Evaluations of the effects of intervention are usually based on time-trend or time-and-place considerations, such as whether the rate has declined following the intervention, allowing enough lag time for effects to

develop. Thus, the epidemiological process comes full circle, starting with descriptive epidemiology suggesting possible cause-and-effect relationships, then to uncovering these relationships through case-comparison and cohort studies, to specific interventions (both planned and unplanned), to the evaluation of these interventions through descriptive epidemiological techniques to verify the effect in a controlled trial.

PRINCIPLES, PROBLEMS, AND LIMITATIONS OF EPIDEMIOLOGY

To conduct a satisfactory epidemiological study whose results would be of material use in risk estimation, consideration must be given to four major criteria:

- The characteristics of the study population must be suitable for the goal of the study.
- The studies must be designed and conducted in a manner that ensures unbiased answers.
- The studies should be large enough to have the power needed to detect small but important effects.
- The exposure data should be of sufficient quality to permit estimation of dose-response relationships.

Several aspects of epidemiological studies affect the above criteria. In an effective study, the study population must be large enough to have a high probability of detecting a true effect as "statistically significant," if it is present. Ideally there should be exposures of consequence only to the substance in question, and no other elements capable of affecting the frequency of the same disease should be present. A control population—ideally comparable in all respects except for exposure to the suspect material—should be available for comparison.

In reviewing the epidemiological evidence on 75 substances used in active commerce that had been found to be carcinogens in laboratory animals, Karstadt et al. (1981) found that fewer than one-third had been or were being subjected to effective epidemiological research. In the industrial settings, they found only small groups of exposed workers and workers who had been exposed to more than one agent. Often, active use of the material had been discontinued, or it had been used so recently that not enough time had lapsed to expect that a meaningful number of cancers would have developed (see also Karstadt and Bobal, 1982). In reviewing the possibilities for conducting epidemiological studies on di(2-ethylhexyl) phthalate, the National Institute for Occupational Safety and Health found similar conditions and did not undertake such a study (Roberts, 1983).

TABLE 7-1 Possible Study Outcomes

	Empirical Finding	
Actual Conditions	Positive	No Effect
Positive	True positive	False negative (Type II error: β)
No effect	False positive (Type I error: α)	True negative

As noted earlier, for valid inferences to be made regarding risk of illness in relation to specific exposure, the populations being examined should ideally be comparable in all respects except for exposure to the particular characteristic under study. In his study of cholera, Snow (1855) achieved comparability by the fortuitous design of the natural experiment he observed: households receiving either polluted or unpolluted water were side by side in the same community and presumably indistinguishable in all other respects. Thus, factors such as differences in socioeconomic status and place of residence were not present to distort the association between etiological agent and biological effect and thus lead to mistaken conclusions. Some statistical techniques permit adjustment when strict comparability is not achieved, but they are not always successful.

Statistical Significance and Power

There are four possible outcomes to any epidemiological study, as indicated in Table 7-1. The study can report an effect (i.e., be positive) or report no effect (the so-called negative study). In reporting an effect or the absence of one, a study can be accurate, i.e., give a true-positive or a true-negative result. If the study produces erroneous findings, it gives a false-positive or a false-negative result. A false-positive result asserts that increased risk exists when in fact it does not (known as a Type I error). A false-negative result asserts that increased risk does not exist when in fact it does (a Type II error). The probability that a study will make a false-positive error is the same as the level of statistical significance or *p*-value given in most reports as 0.05 or less when single comparisons are being made. It is the probability that the event noted could be due to chance alone and not to any cause-and-effect relationship.

The probability of detecting as statistically significant an effect if it is truly present is the *power* of a study. As power increases, the chances of producing a false-negative error decrease. Power is determined jointly by the level of relative risk being assessed, i.e., the magnitude of the effect, and by the number of cases that would be expected in the exposed pop

ulation if there were no increased risk. This expected number depends on the sample size and expected disease frequencies in some appropriate comparison populations. Studies of small populations with common diseases can have the same power as studies of rare diseases in large populations.

TABLE 7-2 Power for Detecting Different Levels of Increased Relative Risk in Relation to Numbers of Expected Cases[a]

Number of Expected	Relative Risk[b]				
Cases	1.2	1.5	2.0	3.0	5.0
1	0.073	0.116	0.208	0.428	0.796
5	0.111	0.262	0.582	0.948	1.000
10	0.149	0.411	0.836	0.999	1.000
25	0.245	0.726	0.994	1.000	
50	0.384	0.937	1.000		
100	0.605	0.998			
200	0.854				

[a] Davis et al., 1985.

[b] Calculations made using one-sided test and significance level of 0.05.

Values of power for different combinations of expected numbers of cases and relative risk values are shown in Table 7-2 (Davis et al., 1985). Not surprisingly, studies of larger samples, and therefore more expected cases, have sufficient power to detect smaller increases in risk. Conversely, studies of small samples (and few expected cases) will have power sufficient to detect only large increases in relative risk. For example, a small study, in which only one case may be expected, will have approximately 0.80 power to identify correctly a relative risk of 5.0 (a fivefold, or 400%, increase in risk), but only about 0.07 power to identify a relative risk of 1.2 (a 20% increase in risk) at a significance level of 0.05. A study with a sample size large enough to expect 200 cases, however, will have about 0.85 power to detect a 20% increase in risk. In designing experimental studies, power should be set at about 0.90 to 0.95. When it is likely that a study will be conducted only once, or at best a few times, as is sometimes the case for epidemiological studies, higher power should be sought in the original design. (For further discussion on determining power see Cohen, 1977.)

The finding of an association between hepatic angiosarcoma and exposures of vinyl chloride polymerization workers illustrates that even small epidemiological studies (in essence, clinical observations of a few people) may have sufficient power to detect a very large relative risk (Creech and Johnson 1974; Heath et al., 1975). In that instance, four cases of hepatic angiosarcoma were epidemiologically sufficient, since the rate of that rare

tumor was several hundred times greater than expected. With this exceptional relative risk, these studies had powers close to 1.0. The biological importance of the finding was greatly enhanced by the earlier independent demonstration that vinyl chloride was carcinogenic in laboratory animals (Maltoni and Lefemine, 1974).

In contrast is the study by Ott et al. (1980), who examined risk of cancer in workers exposed to ethylene dibromide (see Chapter 9). Here, failure to detect an increased risk may be related to the limited sample size (161 workers studied, 5.8 cancer cases expected) and thus, limited power. The study had 0.29 power to detect a relative risk of 1.5—that is, a 50% increase in risk (see Table 7-2 and Apfeldorf and Infante, 1981). The concept of power is especially important in interpreting so-called negative studies. When a specific health effect has been examined in several different epidemiological studies, as they were for studies of health effects from exposure to arsenic in drinking water, results may often seem to be in conflict, some studies yielding positive results (i.e., statistically significant increases in observed risk) and others showing no association or negative results (i.e., nonsignificant variations in levels of observed risk). In this situation, it is important not only to assess the studies for features that might compromise validity or distort analyses but also to examine the power of negative studies to determine if their sample sizes were too small to detect increased risk at the levels medically or biologically worth discovering or observed in positive studies. If power is insufficient, such negative studies cannot be said either to contradict or to support the conclusion that increased risk does in fact exist. Confidence limits based on the excess risk estimates of these small studies can sometimes be regarded as upper limits unlikely to be exceeded in a study of sufficient size and quality. Conversely, of course, studies yielding statistically significant positive results may be found to be false positive.

As examples, short-term studies of skin cancer patterns in small populations exposed to elevated arsenic levels are not likely to have sufficient power to detect an effect (i.e., Harrington et al., 1978). In contrast, the National Cancer Institute is currently analyzing the results of a large case-comparison study on 3,000 cases and 6,000 controls for bladder cancer (Cantor et al., 1985). This study includes data on drinking water. However, if the relative effect of drinking water is rather small, then even this large study will have a low power of detecting any such effect.

Study-Related Limitations

Additional limitations associated with epidemiological studies derive from the intrinsic characteristics of diseases under study. One example is

the difficulty in associating exposure with effect because of the long and variable latency periods for manifestation of chronic illnesses, such as cancers (which are of particular concern because of their potential relationships to low-dose toxic exposures). Moreover, clinical features of chronic illnesses rarely provide any clues to specific etiology. Cases of cancer linked to ionizing radiation, for instance, are usually clinically indistinguishable from those linked to chemical exposures. For some diseases, many agents may lead to the same illness, thus precluding direct linkage of particular cases to particular toxic agents.

It is also difficult to identify suitable control populations. In contrast to laboratory experiments, variables such as nutrition, medication use, or cigarette smoking cannot be easily controlled or matched in populations under epidemiological study. Accurate information on such variables must be collected if they could be potentially confounding elements (Davis et al., 1983). For example, it is inappropriate to try to estimate the risk of lung cancer in a group of smokers in comparison to a general population that includes a substantial number of smokers and former smokers. In addition, comparisons confined to people aged 20 to 65 exclude many people with longer exposures to some toxic chemicals. In view of the fact that half of all cancer cases arise in people over 65, such inappropriate comparisons dilute the real effect of exposure. McMichael (1976) suggested that occupational risk may be understated by as much as a factor of two when using the general population as a control group. For example, it is frequently asserted that the favorable mortality experience of some occupational groups is due to a healthy worker effect. That is, those at work have a lower prevalence of chronic diseases or lower mortality than does the general population, which includes persons not at work due to incapacitating illness.

Epidemiological studies are susceptible to certain biases, which must be minimized. Of particular importance to case-comparison studies is response bias or recall bias. Persons with an illness or with known exposures tend to remember events associated with those illnesses or exposures better than do persons not ill or not aware of the exposure. However large or otherwise well designed an epidemiological study may be, its capacity to provide sound answers to questions of biological risk depends on the accuracy of the data collected. The report of a study must state the extent to which determinations of exposure levels and biological outcomes may be imprecise, subjective, unverified, or unverifiable. For some exposures, the responses may be subjective symptoms that are often rather hard to establish and measure effectively. The greater the uncertainty of exposure or outcome data, the less likely it is that a true association or effect will be correctly identified.

Inference and Quantification

Epidemiological data usually lack the incisiveness of laboratory data, making it difficult to assert with assurance that an epidemiological study has furnished proof of some cause-and-effect relationship. As noted, there are often circumstances surrounding human exposure—sometimes extensive exposures—that do not permit epidemiological investigations. However, the lack of incisiveness can be offset under two sets of circumstances. First, when the effect is very large (as in the relationship between cigarette smoking and lung cancer), the effect can be clearly discerned above the background of confounding elements. Second, if one epidemiological study is possible, often more than one can be conducted. The observed cause-and-effect relationship gains credibility when similar results are obtained in several studies conducted under somewhat different circumstances and affected by different confounding variables of greater or lesser importance.

It is much easier to determine whether or not an effect is a consequence of some exposure than it is to quantify that response, that is, to answer the question: How much response for how much exposure? Many studies are able to distinguish between exposed and unexposed persons, but are unable to say how much exposure there was. This is largely due to the fact that industrial hygiene and environmental monitoring data were rarely collected in the past. Moreover, epidemiological studies necessarily include people who have had multiple toxic exposures. But scientists' understanding of the ways multiple toxic exposures affect health is very limited. This is reflected by the fact that no existing model of carcinogenesis is yet able to deal adequately with the possible interactions or synergism (or antagonism) associated with multiple exposures (see Chapter 5). Molecular or biochemical epidemiology, combining the precision of laboratory measurements of dose and early response with standard epidemiological methods, holds considerable promise in the elucidation of some of these problems (Hattis, in press; Perera and Weinstein, 1982). The molecular assays may elucidate which of the toxicants attain biologically effective levels, how the toxicants interact in the tissues, and what the levels of toxicants are in various organs.

Even for extensively studied materials, such as asbestos, exposure estimates frequently have been developed not from direct measurements of concentrations at time of actual exposure but rather from ingenious reconstructions of earlier working conditions (taking into account ventilation and other factors) in select study populations (NRC, 1984). For inadvertent or ambient exposures of the general population, estimates are even more difficult to obtain. People are often quite unaware of the exposures, if

any, they have had, and the range of exposures in a population may be great. The exposure history for individuals—whether intermittent or continuous, whether at relatively constant levels or with peaks and valleys—is rarely known. Yet, differences in exposure patterns may affect biological response. For example, the age at first or only exposure may also be of considerable importance. Young women exposed to ionizing radiation at Hiroshima were at higher risk of developing breast cancer than women who were older at the time of exposure (Land et al., 1980).

Nature of Populations Studied

Many useful epidemiological studies are conducted on industrial populations for which measurement of exposure can sometimes be obtained. Workers, however, are generally relatively young, healthy males whose exposures are limited to working hours and are governed by workplace conditions. Workers are sometimes able to remove themselves (i.e., find other jobs) if they find exposure disturbing—usually leaving the less affected, less disturbed, or less sensitive workers to receive the greater exposure. Therefore, when results of occupational exposure studies are used for risk assessment, they may not be entirely appropriate for estimating health risks in the general population exposed under different conditions. They may be especially misrepresentative for pregnant women, children, and elderly persons as well as for people with certain illnesses or biological sensitivities that may lead them to be excluded from industrial populations. As for ionizing radiation, the International Commission on Radiological Protection divides by 10 the occupational exposure levels for a 40-hour, 5-day work week in order to produce estimated safe levels for the general population (ICRP, 1977). This approach has not been uniformly applied.

Mechanisms of Biological Action

Knowledge of likely or possible biological mechanisms is of considerable value in evaluating the findings of specific studies. Unfortunately, these mechanisms are rarely understood in full. The model most often used in risk assessment computations for carcinogenesis, and which is used in this report, is the multistage model of Armitage and Doll (1954). This model, discussed in greater detail in Chapter 5, derives from the epidemiological observation that cancer incidence increases with age, implying that the generalized cancer process must be of a multistage nature that leads to rapidly increasing age-specific incidence and mortality curves. This epidemiological observation has a parallel in initiator-promoter lab

oratory studies and in the recent findings that two or more mutations are involved in some carcinogenic transformations (Slaga et al., 1980, 1982).

Knowledge of biological mechanisms has not always been essential for taking public health action. On the basis of an epidemiological association, for example, thalidomide was removed from European markets before its mode of action was completely understood (Sjöström and Nilsson, 1972). When greater biological information exists, however, the precision and depth of epidemiological observations can obviously be much enhanced and can lead to more specific public health measures.

RISK ASSESSMENT

Relative Risk

Epidemiological studies are capable of generating several types of data that might be used to assess risk and to develop public health policy. When there is reason to believe that the exposure-response relationship multiplies the background rates, the increase should be reported as *relative risk*, as with most studies of cancer. The standard mortality ratio (SMR) is a relative risk measure in which the observed number of deaths is compared with the number of deaths expected for a specified population with a comparable age distribution. The SMR is computed as 100 times the number of observed events divided by the number of expected events. Thus, an SMR of 100 implies no difference in risk from that expected in another comparable population. The expected number is usually derived by applying the rates for age-, sex-, and race-specific groups to the numbers of persons in the exposed population in the comparable age-, sex-, and race-specific groups, and then summing the products so derived. For example, the relative risk of death from lung cancer for a white male who smokes a pack of cigarettes a day is about 10 times greater than that of a corresponding nonsmoker. The SMR in this case would be 1,000.

Attributable Risk

Attributable risk can be derived from relative risk when there is information on the proportion of a population that has been exposed. It measures the maximal proportion of disease incidence in a population that can be attributed to a particular factor, such as cigarette smoking. Attributable risk is useful in suggesting how public health resources should be distributed, the assumption being that the higher the attributable risk, the greater will be the health rewards of controlling it.

Originally developed by Levin as a tool to aid decision-makers in understanding epidemiological data (Levin, 1953; Levin and Bertell, 1978),

this concept has gained wide acceptance and is now used extensively. The recent cancer prevention initiative undertaken by the Secretary of Health and Human Services, which advocates specific dietary and life-style practices, is based on assessment of the attributable risk of cancer estimated to be due to certain nutritional practices and cigarette smoking. For instance, up to 90% of the lung cancer cases among men in industrialized countries is now attributed to smoking (Davis et al., 1981; Doll and Peto, 1981).

Additivity

In some circumstances, the biological process resulting from an exposure may not appear to lead to a multiplication of background rates. At times, the risk may be simply a direct addition to, and completely independent of, the background rates. Some mathematical models are used in laboratory studies of carcinogenesis to estimate excess risks above background. The appropriateness of these models to humans will depend upon the specific agent-response interaction.

For low-level exposures, resulting in small increments in risk, the distinction between additive and multiplicative responses (i.e., between additive and relative risks) is not important and probably cannot be observed. Thus, if a relative risk for some demographic group is 1.2 and some exposure produces a further 20% increase in risk (i.e., another 1.2), under multiplicative conditions the risk would be 1.2 x 1.2, or 1.44. If the second risk were additive, the total risk would be 1.2 + 0.2, or 1.4, which is not significantly distinguishable from 1.44. Therefore, for low-level exposures, the increased risks due to additive and multiplicative processes are comparable.

Exposures to more than one substance may result in a synergistic response. For example, cigarette smokers who experience a lifetime of exposure to asbestos in the ambient environment have more than 10 times as much risk as asbestos-exposed nonsmokers of contracting lung cancer (NRC, 1984, pp. 211-222).

Application to Drinking Water Studies

On occasion, results of epidemiological research are useful in the development of drinking water standards. For example, epidemiological studies indicated that adverse health effects developed in humans exposed to foods or water supplies contaminated with lead, cadmium, and mercury (Calabrese, 1983).

Although drinking water constitutes a small percentage of total daily exposure to many substances, it may play a determinative role nonetheless.

TABLE 7-3 Epidemiological Studies of Compounds Reviewed in Chapter 9

Study Findings	References
Dibromochloropropane (*DBCP*)	
Azoospermia or severe oligospermia in DBCP production workers.	Egnatz et al., 1980 Lipshultz et al., 1980 Milby and Whorton, 1980 Potashnik et al., 1979 Whorton et al., 1979
Sperm count reductions in workers exposed during agricultural application of DBCP.	Glass et al., 1979 Sandifer et al., 1979
Sperm count reduction, elevated serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels, and reduced or absent spermatogenic cells in men with history of industrial exposure to DBCP.	Biava et al., 1978 Potashnik et al., 1979
Reduced sperm count, elevated FSH and LH, and decreased testicular volume in a population of workers exposed to DBCP.	Egnatz et al., 1980
Increased frequency of Y-chromosome nondisjunction in the sperm of DBCP-exposed workers.	Kapp et al., 1979
Increased number of sperm containing two Y-chromosomes in DBCP-exposed workers.	Kapp et al., 1979
Increased frequency of spontaneous abortions in wives of DBCP-exposed agricultural workers in Israel.	Kharrazi et al., 1980
No or little recovery in sperm production in DBCP-exposed workers who *ceased* spermatogenesis as a result of exposure. Complete recovery of sperm production in DBCP-exposed workers with *reduced* spermatogenesis when exposure was removed.	Lantz et al., 1981 Whorton and Milby, 1980 Whorton et al., 1979
No change in sperm count in agricultural workers exposed to DBCP concentrations up to 1.8 ppm. NIOSH concluded that a 1-ppm exposure level has no observable effects on male fertility.	NIOSH, 1978

Study Findings	References
Ethylene dibromide (EDB)	
No significantly greater mortality in workers employed at EDB synthesis plants between 1940 and 1976.	Ott et al., 1980
Standardized birth ratios not significantly reduced among EDB-exposed workers and their wives, regardless of amount of exposure.	Wong et al., 1979
Pentachlorophenol (PCP)	
Sweating, weight loss, and gastrointestinal disorders in factory workers exposed to PCP at a Winnipeg plant.	Bergner et al., 1965
Tachycardia, respiratory distress, and liver aberrations in neonates exposed to PCP in diapers and linen. Two of the 20 exposed died.	Robson et al., 1969
Elevated SGOT, SGPT, and LDH, and low-grade infections or inflammations of the skin, eye, and upper respiratory tract in chronically PCP-exposed workers in Hawaii.	Klemmer et al., 1980
No significant difference in chromosome aberrations between PCP-exposed workers and controls.	Wyllie et al., 1975
Plasma protein levels elevated in workers chronically exposed to PCP in Hawaii.	Takahashi et al., 1976
Higher immunoglobulin levels in PCP-exposed workers in the Federal Republic of Germany than in controls.	Zober et al., 1981
Trichlorfon	
Excess of short-lived chromosome breaks and exchange along with a significant increase in stable chromosome alterations in five people exposed to trichlorfon.	van Bao et al., 1974

This is illustrated by the studies of Calabrese and Tuthill (1977), who found that blood pressure measurements differed significantly in matched groups of students living in two communities with different levels of sodium in the drinking water. The investigators also conducted an ex

perimental, 3-month study in which bottled, low-sodium water was supplied to one of the high-sodium community subgroups of students with elevated blood pressure. The study demonstrated reductions in blood pressure, but only in girls (Calabrese and Tuthill, 1980). Since people who previously consumed large amounts of salt are now lowering their intake, additional monitoring of their blood pressure levels and sodium intake, including that from drinking water, could provide useful information regarding the effects of elevated levels of sodium in drinking water on human health.

Table 7-3 summarizes the epidemiological studies on the pollutants assessed in Chapter 9. The committee found no studies on drinking water exposure to the contaminants listed in the table, presumably because of the many limitations to epidemiological investigations discussed above. The committee was therefore forced to extrapolate data from studies of occupational, airborne, and skin exposures to estimate risks for exposure by ingestion of water. Some clinical reports did include exposure to these compounds through ingestion. Several of them related to accidental poisonings or suicides. Such reports can be especially valuable in suggesting important metabolic pathways in humans. As discussed in Chapter 6, knowledge of toxicokinetics derived from studies in humans may ultimately transform the process of risk assessment. In the meantime, epidemiological and clinical studies will continue to play a generally supportive role in the evaluation of risks to humans from drinking water contaminants.

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8

Risk Assessment

The Safe Drinking Water Committee evaluated a number of approaches to assessing the risks of a variety of health effects, including cancer, reproductive and developmental impairments, and neurological diseases. Among the major considerations in any of these approaches are the variety and extent of the variables that will be encountered. For example, different sources of data may be used in the risk-assessment process, ranging from short-term tests for mutagenicity to long-term epidemiological studies of humans. Other variables that make risk assessment difficult and sometimes subject to substantial variation include metabolic differences between species and the variety of ways that exposures can be delivered, e.g., multiple, sporadic, or peak. Other considerations include route of exposure (e.g., inhalation, dermal, or ingestion) and source of exposure (e.g., drinking water, workroom air, or food). In some circumstances, risk assessment can be enhanced when there is an opportunity to evaluate pharmacokinetic mechanisms involved in responses to environmental agents.

In this chapter, the committee first reviews the discussions of risk assessment set forth by previous Safe Drinking Water Committees. It then examines three topics common to all risk assessment: estimation of exposure from various sources and by different routes, pharmacokinetics, and interspecies extrapolation. The remainder of the chapter is devoted to a discussion of risk assessment for different categories of health effects. Cancer is examined first, because risk assessment in this area has the longest history and, therefore, the methods are more highly developed than those for other adverse health effects. However, many of these methods are germane to assessing the risk for noncancer end points as well. The last two sections cover risk

assessment for developmental and reproductive effects and for neurotoxic effects. Conclusions and recommendations drawn from all these discussions are presented at the end of the chapter.

To assess risks associated with exposure to toxic chemicals, agencies have developed a systematic scientific and administrative framework (NRC, 1983). The process commonly begins with the identification of a hazard—often brought to light by data from studies on laboratory animals, from other laboratory procedures, or sometimes from case reports involving humans. The next step is determination of the dose-response relationships between specific quantities of a substance and associated physical responses, such as the development of tumors, birth defects, or neurologic deficits. Then follows exposure estimation and assessment. At that time, a search is made for an answer to the question: To what dose levels or range of dose levels will human populations most likely be exposed? Finally, the dose-response model is applied to the expected exposure levels to produce a quantitative estimate of risk.

To date, quantitative risk assessment (QRA) has been used largely for estimating the risk of developing or dying from cancer, and has been used little in evaluating or estimating noncancer health effects of exposure to materials in the environment. The earliest approaches to QRA for cancer are probably those of Mantel and Bryan (1961). Modifications to these approaches have been published through the years. Recently, the U.S. Environmental Protection Agency (EPA) issued proposed guidelines for assessing the risk of exposure to carcinogens, mutagens, and developmental toxicants (EPA, 1984a,b,c).

A developing, interdisciplinary field, risk assessment is not without unresolved issues, including the extrapolation of results to doses outside the range of observations in the experiments; the choice of the appropriate dose-response model for extrapolating from high-dose animal data to the anticipated low levels of exposure of humans in the ambient environment; the appropriate translation of data from the laboratory animal to humans, which in turn involves questions concerning the influence of body size, life span, and possible metabolic differences; and the potential for confounding (e.g., whether there is synergistic or antagonistic response to exposures to other materials). Problems that lie at the interface of science and policy include selection of test method, selection of animal data and bioassay results for use as the basis for extrapolation to humans, determination of how one should use data on so-called benign as well as malignant tumors, selection of appropriate safety factors for developing standards, and selection of the mathematical model to be used for extrapolation.

QRA methods have been the subject of a number of publications (Anderson and CAG, 1983; California Department of Health Services, 1982;

OSTP, 1985), which will not be reviewed here. They can be used to provide a broad range of estimated risks for the purpose of setting priorities for regulatory action or to identify a specific acceptable or permissible level of human exposure to a carcinogen. Most experts and policymakers agree that current QRA techniques at best indicate a range of risks rather than a precise number. Since 1977, when the first Safe Drinking Water Committee conducted QRAs on waterborne carcinogens, National Research Council committees have consistently highlighted limitations of the methodology (NRC, 1977, 1980, 1983).

This committee endorses attempts to develop, validate, and apply QRA techniques to the evaluation of potential noncancer toxic responses, such as neurotoxicity, reproductive toxicity, and developmental toxicity, as well as liver damage, kidney damage, and respiratory responses other than lung cancer. During the last decade, cancer has been regarded as a nonthreshold phenomenon, whereas most noncancer responses are believed to require a minimum (i.e., threshold) dose before any toxic manifestation will appear. Some recent research has suggested, however, that this distinction may not be a clear one.

PREVIOUS SAFE DRINKING WATER COMMITTEES' VIEWS ON RISK ASSESSMENT

Earlier Safe Drinking Water Committees considered the problems of risk assessment in the first and third volumes of *Drinking Water and Health* (NRC, 1977, 1980). The present committee affirms many of the views expressed by those groups.

Issues Concerning Threshold

After reviewing the many problems inherent in assessing the risks of carcinogenesis and mutagenesis, the 1977 committee acknowledged that many scientists distinguish between injuries produced by chemicals likely to have a threshold dose and effects for which there is likely to be either no threshold (e.g., carcinogenesis and mutagenesis) or no way known to estimate one for large, heterogeneous populations. On the basis of this observation, the 1977 committee concluded, "It is more prudent to treat some kinds of toxic effects that may be self-propagating or strictly cumulative, or both, as if there were no threshold and to estimate the upper limits of risk for any given exposure" (NRC, 1977, p. 25). The report included among self-propagating or strictly cumulative effects those that result from early, chemically induced alterations in cellular DNA that are transmitted by cell propagation and irreparable injuries, such as destruction

of neurons, noting that "destruction of enough neurons leads to a decrease in central nervous system function."

Carcinogenic and Mutagenic Effects

The same committee outlined the following principles that underlie efforts to assess the irreversible effects of long-continued exposure to carcinogenic substances at low dose rates:

1. "Effects in animals, properly qualified, are applicable to man."
2. "Methods do not now exist to establish a threshold for long-term effects of toxic agents."
3. "The exposure of experimental animals to toxic agents in high doses is a necessary and valid method of discovering possible carcinogenic hazards in man."
4. "Material should be assessed in terms of human risk, rather than as 'safe' or 'unsafe' " (NRC, 1977, pp. 53-56).

Noncarcinogenic Effects

For presumably reversible noncarcinogenic and nonmutagenic effects, the committee advised, "For noncarcinogens for which it seems likely that there are thresholds for toxic effects, the acceptable dose should be below the threshold. If a threshold cannot be shown, the acceptable dose must be related to the data from animal experimentation and consideration of the seriousness of the toxic effects, as well as the likelihood and ease of reversibility, the variability of the sensitivity of the exposed population, and the economic and health-related importance of the material" (NRC, 1977, pp. 57-58). The present committee appreciates that there is not a clear distinction between cancer and other toxic responses.

Safety, or Uncertainty, Factors

To this general statement the committee added specific advice on the use of safety, or uncertainty, factors (NRC, 1977, p. 804). An uncertainty factor of 10 was recommended when there are valid results from studies on prolonged ingestion by humans and no indication of carcinogenicity. An uncertainty factor of 100 was recommended when there are few or no toxicological data on ingestion by humans, but there are valid results from long-term studies in animals and no indication of carcinogenicity. An uncertainty factor of 1,000 was recommended when there are no long-term or acute data on humans, only scanty data on animals, and no indication of carcinogenicity.

The Use of Animal Data to Predict Human Risk

The first Safe Drinking Water Committee remarked, "Current knowledge of the proper principles for extrapolating toxicological data from high dose to low dose, and from one species to another, is inadequate" (NRC, 1977, p. 25). In Volume 3 of *Drinking Water and Health* (NRC, 1980), the reconstituted committee reexamined the prediction of risks to human health using acute and chronic toxicity data on laboratory animals. The entire third chapter of Volume 3 was devoted to this issue The committee summarized its conclusion as follows:

> The concentration of most potentially toxic chemicals in drinking water is usually so low that it is difficult to predict potentially adverse effects from drinking the water. In cases of noncarcinogenic toxicity, the preferred procedure would be to make a risk estimate based on extrapolation to low dose levels from experimental curves obtained from much larger doses for which effects can be readily measured. In most instances, such data are not available, and the acceptable daily intake (ADI) approach should be used until better data are obtained. In the ADI approach, "safety factors" based on the quality of the data are applied to the highest no-observable-effect dose found in animal studies.
>
> The [Safe Drinking Water Committee's] Subcommittee on Risk Assessment believes that the ADI approach is not applicable to carcinogenic toxicity, and that high dose to low dose extrapolation methods should be used for known or suspected carcinogens. Six models were evaluated for low dose carcinogenic risk estimation. They were the dichotomous response model; linear, no-threshold model; tolerance distribution model; logistic model; "hitness" model; and time-to-tumor-occurrence model. Because of the uncertainties involved in the true shapes of the dose-response curves that are used for extrapolation, a multistage model was judged to be the most useful. Such a model has more biological meaning than other models, e.g., the probit or logistic model. Moreover, it tends to be conservative in that at low doses it will give higher estimates of the unknown risk than will many others.
>
> More confidence would be placed in mathematical models for extrapolation if they incorporated biological characteristics such as pharmacokinetic data and time-to-occurrence of tumors. Until such data are available, the extrapolation from animals to humans should be done on the basis of surface area (NRC, 1980, pp. 2-3).

As the discussion in that volume indicates, the computation of risk depends upon several assumptions, ranging from the choice of mathematical model for low-dose extrapolation to the minor operating assumptions made within the model itself to implement a specific computer program. The present committee noted that within the multistage model one can compute the dose necessary to develop a given level of risk by using either an experimentally restricted model or a generalized model. The restricted model limits the number of possible stages in the multistage

process to the number of doses at which the experiment was conducted minus 1. The generalized model places no such limit on the possible number of stages but, rather, permits computation of the best fit to the data without the constraint of doses used in the experiment. For example, by using data on response to acrylamide for male A/J mice with lung tumors and the two different computational assumptions concerning stages of carcinogenesis, one can estimate two slightly different risks, as shown in Table 8-1.

TABLE 8-1 Unit Risk[a] Depending on Assumed Stages of Carcinogenesis

Estimate	Stages Experimentally Restricted	Stages Generalized to Data
Maximum likelihood estimate	1.2×10^{-5}	8.8×10^{-6}
Upper 95% confidence limit estimate	2.2×10^{-5}	1.7×10^{-5}

[a] Assuming a daily consumption of 1 liter of water containing the compound in a concentration of 1 µg/liter.

A similar small difference was found in the risks estimated for female mice with lung tumors. Where the data are highly curvilinear, the experimentally restricted approach can assign less of the effect to the linear term, and may even estimate it to be zero. In contrast, the unrestricted form of the computation is more likely to identify a nonzero linear term, which may be more in keeping with current biological knowledge and assumptions. Computations in the third volume were based on both approaches. The differences were only slight and, thus, not important. Following the recommendations of the risk-assessment panel of the Consensus Workshop on Formaldehyde (1984), the computations presented in the present volume are based on the generalized form of the model.

In considering the appropriate scaling for extrapolating data from the laboratory to humans, the 1980 committee remarked:

> The practice among cancer chemotherapists of basing dose on body surface area is useful, particularly for extrapolation from small animals to humans, and is supported by a sizeable body of experimental evidence. Since body surface area is approximately proportional to the two-thirds power of body weight, the anticancer drugs are relatively *more* toxic to the larger animals than to the smaller ones (NRC, 1980, p. 29).

Combined Exposures

The 1980 committee considered information on the combined action of materials found in drinking water, noting first that the joint action could be additive, synergistic, or antagonistic, and that "in general, there is not

likely to be sufficient information on [the action of] mixtures. . . . Consequently, estimates will . . . have to be based on an . . . assumption of additivity" (NRC, 1980, p. 27). The committee pointed out that assuming independence of action of a material in relation to background exposure instead of assuming additivity in dose could, at a low-dose level, easily lead to a 100-fold difference in estimated risk.

In a recent review of the possible effects of exposure to a mixture of materials, Berenbaum (1985), arguing by analogy from the behavior of combinations of antibiotics, remarked, "It is therefore not unreasonable to assume that carcinogens will prove to behave similarly [i.e., will show synergism] . . . and it appears sensible to assume this until proved otherwise." Berenbaum also noted that the "effect of a marked antagonism is to produce a threshold in the curve."

Reliability of Risk Estimation

Considering all the variables encountered in the process of estimating risks, the 1980 committee remarked, "If the estimates of risk from low doses of carcinogens are made with reasonable data and reasonable models, [there will be] a precision of 1 or 2 orders of magnitude in the estimates" (NRC, 1980, p. 60). It has since been pointed out that maximum likelihood estimates (MLEs) are extremely sensitive to the data in that very small differences can lead to large differences in the MLEs (Cohn, 1986; T. W. Thorslund, EPA Office of Health and Environmental Assessment, Washington, D.C., personal communication, 1985). The 95% upper confidence limit estimates are much more stable.

Noncarcinogens

The 1980 committee noted that determination of no-effect levels for noncarcinogens depends upon both data interpretation and the number of animals in the bioassay. It stated, "The likelihood of observing a no-adverse effect at a given dose is statistically greater for experiments with few animals than for larger experiments" (NRC, 1980, p. 31). Thus, if these and other details are not described in published studies, the use of more formal (dose-response) risk-assessment procedures is impeded and it is more difficult to interpret whatever data are at hand. Among the important matters included are whether best-fit or 95% upper confidence limit curves should be used in expressing risk and whether the dose-response model, log-normal model, or log-logistic model should be applied to the dose-response curves. The 1980 committee concluded, "The potential utility of dose-response extrapolation methodology for noncarci

nogenic human risk assessment does exist but has been found to be of limited value for contaminants in drinking water" (NRC, 1980, p. 35).

Crump (1984) has suggested an alternative to the no-observed-effect level (NOEL) for determining an acceptable daily intake (ADI). If it is possible to define as "acceptable" some very small increase over the response level in the control group, then a benchmark dose can be established as the lower (95% or 99%) confidence limit on an exposure that would produce small (or smaller) increases over the control level. Crump's proposal makes more effective use of all the experimental data than does the NOEL approach, taking into account the slope of the exposure-response curve and the size of the experiment. Thus, there is a greater efficiency in the use of the data, and larger experiments should, in general, lead to higher ADIs (rather than the reverse, which the 1980 committee noted is characteristic of the NOEL approach). This procedure is similar to the one used by the committee in developing a recommended range of exposures to aldicarb (see Chapter 9).

The 1980 committee also provided a detailed discussion on the establishment of suggested no-adverse-response levels (SNARLs) for acute 24-hour or 7-day exposures to noncarcinogenic materials. It based its calculations on assumptions that "100% of the exposure to the chemical was supplied by drinking water during either the 24-hour or the 7-day period" (NRC, 1980, p. 68). To calculate chronic SNARLs, the committee arbitrarily assumed that drinking water provided 20% of the intake of the chemical of concern.

Estimates of Exposure from Different Sources and Various Routes

All sources of exposure must be considered when assessing risk or setting safety factors for estimating ADIs of chemicals in water, regardless of the biological end point under consideration. The population may be exposed to chemicals through air and food as well as through water. For some population subgroups and for certain toxic chemicals, the air and the workplace may be the major sources of exposure. For others, drinking water may be the primary source.

The various routes of exposure from these sources must also be considered. Systemic absorption resulting from simultaneous exposure via multiple routes has received little attention to date. Shehata (1984) presented a modeling approach that may be useful in estimating the relative contribution of multiple exposure routes to body burdens of volatile organics. However, further research is required to validate these multiple route models.

The present committee finds it worthwhile to distinguish between dose response and exposure response. Dose response may be defined as the

response to the dose of toxicant actually delivered to the target site. Generally, determination of dose response requires knowledge of the fate and distribution of the administered material, including pharmacokinetic behavior and metabolic activation or deactivation. Exposure response is easier to determine, since information about exposure or nominal dose is more accessible and exposures are subject to potential regulation. Adequate understanding and elucidation of the mechanisms of toxicity will require knowledge of dose-response relationships. For regulatory purposes, an adequate description of the exposure-response relationship is more important.

Water

For chemicals with a threshold dose, the ADI is based on animal toxicology data by applying a safety factor to a NOEL. To determine the maximum exposure from water that should be allowed, contributions to total exposure made by sources other than drinking water should be subtracted from the permissible exposure (maximum permitted intake is usually the ADI times assumed adult body weight of 70 kg). The maximum permissible level in water can then be determined from the permissible exposure through drinking water and the standard volume of daily water consumption, which is normally assumed to be 2 liters in a 70-kg adult male and 1 liter in a 10-kg child (Kelly, 1980; NRC, 1977). Where the standard volume of consumption is set high in the range of that consumed in a population, an action level of exposure may be safely set without any increase in risk. In fact, actual water intake varies considerably with physical activity, environmental temperature, and relative humidity, which in turn vary by region of the country. At this time, however, the committee was unable to estimate the contributions of these other factors and sources other than drinking water and encourages the development of better data and models on these variables.

Valid arguments may be constructed for setting the standard daily consumption volume for drinking at the population mean, the median, the ninth decile, or some higher standard volume. If set at the population mean or median, roughly half the population will be expected to exceed this level. This choice must be related to recommended maximum permissible levels in water.

Although ingestion is the chief route of exposure to chemical contaminants in drinking water, inhalation and dermal exposure may also contribute to systemic absorption of waterborne contaminants. For example, large amounts of volatile organic chemicals may be inhaled from boiling water or hot showers. Since many organic compounds are poorly soluble

in water and are quite volatile, a substantial portion of the contaminants in water may evaporate under certain usage conditions in the home, e.g., from washing machines, dishwashers, sinks, bathtubs, and showers. During showers with chemically contaminated water, the combined action of the spray and high temperature could result in the generation of relatively high vapor levels in confined areas. Seasonal changes in household ventilation may also greatly influence the extent to which volatilized chemicals are retained in the household air.

Brown et al. (1984) proposed that absorption through the skin may contribute significantly to the total dose of volatile organic compounds received during normal daily use of contaminated water. Their calculations were based on skin absorption rates for toluene, xylene, styrene, and ethylbenzene, which were measured in human exposure studies by Dutkiewicz and Tyras (1967, 1968). The reliability of the models and the accuracy of such predictions must, of course, be verified through experimentation.

For less volatile water contaminants, there are no definitive experimental data on inhalation and dermal exposures. Thus, doses from these routes of exposure cannot be reliably estimated at this time. It may be prudent to assume that in addition to the standard 2 liters of water consumed orally each day, daily exposure to another 2 liters results from inhalation and skin absorption during bathing, showering, cooking, washing, and other activities involving water usage. The adoption of this concept would, of course, have the effect of lowering to one-half the permissible concentration of a chemical in water.

Food

Traces of pesticides and other chemicals that contaminate drinking water also contaminate foods. Exposure through food (not including any contribution from the water used in cooking) varies considerably, depending on diet. The estimated tolerances to a pesticide or recommended action levels for other chemicals in foods may be used to calculate a theoretical maximum residue contribution (TMRC) through food (Ariëns and Simonis, 1982; Hathcock et al., 1983). These TMRC values constitute an upper limit on the exposure through food. They are difficult to use, however, because they usually exceed the measured intakes by one to several orders of magnitude. Moreover, estimates of the proportion of the diet accounted for by specific foods are often not current and there is little information on variation in diets by region, ethnicity, age, sex, or other factors (NRC, 1982).

Air

The background level of exposure to many toxic chemicals in the ambient air is usually even more variable and difficult to determine than the exposure through food. The extent of both indoor and outdoor exposures through air depends strongly on occupation, region (urban levels exceeding rural levels for many substances), climate, and life-style (e.g., whether sedentary or active, length of time spent indoors and outdoors) (NRC, 1985).

Workplace

In occupational settings, workers may be exposed to toxic chemicals by routes other than ingestion of water, including inhalation, direct contact with skin, or ingestion after contamination of hands, cigarettes, or food. Contaminants in ambient air and in settled dust may account for much of the total workplace exposure. Contaminants may come into contact with skin from clothing contaminated by vapors, dust, or spills.

Household

The Total Exposure Assessment Measurement (TEAM) study conducted by the EPA (Pellizzari et al., 1984) has shown tremendous variation in personal indoor exposures to toxic chemicals. Until there is a better understanding of the doses provided by all the different indoor sources of pollutants, estimates of average national exposures to drinking water contaminants in the home will not be useful in determining total exposures to the chemical of interest. Upper limits of likely ranges of exposure may be more useful than the boundaries selected.

PHARMACOKINETICS

An understanding of pharmacokinetic principles is necessary to the successful extrapolation of data from high to low doses. (See Chapter 6 for a detailed discussion of models based on pharmacokinetics.) The Office of Science and Technology Policy (OSTP) (1985), elaborating on a conclusion reached by Hoel (1980), noted, "Even if only a small portion of the background incidence [of the toxic response] is associated with the same mechanistic process as the study chemical, linearity will tend to prevail at sufficiently low doses" (OSTP, 1985, p. 81).

Some arguments to the contrary have been raised. An unresolved issue that may have a bearing on the possible existence of a threshold is the dissimilarity of the kinetics of toxification-detoxification at high in com

parison to low doses. Some scientists believe that chemical carcinogens can be crudely classified into two groups:

- those that attack DNA directly in the nucleus (e.g., as assayed by adduct formation or mutation) and
- those that do not appear to modify DNA directly.

There is substantial disagreement about how to make this distinction, as discussed later in this chapter and in Chapter 5.

Most of the substances in the first category discovered to date are not carcinogenic *per se*, but are instead activated enzymatically in the cell to produce unstable, chemically reactive intermediates (e.g., polycyclic hydrocarbons, alkanes, alkyl halides, and aryl amines). Mixed-function oxidases (i.e., the cytochrome P450 system and the monoamine oxidase system) are responsible for such activation (Miller and Whitlock, 1982; Nebert and Gelboin, 1968). The primary function of both systems appears to be the detoxification of cells exposed to lipid-soluble molecules. Such molecules are metabolized (typically to form epoxides or acetoxy compounds), and then condensed catalytically with water-soluble substrates, such as glutathione (Gelboin, 1980).

This metabolic pathway has three important features:

- The oxidases are inducible by a mechanism that directly increases the level of oxidase-specific messenger RNA, resulting in a 10- to 100-fold increase in the activity of the enzyme (Bresnick et al., 1981).
- The inducibility and the absolute basal activity of the oxidases and transferases that are fundamental enzymes for cell detoxification vary as much as 100-fold among different tissues and species (Miller and Whitlock, 1981).
- In such a coupled activation condensation pathway, the steady-state concentration of activated, potentially toxic carcinogen (d^*) is very sensitive to the relative concentration and the relative activity of the various enzymes in the coupled pathway (Hoel et al., 1983).

As a result, there is a fundamentally nonlinear relationship between the administered dose (d) and the effective dose (d^*) at the molecular targets, although under certain circumstances it can become linear at low doses (Hoel et al., 1983). Figure 8-1 depicts four consequences of coupling between activators (oxidases) and condensing enzymes. Part A of the figure shows a noninduced oxidase, where the transferase reaction has a high K_m compared to the oxidase. The dose d^* available to attack DNA displays ordinary Michaelis-Menten enzyme kinetics. When the transferase reaction has a low K_m, the reaction will become saturated at a relatively low applied dose d (part B). Under those conditions, the relationship between d and d^* is very flat at low doses but converges at a higher dose

to behavior that is more like that of a simple enzyme, with an apparent K_m corresponding to that of the oxidase alone.

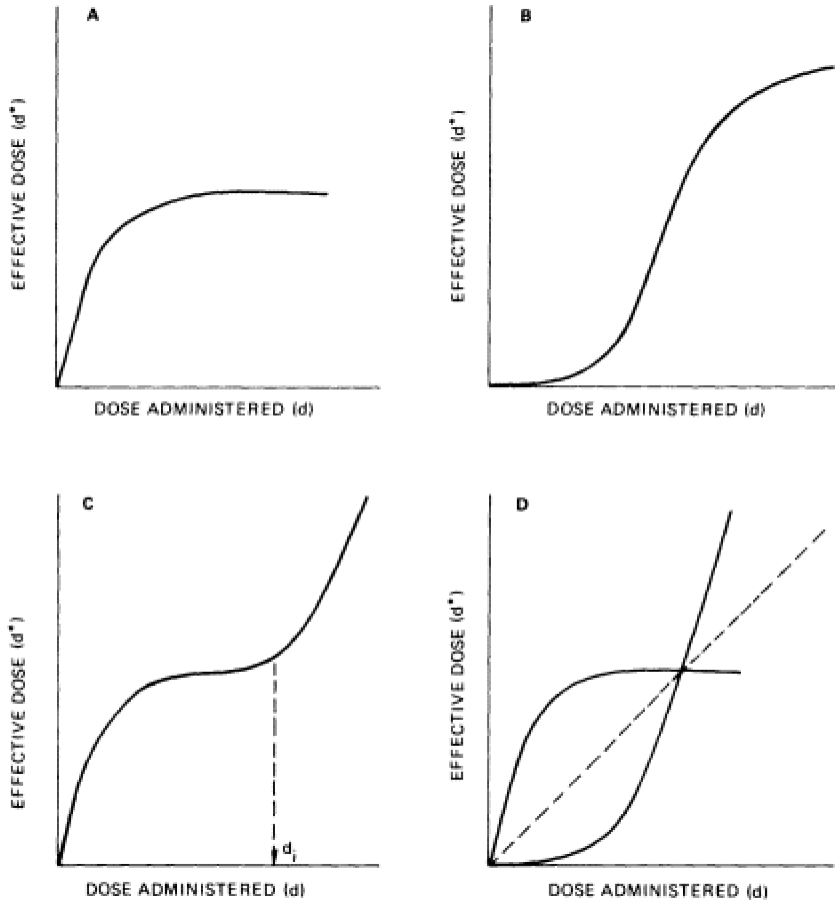

Figure 8-1
Four consequences of coupling between activators (oxidases) and condensing enzymes.

The inducibility of the oxidase (and perhaps the transferase) in these kinetic schemes further complicates the picture. Part C of the figure shows the simplest behavior expected for an inducible oxidase, corresponding to the enzyme kinetics in part A, except that at a dose d_i, the cell responds to the inducer by producing more enzyme. As seen in part C, the applied dose to d^* response becomes biphasic with a characteristic inflection point at d_i. Part D is a combination of parts B and C.

To gain an understanding of dose extrapolation for carcinogens in terms of these figures, it is important to recognize that long-term animal testing

must generally be performed at doses within a factor of 10 of the level that produces immediate tissue damage (the maximum tolerated dose). Given that the hydroxylase-transferase system is the principle mechanism by which cells are detoxified, such dose schedules are almost certainly positioned on the right side of the dose responses described in Figure 8-1.

In any biological or biochemical process, an experimentalist looks for specific characteristics. In enzymology, such a characteristic would be the substrate concentration at half maximal velocity K_m. Below that critical concentration, the reaction rate is roughly linear with respect to substrate concentration. Above K_m, reaction velocity becomes level and independent of the substrate. By contrast, at the limit of high substrate concentration (such as those that might be expected at common environmental exposures), the enzyme reaction rate depends only upon rate constants for processing bound substrate. At the limit of low substrate concentration, the measured rate depends upon substrate-binding and enzyme-processing rates.

An important experimental fact should be evident from these considerations: the information necessary to extrapolate from high to low doses cannot be obtained from observations of enzyme behavior at high doses, even if the formal relationship between dose and response is clearly understood. The reason for this is that the behavior of an enzyme system at high doses is (or may be) dominated by factors different from those that determine behavior at low doses.

Consequently, by analogy with a simple enzyme kinetics process, it is a distinct possibility that the experimental induction of a tumor at high doses may be dominated by system characteristics that differ from those directing behavior at low doses. Again, a functional form can always be fit to high-dose data. However, even if that function precisely describes the relationship between tumor and dose, it will not yield a meaningful low-dose prediction if the system has become saturated, or approached saturation, with respect to crucial enzymatic processes.

The dose responses described in parts A and B of Figure 8-1 appear to be very different, but they result from exactly the same coupled enzyme pathway; only the enzyme parameters have been altered. Given the substantial tissue-specific (Nebert and Jensen, 1979), species-specific (Gregus et al., 1983), and individual-specific (Nebert et al., 1975) differences that have been described for procarcinogen oxidases, it is likely that dose-dependent behavior as different as that shown in parts A and B of Figure 8-1 may occur in different organs in the same person, or in different people, or at different stages in cell differentiation.

As a class, carcinogens that do not bind to DNA are even less well understood. The best studied of these compounds is dioxin, which is a potent activator of aryl hydrocarbon hydroxylase activity and therefore

increases the rate at which procarcinogens are transformed to a carcinogenic state (Miller et al., 1983). Such activation could substantially alter the active dose of carcinogen in the nucleus, resulting in tumor formation.

A second type of indirect carcinogenic effect has recently been suggested for promoters of carcinogenesis such as the phorbol esters, e.g., 12-*O*-tetradecanoylphorbol-13-acetate (TPA). These compounds appear to behave as mitogens, disrupting the ordinary pattern of cell-cycle control, and in turn possibly disrupting the expression of genes crucial to tumor formation (Michell, 1984; Nishizuka, 1984).

Although the dose response for dioxin activation or TPA modification of cell-cycle control may be linear at low applied doses, the events are complicated enough that the dose response may depart substantially from linearity under experimental conditions. As for DNA-binding carcinogens, experiments conducted at high doses may be affected by saturation and for that reason may not be extrapolated to low doses in a simple fashion.

Given the data at hand, extrapolation for carcinogens such as dioxin or for promoters such as TPA cannot be made with certainty at this time. Experimental data could be fit empirically by equations describing a multistage model or a Weibull model for generating a low-dose extrapolation. At present, however, there is no firm experimental evidence to support the selection of any particular extrapolation protocol, although the multistage model was derived as a way to explain or describe the age patterns of cancer in humans. As indicated earlier, the OSTP does not consider "goodness of fit" as an adequate basis for choice among models (OSTP, 1985).

INTER-AND INTRASPECIES EXTRAPOLATION

Calabrese (1983) has reviewed both qualitative and quantitative differences between species. These include differences in physiology (e.g., rats are obligate nasal breathers and have a nonglandular stomach but humans do not, and the volume of blood flowing to various organs in rats is different than in humans); biochemistry (e.g., differences in basal metabolic rates, pharmacokinetics, enzyme activity, and receptors); size; life span; and the nature, routes, and duration of exposures. Although it may be possible to identify test species both qualitatively and quantitatively similar to humans in some respects, it has not been possible to identify which similarities are most important for the comparison of long-term chronic effects such as carcinogenesis. For the near future, chronic studies in laboratory animals are likely to remain the primary means of evaluating chronic toxicity and carcinogenicity.

Enzyme function is highly efficient among vertebrates. However, there can be enormous quantitative differences in enzyme activity, even among

evolutionarily conserved pathways. There are three basic types of diversity in enzyme activity: differences among species, differences among individuals, and organ-or tissue-specific differences.

Gregus and colleagues (1983) have catalogued differences in the activity of liver enzymes. Among eight different vertebrates tested, the activating and condensing enzymes can vary 10- to 30-fold. More importantly, there is no systematic evolutionary relationship: trout display an enzymatic fingerprint that is as similar to that of dogs as that of cats. Thus, in the absence of quantitative enzymology, it is not necessarily valid to presume that higher vertebrates will be more similar to humans in terms of their dose response.

Substantial intraspecies differences in sensitivity to carcinogens have been reported (Mohrenweiser and Neel, 1982). The extent of such variation has been elucidated in a dramatic fashion by Miller and Whitlock (1982), who identified two natural variants within a Hepa-lclc7 mouse clonal hepatocyte population:

- 1% to 2% of the hepatocytes had *no* basal aryl hydrocarbon hydroxylase (AHH) activity (they cannot metabolize procarcinogens);
- 1% to 2% of them had basal enzyme activity about 40 times greater than the population average.

Differences of that sort can also be identified in intact animals. At the biochemical level of analysis, for example, C57BL/6 mouse strains have a highly inducible liver cytochrome P450 AHH system, but DBA/2 mice do not (Gielen et al., 1972). This variation has been localized genetically to the *Ah* locus, which appears to encode a gene for a cell surface receptor (Tukey et al., 1981). To date, there is no equivalent genetic information on humans, but it is very unlikely that genetic diversity in the carcinogen-processing enzymes is specific to mice.

When there are results from more than one valid and well-conducted bioassay, data are usually selected from the bioassay in which the most sensitive species was used (California Department of Health Services, 1982). This decision is based in part on the widely accepted assumption that in the absence of data to the contrary, humans should be considered as sensitive as the most sensitive species. Some evidence suggests that humans are roughly as sensitive or in some cases more sensitive than experimental animals (California Department of Health Services, 1982; Crouch and Wilson, 1979; NRC, 1975, 1977).

To predict carcinogenic risk to humans from data on animals, one should consider all the available data on the type of tumor produced, number of tumors, and time-to-tumor induction. Attention also needs to be paid to interspecies differences in body size and life span; the duration, nature, and route of exposure; and possible variations in metabolic and pharma

cokinetic patterns and rates as well as in inherent susceptibility. In practice, conversion factors have been generally applied for size and exposure differences. For the other variables, humans and experimental animals are generally assumed to be similar; for example, a lifetime of exposure of a laboratory animal is considered equivalent to a lifetime of exposure of humans.

Conversion factors for size are usually based on surface area or body weight. When surface area is used to scale dose rates from experimental animals to humans, the projected human risks in rats and mice are 6- and 14-fold higher, respectively, than rates derived from body weight (OTA, 1981). In assessing the risk of waterborne carcinogens, different committees of the National Research Council have used surface area rather than weight (NRC, 1977, 1983), which ensures the provision of risk estimates with the greatest potential for protecting health.

CARCINOGENESIS

Because of the complexity of carcinogenesis, the selection of the most appropriate risk-assessment method is not a simple matter. As a general rule, nonthreshold models should be used. It is reasonable to use models that incorporate background additivity and, hence, low-dose linearity, unless there are convincing experimental and human data showing that such an assumption is inappropriate.

Long-term tests designed to determine the response of laboratory animals to possible carcinogens are performed on relatively small populations of approximately 100 treated animals per sex per species. Because of inherent sampling error, a suspected carcinogen must be administered in doses usually many times greater than those expected in the environment to obtain a statistically significant response in such small test groups. For that reason, the process by which a low-dose relationship is inferred has been (and will continue to be) the subject of intense debate.

The NOEL

Volume 1 of *Drinking Water and Health* contained a detailed discussion of the relationship between classical toxicological testing and the extrapolation to low doses. In a classical testing protocol for a small sample, it is always possible to identify an applied dose d_0 small enough that the elicited response cannot be distinguished from that detected in an unexposed control population. That dose—the NOEL—should be interpreted carefully.

If the probability of a toxic event occurring at some dose d_0 is no greater than background, an upper limit to the real excess probability of that toxic

response can be calculated by using sampling theory (Young, 1962). First, an upper confidence limit is specified. This is the chance that the calculated response probability is not a true upper limit to that specified by the data. Typically, a confidence limit of 95% is selected. That is, given the observed data, the true excess toxic response probability will be at or below the calculated upper probability 95% of the time.

When there are no responders in a population of N test animals (i.e., at the NOEL), the formal relationship between parameters is

$$(1 - Pr)^N = 0.05,$$

where Pr is a calculated upper limit to the toxic response probability at dose d_0 (Young, 1962). For a population of 100 test animals ($N = 100$), $Pr = 0.03$, i.e., a dose d_0 produced zero toxic responses in 100 animals, implying that there is a 5% chance the true response at that dose is greater than 0.03.

If this kind of computation were applied to cancer, we recognize at once that a 3% incidence rate is very high (between a thousand and a million times greater than many spontaneous tumor rates). Therefore, in addition to the detailed considerations required in relating human sensitivity to that of small test animals, estimation of cancer risk from environmental exposures requires that experimental data (i.e., estimates of risk in animals) be extrapolated to doses far lower than the usual NOEL determined for threshold-type responses.

Quantitative Assessment

A set of experimental data relating applied dose d to tumor probability $Pr(d)$ can be fit mathematically by many functional forms nearly equally. But calculations of probability of tumor occurrence at low doses based on those equations can vary widely. In Volume 3 of *Drinking Water and Health* (NRC, 1980), the committee noted that "goodness of fit" was not an appropriate criterion for the selection of an extrapolation model. Therefore, data from chronic studies in animals cannot be used to determine the correct relationship between dose and response at low-dose levels. Models need to be evaluated in relation to basic knowledge of the tumor formation process.

Epidemiological (ICPEMC, 1983), clinical (Lee and O'Neill, 1971), and biochemical (Land et al., 1983) evidence combined suggests that tumors occur after a cell has experienced two or more lesions, or hits. These lesions need not be environmentally induced. For example, one or both may be the consequence of an inherited genetic trait. In many instances, the crucial hits may occur in DNA or in the cellular machinery responsible for gene control. In such a genetic model for tumor growth,

it is presumed that transformation follows one or more hits, after which a single cell can proliferate to form a tumor.

These experimental findings limit the selection of the model to be used for low-dose extrapolation. In addition to the consequences of the existence of some background level of incidence, if two or a few hits within a single cell are sufficient to initiate tumor growth, then there can be no absolute threshold in the dose-response relationship; i.e., at any dose, there will be some probability that a cell can be transformed, thereby initiating tumor growth (Crump et al., 1976). A multistage model of carcinogenesis proposed several years ago (Moolgavkar and Knudson, 1981) provided a way for taking into account the birth and death of cells in any preneoplastic compartment. Thus, the model appears to be consistent with current biological knowledge and should be able to elucidate whether an agent affects transition rates, tissue growth, or tissue differentiation. By including the differential birth and death process, the model should be able to account for nonlinearities seen in some dose-response data.

A major problem in understanding the etiology of cancer lies in the choice of the dose metameter. If the probability of the development of cancer is linearly related to dose, then the appropriate measure to consider would seem to be lifetime cumulative exposure. Most risk-assessment models use this cumulative exposure as the dose. However, there are data that do not conform to such an explanation, implying some nonlinear mechanisms. For example, in reviewing the dose-times-rate effects of the administration of 2-acetylaminofluorene (2-AAF), Littlefield and Gaylor (1985) noted that the higher dose rates for shorter periods led to a higher incidence of tumors, although total dose was equivalent. These results strongly suggest that for some materials dose rate may be a more important measure than total dose. This, in turn, raises questions about the age of the animals (or persons) at the time of exposure—perhaps suggesting that the administration of a sufficient dose at a particularly sensitive time in the life of an animal may lead to a higher incidence of cancer. This possibility then raises questions concerning the design of experiments for testing to identify or elucidate the effects of erratic or sporadic exposures of the kind that humans experience.

The Multistage Dose-Response Model

Crump et al. (1976) devised a mathematical approach for fitting a linear dose-response curve at low doses, which is consistent with a no-threshold model. This model presumes that the probability of tumor occurrence is related to dose d, as a product of exponential terms, each in some way related to the probability of the occurrence of a necessary stage in carcinogenesis.

Equations such as the one given below have several features that make them attractive to policymakers. The equations become very close to linear when the probability of cancer incidence, $Pr(d)$, is within a factor of two of the background, independent of the number of steps k built into the formalism or the value of the coefficients q_k (Crump et al., 1976). The model leads to a no-threshold extrapolation procedure (predicting a finite excess risk of cancer at any dose):

$$Pr(d) = 1 - e^{-Q(d)},$$

where

$$Q(d) = q_0 + q_1 d^1 + q_2 d^2 + \ldots q_k d^k; \; q_i > 0, \text{ and}$$

q_k are coefficients to be fit to the data and d_k is applied dose raised to the kth power. When applied to data, k can be chosen arbitrarily as being equal to (or one less than) the number of independent data points to be fit or can be allowed to be unrestricted and, thus, be determined by the data. Formally, each of the k terms in Q are believed to be equivalent to a transition between individual steps in a multistep pathway leading to tumor growth. The equation presented above formally corresponds to the multistage model of carcinogenesis, such as that of Armitage and Doll (1954).

Threshold Models

One of many alternative threshold models that have some heuristic, but less biological, appeal is the Weibull model:

$$Pr(d) = 1 - \exp[-(\alpha + \beta d^m)],$$

where $Pr(d)$ is the lifetime probability of developing cancer when exposed at a dose rate, d; α is the natural background incidence; β is a slope or potency coefficient (corresponding roughly to the q_i in the multistage model); and m is a shape parameter. Thus, $m < 1$ implies a response curve concave down (hyperlinear), $m = 1$ implies a linear dose-response curve, and $m > 1$ implies a convex (rising at high doses more rapidly than linear) dose-response curve (hypolinear).

The shape parameter m for the dose indicates the severity of the end point s. The more severe the end point, the steeper the dose-response curve (Carlborg, 1982). The Weibull model has several advantages:

- It often fits the laboratory data better than does the multistage or linearized multistage model in the high-dose region where most laboratory experiments are conducted.

- It can be modified to permit consideration of time-to-tumor occurrence and time to death.
- It coincides with earlier (Druckrey, 1967) formulations of the carcinogenic process relating median time-to-tumor occurrence and dose (i.e., higher dose leading to lowered median time-to-tumor occurrence).
- It can be modified in a manner similar to the multistage model to give an upper limit (i.e., 95% or 99%) to the estimated risk at some low dose.

When the only coefficient of consequence in the multistage model is q_1, and the shape parameter m of the Weibull model equals unity, the multistage and Weibull models give the same low-dose estimates of risk.

As discussed by Crump (1985), an estimating or predicting equation is built on three basic premises:

- Tumor production is likely to be a multistage, or multistep, process.
- Chemical carcinogens do not behave uniquely. Many natural and synthetic substances appear to act in a similar manner. Therefore, the background probability of tumor incidence is never zero.
- Tumors can arise from a single transformed cell.

In effect, these premises preclude the existence of a dose-response threshold. However, there is substantial evidence suggesting that in mammalian cells, or in whole animals, there can be a dose response that is experimentally indistinguishable from that which would be predicted if there were a threshold. For instance, in the largest study performed in mice to date (635,000 mice overall), Russell et al. (1982) have shown that testicular mutations produced in response to doses of ethylnitrosourea (ENU) vary in a sigmoidal fashion with dose. In their report, they cited unpublished observations indicating that at each dose, the amount of ENU reaching the testes remains constant. Such behavior resembles a classical threshold dose-response model and suggests that if carcinogen-induced mutation is a necessary first step in carcinogenesis, and external exposure is the only source of the mutation, then there may be a dose-response threshold for tumor formation in mouse testes in response to ENU.

Ehling and colleagues (1983) reviewed evidence concerning the existence of thresholds for carcinogens. They found that among the eucaryotic systems studied to date, a threshold or apparent threshold dose response appeared in fewer than 23% of the tested cases. The log-probit model described by Mantel et al. (1975) is one widely discussed threshold model for low-dose extrapolation. This model is based on a classical toxicological description of the dose response and is one of the tolerance models described earlier. It presumes that each member of a population has a personal carcinogen dose threshold: at a dose above the threshold, they will develop

one or more tumors, and at an individual subthreshold dose, they are unaffected. Such a model is appealing because it considers individual diversity within a population and is consistent with the traditions of classical reversible toxicology (e.g., many toxicants have a well-defined threshold dose: two aspirin tablets are a mild analgesic, whereas 200 aspirin tablets are lethal). The model, however, has been severely criticized as having little or no biological basis in carcinogenesis (NRC, 1980).

There is some philosophical equivalence between the tolerance distribution models and the no-threshold models—if one adds the concept of individual differences in probability of response. In the no-threshold models there is no dose level at which there is a zero probability of response for all members of the population. This probability of response, however, may be larger or smaller for specific individuals. Current developments in cancer research related to the presence and distribution of oncogenes in individuals may be expected to lead to cancer incidence models that take into account both the initiator-promoter concepts and the potential for response in individuals, as a consequence of the distribution of numbers and kinds of oncogenes.

In general, models that include thresholds for individuals predict a dose response that may become very flat at low doses. After being fit to high-dose data, such a model may predict low-dose risk values substantially lower than those calculated from a linear or linearized multistage model (Swartz et al., 1982).

DEVELOPMENTAL AND REPRODUCTIVE EFFECTS

As indicated in Chapters 2 and 3, reproductive impairments of one kind or another are frequent and widespread. Nonetheless, formal risk assessment has seldom included measurements of adverse effects on reproduction. Under the Toxic Substances Control Act, information on reproductive effects is not required before a product is marketed.

Koëter (1983) recently showed that, in comparison to data from subchronic studies, data on reproductive toxicity produced lower estimates of the lowest-observed-effect level (LOEL) for 35% of the compounds tested, the same estimates for another 35% of the compounds, and higher estimates for the remaining 30%. For 8 of the 37 compounds tested (>20%), fertility or reproduction toxicity end points were the most sensitive and thus solely determined the LOEL. Thus, the inclusion of reproductive toxicity effects in a standard test battery would have produced a lower "safe" dose in one-fourth to one-third of these materials. Koëter concluded that reproductive function is highly sensitive to impairment and should be examined at earlier stages of safety testing.

At present, there is limited agreement about how to apply the results of animal reproductive and developmental toxicity studies to assess the risk of exposure to a compound. This is partly because an understanding of the underlying events leading to reproductive toxicity is usually missing.

There are no agreed-upon standards or quantitative methods for cross-species extrapolation for reproductive or developmental effects. However, there are some conditions under which reproductive toxicity data from animal studies can be used to estimate risk to humans. In the following sections, consideration is given to concepts of susceptibility, timing of exposure, patterns of dose response, interpretation of animal data, and possible methods for cross-species extrapolation.

The NOEL

If the data are of sufficient quality and quantity, it should be possible to identify a NOEL or LOEL, the maternally toxic dose levels, and the specific types and incidences of adverse effects. Data sets that fail to identify these responses or dose levels are inadequate for quantitative risk assessment. Even without these data, however, a qualitative ranking of agents as having high, moderate, or low potential for developmental toxicity in humans can at times be made. Agents that selectively induce irreversible developmental toxicity in animals at low, nonmaternally toxic doses are assumed to have the highest potential for causing developmental toxicity in humans. If a high-level, maternally toxic exposure to an agent causes irreversible developmental toxicity, or if a low-level exposure that is not maternally toxic causes reversible variants or minor malformations in animals, the agent should be considered a moderate risk to humans. A low risk to humans can be anticipated if prolonged exposure to high levels of the agent in a well-conducted experiment of sufficient size does not result in any developmental toxicity in animals.

The decision to use a NOEL or LOEL approach to risk assessment is based largely on which value can be most accurately identified from the data base. It is usually easier to identify a LOEL from experimental data, because this value can be observed directly, whereas the NOEL can be orders of magnitude below the lowest experimental exposure level observed to induce developmental toxicity. The LOEL is not restricted to a dose at which responses are statistically significantly different from control responses. Trends in the data indicating biologically relevant increases in the incidence of adverse effects at low doses can be used to establish a LOEL if there is also an increased incidence of the same effects at high doses or if there is a statistically significant dose-response relationship. LOELs are most accurately identified under conditions where the response is minimal and the end point involves reversible developmental toxicity,

indicating that a NOEL is being approached. In the absence of statistical significance, which may often be the case because of sample size considerations, it is sometimes possible to define a minimal response such as a doubling of the low background rate of the particular response. Protection against doubling of an adverse background rate can be achieved by using a large safety factor for the NOELs selected under these conditions, recognizing that doubling may produce an unacceptably large increase. For major malformations, a doubling would represent an unacceptable increase in the United States from approximately 60,000 malformed infants to 120,000 per year.

Quantitative Assessment

Some work is under way to develop a quantitative index for comparing developmental toxicity across species taking concurrent maternal toxicity into account (Fabro et al., 1982; Johnson, 1980). This approach derives from the perceived need to distinguish between compounds that are uniquely toxic to the embryo and those that induce developmental toxicity only at exposure levels that are also toxic to the mother. Maternally toxic materials would need to be regulated on the basis of their adult toxicity, whereas regulations for compounds toxic to the embryo only at low doses would be based on that unique embryotoxicity. In attempting to provide a quantitative index to separate these two types of compounds, Johnson (1980) has defined a "teratogenic hazard potential," which is the log of the ratio of doses that produce adult and developmental toxicity:

$$\text{Log} \ \frac{\text{lowest adult toxic (lethal) dose}}{\text{lowest developmental toxic dose}}.$$

The higher this so-called *A/D* ratio, the more likely it is that the material has special embryotoxicity. Johnson calculated this ratio for more than 70 compounds using data from an *in vitro* system of adult and embryonic tissues from *Hydra attenuata*. (It has been reported that the *A/D* ratio from the hydra assay has ranged from one-tenth to 10 times that of the mammalian *A/D* ratio.) Most compounds had ratios near 1. Several of them had ratios larger than 5, but very few had ratios larger than 10 (Johnson and Gabel, 1983). The use of this ratio has been proposed as a method for setting priorities for further testing of agents in mammalian developmental toxicity studies.

Fabro et al. (1982) have begun to explore the quantitative characteristics of a similar type of index in mammalian studies. Dose-response data for adult lethality and fetal malformations were fitted separately (probit of response against log of dose) for eight compounds. The observed log

probit dose-response lines for lethality and teratogenicity were not parallel, nor was there a constant ratio between the slopes for the two lines. Consequently, a simple ratio between the median effective lethal and teratogenic doses (i.e., LD_{50}:tD_{50}) could not be used. To calculate a relative teratogenic index, Fabro et al. chose one point (i.e., a dose corresponding to some arbitrary percent response) from each dose-response line and generated a ratio from these two points. The LD_{01} value was chosen to represent adult lethality based on the argument that using a low LD value would help guard against compounds that have a shallow dose-response curve for adult lethality. The tD_{05} value, which is the dose causing a 5% increase in the malformation rate above background, was chosen to represent teratogenicity. It was believed that the $tD0_5$ value could be estimated with confidence for most teratogens because frequency of induced malformations often ranges between 1% and 20% in animal studies. This approach appeared to be satisfactory for ranking the candidate compounds according to teratogenic potency provided the dose-teratogenic response relationship was not complicated by significant adult lethality. The selection of other dose levels for computing the ratio could change the relative ranking of the compounds evaluated.

This ranking system was developed to assist in evaluating the structure-teratogenicity relationships between structurally related compounds. For this purpose, the Fabro index may be adequate. The usefulness of this index for interspecies comparisons and risk estimation, however, has not been established. In their evaluation of the index, Hogan and Hoel (1982) argued that due to the lack of parallelism between the fitted probit lines for lethality and teratogenicity, the index will not be invariant to the selection of other LD and tD values; e.g., if a ratio of LD_{10} to tD_{05} were selected instead of an LD_{01} to tD_{05} ratio, a different ranking of the compounds could occur. In addition, the index would be subject to the established deficiencies of the probit model, which tends to be insensitive in the low-dose region. Therefore, until the index is more extensively applied and evaluated, it should not be used for formal risk assessment. If a uniform method for ranking agents according to their embryotoxic potential were based on selective toxicity to the conceptus, it might provide a yardstick for comparing all agents and, thus, possibly standardize a procedure for the selection of the appropriate NOEL or LOEL for risk-assessment purposes. The safety factor could then be selected to reflect the severity of the end point.

Existing models for quantitative risk assessment do not appear to be appropriate for data on developmental toxicity, for which there are probably threshold doses (Wilson, 1973). To establish safe levels for polychlorinated biphenyls, EPA (1983) examined a number of models based on developmental toxicity data. The safe dose for one set of data varied

by a factor of 7,000, depending on the model used. Rai and Van Ryzin (1985) have proposed a dose-response model for teratological quantal response data where litter size is considered in evaluating the probability of response for one animal in a litter. Their model extends the one-hit model of Hoel et al. (1975), while recognizing that the probability of response for an offspring varies among females exposed to the same dose. Litter size is used as a measure of the female-to-female variability, based on the assumption that the more sensitive animals will have smaller litters. An interagency governmental group concluded that existing mathematical models were inappropriate for assessing developmental toxicity data and that the safety factor approach is appropriate for establishing exposure levels expected to yield acceptable levels of risk (EPA-ORNL, 1982). Nonetheless, the development of models for assessing reproductive toxicity should be encouraged.

The Food and Drug Administration has also indicated that it will use the safety factor approach in developmental toxicity risk assessment, but it has not given specific details on how safety factors will be chosen. The agency has reported, however, that it will apply safety factors ranging from 100 to 1,000 to NOELs identified in developmental toxicity animal testing for drug residues in human food. According to Norcross and Settepani (1983), smaller factors will be used when the prenatal effect can be ascribed to nonspecific maternal toxicity. In the absence of other widely accepted approaches, the use of safety factors seems to be a reasonable approach for the establishment of safe levels of exposure to materials that may result in developmental toxicity.

The considerations discussed above suggest the following criteria for selecting safety factors for developmental toxicity data:

- A minimum quality and quantity of data are required to perform a quantitative risk assessment. Thus, compounds not having a sufficient data base should only be qualitatively assessed for high, moderate, and low potential to cause developmental toxicity in humans, and should be assigned high, moderate, and low safety factors accordingly.
- Human populations should be considered to include individuals who are at least 50 times more sensitive than laboratory animals to agents causing well-defined developmental toxicity.
- Compounds that lead to developmental toxicity at levels lower than those causing maternal toxicity constitute a greater potential hazard than compounds that cause developmental toxicity only at maternally toxic doses. This potential hazard implies the need for a larger safety factor.
- The potential hazard associated with a compound is related to the severity of response and both the time and route of exposure. The greatest

potential hazard is presented by compounds causing serious effects under conditions of exposure that may be encountered by humans.

NEUROTOXICITY

With the exception of lead, there has been little effort devoted to quantitative risk assessment of chemical neurotoxicants. Risk assessment is substantially more complex for neurotoxicants than for carcinogens because of the many end points associated with neurotoxicity. Moreover, numerous chemicals affect the nervous system of animals when administered in sufficiently high doses. Clinical experience with therapeutic substances has demonstrated that hundreds of pharmaceutical products can induce a variety of neurological and psychiatric disorders at prescribed therapeutic dose levels. By contrast, there are only 30 chemicals or industrial processes for which the International Agency for Research on Cancer (IARC) considers there is "sufficient" evidence of carcinogenicity in humans (IARC, 1982).

Few of the substances that produce neurotoxic effects in humans were studied in the experimental laboratory before they were marketed and subsequently observed to produce these effects in humans. This experience stands in marked contrast with that of experimental chemical carcinogenesis, where several hundred substances have been reported as carcinogens in experimental animals. Although toxicologists have long recognized the susceptibility of the nervous system to toxic perturbation, there has been no coordinated program of animal bioassay to seek out and regulate environmental neurotoxicants other than organophosphorus pesticides.

When administered in large doses, many chemical substances produce marked changes in human behavior and neurological function, such as depression of the central nervous system, that disappear rapidly and with no apparent sequelae after exposure has ceased. The same compounds may produce another type of neurotoxic effect in persons chronically exposed to lower doses for long periods. The mechanisms underlying these two (or more) neurotoxic effects may be entirely different. Some of these compounds, such as *n*-hexane and toluene, cause various types of neurodegenerative diseases after prolonged exposure; others, such as methyl ethyl ketone and acetone, do not appear to induce such chronic effects.

Evidence that environmental agents produce adverse effects on the human nervous system can be provided through clinical evaluations of exposed humans and experimental animal studies. Definitive demonstration that a substance is neurotoxic comes from complementary investigations showing that the suspect agent produces the same type of disorder in humans and in one or more appropriate test species. In the absence of data on humans, convincing demonstration that a substance is neurotoxic

in an appropriate animal species can be taken as evidence that the agent is probably neurotoxic in humans. For example, certain well-studied classes of chemicals, such as the organophosphates, reliably produce humanlike disorders in fowl.

Chronic neurotoxicity may develop many years after exposure to certain chemical substances (e.g., phenothiazines). Negative results obtained from studies conducted over shorter periods should be interpreted carefully, since chronic (lifetime) studies to evaluate agents for neurotoxic properties have rarely been undertaken.

Short-term *in vitro* methods for assessing neurotoxic activity are under development. Tissue culture systems, especially those that exploit a complex cellular structure and function comparable to that found in parts of the human nervous system, may ultimately provide surrogates for *in vivo* animal bioassays of potential neurotoxicants. Organotypic tissue cultures composed of structurally and functionally coupled spinal cord, dorsal root ganglia, peripheral nerve, and muscle develop specific types of pathological change (e.g., sensory neuronopathy, axonopathy, myelinopathy) that are also seen in humans and animals challenged with the same substances. These tissue-culture systems also have been used to study alterations of neurotoxic response to one agent (*n*-hexane) by concurrent exposure to a second (e.g., methyl ethyl ketone, ethanol, toluene).

In some cases, structure-activity considerations may be useful in assessing whether a substance may pose a hazard to the human nervous system. Examples of compounds with structural similarities that constitute neurotoxicants include some organophosphorus esters (phosphates, phosphoramidates, phosphonates), pyrethrins, and 1,4-dicarbonyl aliphatic hydrocarbons. Factors likely to modify human neurotoxicity also may be predicted on those rare occasions when the mechanism is largely understood. Examples include the protective action of sulfonates, phosphonates, and carbamates against organophosphorus neuropathy.

The NOEL and LOEL

Information on general dose response for neurotoxicity in human populations exposed to environmental chemicals is extremely limited. For the one exception, lead, responses have been well characterized with reliable biological markers of toxicity that may be readily determined by assay of blood or other tissues. The LOELs for different neurological effects are shown in Table 8-2.

For populations with special susceptibilities, there are uncertainties about the applicability of general epidemiological findings. For example, children and women of reproductive age were found to be most susceptible to the toxicity leading to spastic paraparesis during an outbreak in Mo

zambique, which was attributed to subacute cyanide intoxication from the consumption of raw cassava (a cyanogenic plant) during a famine (Ministry of Health, Mozambique, 1984). The extent to which these findings can be extrapolated to a well-nourished population composed of males and females of all ages is unknown.

TABLE 8-2 Lowest-Observed-Effect Levels for Lead-Induced Neurological Effects in Children and Hematological Markers of Exposure[a]

Lowest-Observed-Effect Level (PbB[b] in µg/dl)	Neurological Effects	Hematological Markers
80-100	Encephalopathic signs and symptoms	
70		Frank anemia
50	Peripheral neuropathies	
40		Reduced hemoglobin synthesis Elevated coproporphyrin Increased urinary δ-aminolevulinic acid
30	CNS cognitive effects (e.g., IQ deficits) Peripheral nerve dysfunction (slowed nerve conduction velocities)	
15		Erythrocyte protoporphyrin elevation
10	Altered CNS electro-physiological responses	Inhibition of δ-aminolevulinic acid dehydratase Py-5-N[c] activity inhibition

[a] Adapted from EPA, 1986, p. 13-34.
[b] PbB = blood lead concentrations.
[c] Py-5-N = pyrimidine-5'-nucleotidase.

When there are no data on human populations, animal bioassay data are useful for estimating dose response for substances with toxic properties. Accurate models of human neurological disorders develop in most adult animals challenged with selected chemical substances administered at a suitable dose for an appropriate period. However, extreme caution is necessary when using data on animals since some animal species are relatively refractory to potent human neurotoxicants. For these substances (many of which are probably unknown), evidence of dose response in two or more quite different species (e.g., the rat and chicken) is desirable.

Since neurotoxic disorders usually increase in severity as a function of dose and duration of exposure, the earliest change directly linked to the disorder under scrutiny represents the most appropriate toxic end point from which to estimate dose response for neurotoxic substances. This assumes that by protecting the target most sensitive to the agent in question, one would be protecting all important functions.

Because of the availability of continuously measurable (as opposed to categoric) end points, risk assessment for neurotoxicity has the potential for greater precision and accuracy than is currently attainable in risk assessments for carcinogenicity. Whenever possible, therefore, continuously measurable end points should be selected for study.

From a regulatory viewpoint, it is simpler to set exposure limits for noncarcinogens with a clear threshold dose below which no adverse neurotoxic response is observed (the NOEL). At low doses of a given chemical, for example, the response may lie within the body's ability to maintain homeostasis so that no overt effect occurs. In the presence of additional insults, however, the body's ability to maintain homeostasis may be reduced, which should be considered as a secondary and possibly unmeasurable toxic effect of the chemical. If there is a threshold dose for this secondary effect, it would most likely be lower than the threshold for the primary effect. Thus, although there may be a threshold dose (LOEL) for a given effect in a particular animal, it is not likely that there will be a single threshold dose for all end points, nor is it likely that a single dose level will constitute the threshold for every animal in a population. When the measurement device, scale, or experiment is insufficiently sensitive, a threshold may be incorrectly inferred where none exists. Conversion of a continuous effect to a categoric scale (e.g., no effect, tremor, convulsions) can lead to such a false threshold.

There appears to be no reason why a neurotoxic response is unlikely to follow common sigmoidal dose-response relationships; however, empirical data on the dose response of environmental neurotoxicants are urgently needed to support or refute this conclusion.

Quantitative Assessment

Since there are no uniformly accepted methods for assessing risks of exposure to neurotoxicants, the following section discusses the theoretical requirements of possible methods. Two intermediate goals of risk assessment are to learn whether a given chemical produces a neurotoxic effect and, if so, to determine the nature of the toxicity and the risk associated with various doses. Carcinogenicity studies can be optimally designed to evaluate whether neurotoxic as well as carcinogenic effects occur. However, the calculation of a dose-response relationship for low doses of

neurotoxicants is problematic, as indicated in the preceding discussion. Ideally, separate experiments could be conducted to detect neurotoxicity and to estimate risks at specific doses. When resources are limited, consideration should be given to developing a statistical design that is a compromise between optimal detection of an effect and estimation of a dose response. In the case of continuous dose-response curves for neurotoxicity, exposing small groups of animals to a wide range of doses (e.g., 4 animals exposed to each of 12 doses rather than 12 animals to each of 4 doses) is likely to impair detection only slightly while possibly producing a large amount of model-specific information for use in risk estimation.

Dose-response relationships can be regarded in two ways: responses of a more serious nature in a person exposed at increasingly higher doses or a greater percentage of a population having a specified adverse effect of a given intensity as dose is increased.

Biologically plausible mathematical models of an individual animal's dose-response relationships can be developed. These, coupled with fairly general assumptions, will lead to log-normal or normal distributions of response severities across a population of animals. When it is inappropriate to use such distributions, distribution-free (nonparametric) or other robust statistical procedures can be used.

The fidelity with which animals can serve as models for neurotoxic responses in humans suggests that the same class of mathematical functions for modeling dose-response relationships for humans would also be appropriate for animals. Experiments on two or more animal species could be conducted to provide information on the species-to-species variability of the parameters for a specific neurotoxicant. This knowledge could then be used to assess the uncertainty surrounding the extrapolation of animal data to determine the dose-response relationship in humans, and any safety factor (or confidence limit) could be adjusted accordingly.

A biologically plausible mathematical model (relating each possible dose to a corresponding level of response severity) for a particular animal is dependent upon a comprehensive understanding of the mechanism whereby a specific neurotoxicant causes a given response or of data from experiments specifically designed to help choose among appropriate models. It is reasonable to anticipate that a small class of mathematical functions (characterized by one or possibly two animal-specific parameters) will suffice to describe the dose-response relationship for each animal in a population. Once such a class of functions has been proposed, the variability across the population can be modeled by assuming a probability distribution for the parameters. (Thus, random selection of an animal for an experiment or of a human for exposure to a pollutant corresponds to

random selection of one dose-response curve from the small class of mathematical functions.) This combining of the two types of dose-response relationships into a single model creates a random coefficients model, a current topic of research published in the biostatistics and econometrics literature. Application of such models to a data set requires estimation of different aspects of the probability distributions.

A class of mathematical functions (involving possibly as few as three or four parameters) should suffice to model the animal-specific dose-response relationships for a broad class of neurotoxic substances. Different types of neurotoxic end points may require different classes of mathematical models.

If resources are dedicated to experiments designed to identify a class of functions sufficient for modeling dose-response relationships for a group of neurotoxicants, benefits for future risk assessments for neurotoxins in the group will include the replacement of two major sources of uncertainty: (1) the problems involved in selecting a model for low-dose extrapolation will be lessened by the development of a procedure with measurable (via confidence limits, for example) uncertainty of lesser magnitude and (2) the uncertainties surrounding species-to-species extrapolation will be reduced.

The concentration of the chemical to which humans are exposed may be measured directly. More typically, however, exposure data are incomplete and must be estimated. Among the neurotoxicants for which there are rather accurate estimates are lead, mercury, pesticides, and certain biological agents in drinking water. Chemical type, effect, route, duration of exposure, and exposure to possibly interacting materials are other important considerations in exposure assessment.

The intake by groups especially susceptible to neurotoxic agents must also be determined. Some compounds interfere selectively with the development of the nervous system; others only affect adult populations, especially aged individuals with normal deterioration of the nervous system. Other persons may show genetic susceptibilities to certain compounds (e.g., slow acetylators exposed to isoniazid). Those with metabolic, psychiatric, or other disorders associated with neurological changes might have their disease unmasked or exacerbated by concurrent exposure to a neurotoxic agent. Susceptibility to neurotoxicity might also be heightened by medical (drug) treatment, alcohol abuse, or renal or hepatic compromise. Malnutrition is another susceptibility factor that alters the risk of neurotoxic disorders both during development and in adult life. People concurrently exposed to two or more neurotoxic agents (e.g., streptomycin and noise) are sometimes at a greater risk for developing neurological disorders (i.e., ototoxicity).

CONCLUSIONS AND RECOMMENDATIONS

Carcinogenicity

Carcinogenesis is a complex process involving multiple steps of initiation, promotion, and progression as a cell proceeds from the normal to the tumor state (see Chapter 5). Initiating agents are genotoxic and exhibit cumulative, nonreversible effects. Certain promoting agents also exhibit genetic toxicity, but the precise contribution of this effect to promotion is not yet known. Promoting agents are not directly genotoxic and can produce reversible effects. Data from studies of radiation-induced mutations indicate that there is no threshold for initiation. No matter how low the dose of a mutagen, there remains the finite possibility of producing the mutational event. Thus, because cancer derives from the multiplication of a single cell and because there is background disease, the risk assessment models chosen for cancer assume no threshold.

Multiple applications of experimental promoters are generally required to produce activity, suggesting that some, but not necessarily all, of the steps in promotion may be reversible (Slaga et al., 1982). Some compounds induce cell replication as a result of regenerative growth following severe cell toxicity, whereas others induce hyperplasia directly (Miyazawa et al., 1980; Recknagel, 1967). Increased DNA synthesis following increased regenerative growth may result in an increase in spontaneous mutational events, promotional effects, or alteration of expression of those genes controlling cell division. Thus, forced cell proliferation may play a contributory, but not necessarily sufficient, role in the process of carcinogenesis. Mouse liver cells may be particularly sensitive to this type of action, which may explain the observation that mouse liver tumors have been induced with agents that did not produce tumors at other sites or in other rodent species and which apparently exhibit minimal or no genetic toxicity (Doull et al., 1983; Ward et al., 1979). These observations have led some to propose that nongenotoxic agents should be regulated because there are likely to be thresholds for these compounds and because the assumptions inherent in linear mathematical risk models do not apply (Kroes, 1979; Stott et al., 1981; Weisburger and Williams, 1980, 1981). Included in this category are diethylstilbestrol (DES), azathioprine, phenobarbital, chlorinated hydrocarbons such as dichlorodiphenyltrichloroethane (DDT), and the phorbol esters (Weisburger and Williams, 1980, 1981).

The committee identified four major objections to the proposed dichotomous approach to risk assessment for carcinogens (IARC, 1983; Perera, 1984; Weinstein, 1983):

1. The terms *genetic*, *genotoxic*, *epigenetic*, and *nongenotoxic* have been used in the recent past as operational terms implying that different chemicals

act via separate, distinctive, and well-defined mechanisms. However, the lines are increasingly blurred as substances previously characterized as nongenotoxic are being shown to alter the structure or sequence of the genetic material. For example, carbon tetrachloride is a weak hepatocarcinogen (IARC, 1979), but it is extremely hepatotoxic and produces a dramatic increase in cell turnover as a result of regenerative growth (Mirsalis et al., 1982; Recknagel, 1967). Carbon tetrachloride has also been reported to be nonmutagenic in bacteria (McCann et al., 1975) and in mammalian cells (Dean and Hodson-Walker, 1979; Stewart, 1981), and it fails to induce DNA repair in the hepatocytes of treated animals (Mirsalis et al., 1982). Yet, other studies have shown that carbon tetrachloride binds to DNA, RNA (Cunningham et al., 1981; DiRenzo et al., 1982; Rocchi et al., 1973), and protein (Bolt and Filser, 1977) and induces mutation in yeast (Callen et al., 1980). Other substances that were at one time believed to be acting by so-called epigenetic mechanisms, including TPA, DDT, DES, asbestos, saccharin, and phenobarbital, have since been reported to exhibit genetic toxicity in some assays. A distinction has been made between primary genotoxicity and secondary genotoxicity resulting from another activity. This distinction may only be academic, however, because the latter also damages the genome (Becker et al., 1981; Birnboim, 1982). Furthermore, new information suggests that the promoter TPA has a memory effect lasting for at least 8 weeks (Fürstenberger et al., 1983). Thus, a lack of adequate testing or a limited knowledge of the mechanisms involved could lead to the misclassification of a substance (see Chapter 5).

2. Somatic cell mutation is not necessarily the most important mechanism in carcinogenesis, nor are genetic and nongenetic mechanisms mutually exclusive. Many concurrent factors (both genetic and epigenetic) may take part in the process of tumorigenesis. These include chromosome abnormalities, gene rearrangements, oncogene activation, disorders of differentiation, DNA damage, and disruption of DNA repair (see Chapter 5).
3. The threshold issue concerns the shape of the dose-response curve at increasingly small doses where little or no information is available. At present, we do not know the shapes of the low-dose curves or if there is or is not a true threshold for an animal or human population for any carcinogen (Ehling et al., 1983). There are no convincing data to support a nonlinear dose-response curve or threshold at very low doses for any carcinogen (Hoel et al., 1983; Weinstein, 1983). This information is not available even for DES and 2,3,7,8-tetrachlorodibenzo*p*-dioxin (TCDD), two of the better-studied compounds on the conventional lists of epigenetic agents (Hertz, 1977; Weinstein, 1983).
4. Experimental data demonstrating that agents such as TCDD can be carcinogenic by themselves and may have greater carcinogenic potency

than many initiating agents contradict the implied assumption that epigenetic agents carry lower risks. The striking reductions in human cancer risk following decreases in exposure to promoters, e.g., by cessation of smoking and by reduction or elimination of some estrogen therapy (Day and Brown, 1980), strongly support rigorous control of epigenetic agents.

In general, quantitative biochemical information is not sufficient for low-dose extrapolation. For carcinogenic risk assessment, the data suggest that a multistage model is consistent with certain qualitative aspects of cancer biology. This model is attractive because for most experimental data, the curve becomes linear at low doses. However, the biochemistry also suggests that regulatory agencies should not be complacent about such a dose-response model, despite its simplicity and its apparent conservative approach to extrapolation to low doses. The dose response may be fundamentally nonlinear at low doses, and a linear extrapolation may underestimate risk for certain individuals, species, or tissues. Even more importantly, basic biochemical rate concepts suggest that when experimental dose-response data are accumulated at doses near the maximal tolerated dose, carcinogen activation, detoxification, and repair pathways may become saturated. Under those circumstances, measured dose-response data might not contain the information required to make a low-dose extrapolation, even if the precise mathematical relationship between dose and response were known independently.

In light of such considerations, a generalized multiparameter-fitting protocol may be a reasonable mechanism for generating a low-dose extrapolation. However, it is impossible to determine if this is a consistently conservative procedure, which is the type most generally favored by regulatory agencies. The agencies prefer the risk-assessment approach with the greatest potential for protecting human health, i.e., treating all carcinogens in a similar manner (EPA, 1976; NTP, 1984; Perera, 1984; Weinstein, 1983). Although it may not be possible to use mechanistic data by extrapolation at this time, the committee hopes that as our understanding of the carcinogenic process increases so will our ability to make better risk assessments. For now, any information on mechanisms of cancer induction that bears on the risk-assessment process should at least be noted by those doing the evaluation.

Developmental and Reproductive Toxicity

Assessing developmental and reproductive toxicity is especially complex due to the great variety of possible toxic end points and the likely involvement of threshold doses. At present there is limited agreement about how to apply the results of animal reproductive and developmental

toxicity studies to assess the risk of exposure to a compound. This is partly because an understanding of the underlying events leading to reproductive toxicity is usually missing. There are no agreed-upon standards or quantitative methods for cross-species extrapolation. Nonetheless, there are some conditions under which the committee believes that reproductive and developmental toxicity data could be used to estimate risk in humans. When there are sufficient data, the NOEL approach is the most reasonable approach at this time (EPA-ORNL, 1982). It is usually easier to identify a LOEL from experimental data because this value can be observed directly, whereas the NOEL can be orders of magnitude below the lowest experimental exposure level observed to induce a toxic effect. LOELs are most accurately identified under conditions where the response is minimal and the end point involves reversible effects, indicating that a NOEL is being approached.

There are several considerations in the selection of an appropriate safety factor to be used with NOELs or LOELs for reproductive toxicity. The committee recommends that humans should be considered to be at least 50 times more sensitive than laboratory animals to agents causing well-defined developmental and reproductive toxicity. Substances that lead to developmental toxicity at levels lower than those causing maternal toxicity constitute a greater potential hazard than substances that cause developmental toxicity only at maternally toxic doses. The size of the safety factor should reflect the potential hazard and should consider not only the severity of the response but also the time and route of exposure. The greatest potential hazards are presented by substances causing serious effects under conditions of exposure that may be encountered by humans.

Existing models for quantitative risk assessment have not been sufficiently well developed to be applied to reproductive toxicity data. However, some encouraging work in progress may result in the production of acceptable models for some types of data. The advantage of modeling reproductive toxicity data over the use of NOELs is that the modeling approach takes into account the slope of the exposure-response curve and the size of the experiment. An example of modeling reproductive data is shown in Chapter 9, where tolerance distribution models were applied to the developmental toxicity data for nitrofen.

When insufficient data are available for the NOEL approach or possibly the modeling approach, a ranking system or quantitative index may be used. Underlying this approach is the need to distinguish between substances that are uniquely toxic to the embryo and those that induce developmental toxicity at exposure levels that are also toxic to the mother. Agents in the latter category should be regulated on the basis of their adult toxicity, whereas those in the former would be regulated on the basis of their unique toxicity to the embryo.

Neurotoxicity

In the four-step process concluding with a risk assessment described by a National Research Council committee (NRC, 1983), the work evaluating neurotoxicity appears to present serious difficulties that are a natural consequence of the complexities surrounding neurotoxicity. The many neurotoxic effects that can be induced by different exposures range from barely perceptible sensory deficits to gross behavioral or functional abnormalities. With increasing dose and duration of exposure, one specific effect may be manifested in a larger and larger proportion of the population, or the number of affected people may not increase but the effects may become more and more serious and incapacitating. Intense short-term effects may occur, but no residual effects may be detectable after exposure ends. At the other extreme, an exposure may produce no observable consequences yet may leave the exposed person highly vulnerable to a subsequent exposure to the same, or even an unrelated, neurotoxicant. Nutritional status has been found to strongly affect responses. A very large number of substances are known to produce neurotoxic effects in humans, in contrast to cancer, for which 30 causative materials or industrial processes have been implicated (IARC, 1982).

Because of the many neurotoxic end points, implying many different mechanisms of action, there are essentially no general mathematical models of neurotoxicity leading to quantitative risk assessment. Models may have to be constructed on a material-by-material basis. In some evaluations for lead, levels of exposure were related to neurologic effects, many of which can be associated with hemolytic markers (Table 4-2).

The difficulty in measuring exposures is illustrated by the need to use secondary markers of exposure such as blood lead levels. Regulatory actions are usually based on what have been identified as nominal exposure levels that are objective, measurable external quantities such as parts per million in an air sample. Exposure to these nominal levels, of course, is not the same as the dose, at least not in the biological sense. Lead found in the blood can be regarded as an internal dose (the amount of the substance or its active metabolites in body tissues) or as the biologically effective dose (the amount of the active material that interacts with the tissue or organ). These biological doses are rarely measured; very likely vary with age, sex, and genetic background; and at times may even be unmeasurable.

Because quantitative dose-response models or even adequate measures of exposures do not exist, safety factors must be used. A NOEL or a LOEL must be identified and the observed doses divided by an appropriate safety factor. The intensity or seriousness of the response, age-sex vari

ation, experiment size, and similar factors need to be taken into account in setting the appropriate safety factor. Use of these safety factors is intended to lead to exposure levels that will be safe for the most sensitive individuals. In general, however, they are usually determined on a material-by-material basis and do not take into account aspects of bioaccumulation, sensitization, or multiple exposures—all factors that need specific consideration.

The committee's approach to a quantitative estimate of a safe exposure level through a modified LOEL approach is described for the cholinesterase inhibitor aldicarb in Chapter 9. The approach is based on the argument that if no clinical manifestation of cholinesterase inhibition is observed unless cholinesterase levels are reduced by at least 20% or 30%, then the lower 95% confidence limit on the dose that produced such an inhibition could be looked upon as the acceptable, or maximum permissible, exposure level.

To assess risk for neurotoxic end points, research must be conducted to develop measures of exposure, including biological markers; better laboratory techniques, including short-term tests for identifying neurotoxicants; and quantitative models for low-dose extrapolation, reflecting the different types of effects and species-to-species variability. Studies must also be undertaken to learn whether or not structure-function relationships can be predictive. The effects of interactions and modified (or sporadic) exposures must also be examined. Furthermore, the relationship of morphological changes to neurological or neurotoxic responses should be explored.

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9

Toxicity of Selected Contaminants

The 14 compounds reviewed in this chapter were evaluated at the request of the EPA to assist the agency in regulating contaminants in drinking water. In selecting compounds for review, the committee was guided both by EPA's regulatory agenda and by concerns about important current toxicological issues within the research community. The 14 substances selected were, in order of discussion, acrylamide, aldicarb, diallate, sulfallate, dibromochloropropane, 1,2-dichloropropane, 1,2,3-trichloropropane, 1,3-dichloropropene, di(2-ethylhexyl) phthalate, mono(2-ethylhexyl) phthalate, ethylene dibromide, nitrofen, pentachlorophenol, and trichlorfon. Ten of the 14 contaminants are reviewed by a Safe Drinking Water Committee for the first time. The other four compounds, which were discussed in previous volumes of *Drinking Water and Health*, are reevaluated in this volume. Whenever possible, the committee evaluated published, peer-reviewed literature pertaining to the compounds under study. For trichlorfon and di(2-ethylhexyl) phthalate, however, it examined reviews prepared by the World Health Organization, followed up by telephone calls to the investigators or knowledgeable sponsors. For acrylamide, aldicarb, and nitrofen, important new information was made available to the committee by researchers with projects under way. The committee conducted its own peer review of the unpublished studies and in some cases subjected the data to independent review.

At the first stage of evaluation, an intensive literature review was conducted for each substance. In addition, data summaries were obtained from several offices of EPA, including the Office of Drinking Water and the Office of Pesticides. These summaries were used as an initial indication

of the range of available toxicological data. In some cases, foreign literature was translated and evaluated. Much of the data are the results of 2 year chronic feeding studies in rodents, reflecting past interests in carcinogenesis testing. However, the committee carefully examined toxicological data on other effects, such as teratogenesis, mutagenesis, reproductive effects, and metabolism. In addition, it reviewed the relatively sparse data on current production, manufacture, environmental distribution, and environmental monitoring.

The committee recognized that ingestion may not be the sole route of exposure to contaminants in drinking water. Cooking, showers, bathing, swimming, and other activities could theoretically provide important toxic contributions; however, given the absence of data on these noningestion routes, the committee declined to develop specific estimates of exposure for them. In addition, drinking water is not the only means of exposure to many of the compounds evaluated here. All sources of exposure must be considered by regulators in setting acceptable levels of exposure to contaminants in water, regardless of the biological end point under consideration. To allow for exposures through other routes, the committee generally assumed that drinking water provided 20% of the total exposure to a given compound.

Following its review of the toxicology data, the committee classified compounds according to whether they were or were not known (or suspected) carcinogens. For carcinogens, the risk to humans was expressed as the probability that persons weighing 70 kg would develop cancer some time in their lives as a consequence of ingesting 1 liter of water containing 1 µg of the substance daily over a lifetime of 70 years. Although risks to the 10-kg child were not calculated, the disproportionately high intake of drinking water by children as compared with that of adults would place them at greater risk.

The committee then examined models for extrapolating from the high doses used in animal studies to the lower doses common in the environment of humans, and concurred with many experts who believe that several risk quantification techniques should be utilized to produce an estimated range of risks rather than a single number. The selection of models for low-dose extrapolations must be somewhat arbitrary except for the multistage model, which is the only one with firm biological criteria at this time. Nonetheless, the committee recognizes that risk quantification remains an essential tool for rationalizing regulatory actions.

The computation of risk depends on several factors, ranging from the selection of a mathematical model for low-dose extrapolation to the assumptions made within the model to fit a specific computer program. Because of the uncertainties involved in determining the true shapes of the dose-response curves used for extrapolation and because recent re

search indicates several stages in cancer induction, the committee decided that in general the multistage model is the most useful. It appears to have more of a biological basis than most other models and in most cases is more conservative, giving higher estimates of risk at low doses than most other models. The model incorporates the reasonable assumption of background additivity and is thus linear at low doses.

Within the multistage model, one can compute the dose associated with a given level of risk by using either a restricted model or a generalized model. In the restricted model, the number of possible stages in the multistage process is limited to the number of doses at which the experiment was conducted minus 1. The generalized model places no such limit on the possible number of stages; rather, it permits computation of the best fit to the data without this constraint. A more detailed discussion of the committee's reasoning in selecting the generalized multistage model and its overall framework for risk assessment appears in Chapter 8.

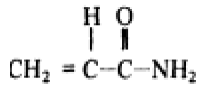
Although, as stated in Chapter 8, the committee believes that an understanding of pharmacokinetic principles is useful to the extrapolation of response at high doses to estimate response at low doses, the relative paucity of pharmacokinetic data is apparent in the risk assessments made in this chapter. Adequate data of this type were not available for any of the compounds studied. The committee recommends a review of the needs and potential gains possible through the use of pharmacokinetic data and, where appropriate, stimulation of the acquisition of such data for compounds under consideration for future risk assessments.

For agents not identified as known or suspected carcinogens and for which there were adequate toxicity data from prolonged ingestion studies in humans or animals, the committee calculated an acceptable daily intake (ADI), using methods developed in earlier volumes of this series and estimating dose-response relationships when data were sufficient. This conventional approach was taken by default in the absence of suitable low-dose extrapolation models and because a "safe level" has not been demonstrated for these noncarcinogenic effects. For carcinogens that produced other toxic effects at low levels, the committee also estimated the minimum exposure levels at which such effects might be expected to occur. The ADI is derived by estimating the no-observed-effect level (NOEL) for any given compound and then dividing it by an uncertainty or safety factor. Aware of the pitfalls encountered in estimating NOELs, the committee carefully weighed the evidence supporting this level in any given study. Also sensitive to possible misinterpretations concerning the use of "safety" factors, the committee recognized that these factors properly indicate levels of confidence in the underlying studies. For some compounds, the data base was adequate to permit an estimate of the magnitude of inter-or intraspecies variability and suggest a safety factor

based on that estimation. Where such an estimation was not possible, the committee used safety factors provided in previous guidelines: 10 when satisfactory data from chronic epidemiological or clinical studies were used; 100 for well-conducted long-term animal studies; and 1,000 for short-term studies or studies with some potential inadequacies. The polymorphism of human drug metabolism indicates that the range of intrahuman variability may be as high as 100-fold, implying that the uncertainty factor of 10 may not be adequately conservative. Furthermore, when extrapolating risk to the general population from epidemiological data, lack of quantitative exposure data may necessitate a further uncertainty factor.

ACRYLAMIDE

2-Propenamide
CAS No. 79-06-1
RTECS No. AS3325000



Acrylamide, the neurotoxic monomer of a commercially important polymer, polyacrylamide, is a highly reactive agent that spontaneously reacts with hydroxyl-, amino-, and sulfhydryl-containing compounds (Hashimoto and Aldridge, 1970). It has a molecular weight of 71.08, a melting point of 84.5°C, and a vapor pressure of 0.007 mm mercury at 20°C. Its solubility in water is 215 g/100 ml at 30°C. Total U.S. production of acrylamide in 1978 was 34,000 metric tons (MacWilliams, 1978). Acrylamide polymers are used as additives to enhance oil recovery, increase dry strength in paper products, dissipate fog, and stabilize soil. They are also used in grouting operations, clarification of potable water, and treatment of municipal and industrial effluents.

The biodegradation and environmental fate of acrylamide have been examined in both water and soil. Cherry et al. (1956) found that acrylamide degraded in filtered river water in 10 to 12 days. However, Croll et al. (1974) found more rapid degradation in river water of approximately 4 days. When acrylamide was added to soil (Lande et al., 1979), complete degradation occurred in approximately 6 days; a maximum of 60% of the acrylamide was degraded to carbon dioxide. Acrylamide should not significantly accumulate in the environment because of its high water solubility (Dow Chemical USA, 1984).

Metabolism

Following administration, acrylamide is rapidly distributed to all tissues, metabolized, and excreted (Edwards, 1975; Miller et al., 1982). After a single dose of ^{14}C-labeled 2,3-acrylamide, Miller et al. (1982) found equivalent concentrations of acrylamide in all tissues except in erythrocytes, where acrylamide appears to accumulate (Pastoor and Richardson, 1981). Nervous system tissues accumulated less than 1% of the dose of acrylamide. The tissue content of radiolabeled acrylamide decayed in biexponential fashion (half-life of approximately 8 days), except in erythrocytes, where shortly after dosing a plateau was reached with a half-life of approximately 10.5 days (Pastoor and Richardson, 1981).

The major route of biotransformation of acrylamide is conjugation with the tripeptide glutathione (Miller et al., 1982; Pastoor et al., 1980); it is eventually excreted in the urine as *N*-acetyl-*S*-(3-amino-3-oxypropyl)cysteine. This route appears to be detoxifying since depletion of the nonprotein sulfhydryl content increases the neurotoxic potency of acrylamide.

In addition to conjugation with glutathione, acrylamide appears to undergo partial microsomal-mediated metabolism.

Health Aspects

Observations in Humans

Although most human exposures to acrylamide result from dermal absorption or ingestion of dust, one report documented exposure and toxicity resulting from drinking water contaminated with acrylamide. Igisu et al. (1975) described a Japanese family of five who ingested well water contaminated with acrylamide from a nearby grouting operation. The concentration of acrylamide in drinking water was found to be approximately 400 mg/liter. The children, who consumed acrylamide-free water while at school, developed mild gait disorders and sleep disorders. The parents, who consumed the acrylamide-contaminated water exclusively, developed slurred speech, unsteady gait, memory loss, irrational behavior, and visual, tactile, and auditory hallucinations.

In other studies in humans, all the investigators (Auld and Bedwell, 1967; Davenport et al., 1976; Fullerton, 1969; Garland and Patterson, 1967) described neuropathy with a consistent set of symptoms. The first clinical manifestation of acrylamide neuropathy in humans is slowly progressing symmetric distal sensory abnormalities and motor weakness. Subjects commonly reported skin sensitization (contact dermatitis); cold, blue hands; unsteadiness; muscle weakness; paresthesia; and numbness of the

hands or feet. Tendon reflexes disappear and vibrational sense is lost, but heat, pressure, and other objective sensory modalities remain intact. Recovery from mild forms of acrylamide neuropathy is usually complete, occurring within a few months. Patients with severe neuropathy may never completely recover, but may experience residual ataxia, distal weakness, and sensory loss.

Observations in Other Species

Acute Effects

The oral LD_{50} for acrylamide is 150 to 180 mg/kg body weight (bw) in rats, guinea pigs, and rabbits and 100 mg/kg bw in cats and monkeys (McCollister et al., 1964). Symptoms of intoxication in cats after high doses of acrylamide include behavioral disturbances, clonic seizures, severe ataxia, tremors, and death due to respiratory failure (Kuperman, 1958). Similiar responses are reported for rats, guinea pigs, rabbits (McCollister et al., 1964), and chickens (Edwards, 1975). Miller et al. (1983) demonstrated inhibition of retrograde axonal transport in peripheral nerves within 24 hours after intraperitoneal administration of acrylamide to rats at 40 mg/kg. Increased numbers of dopamine receptors have been reported in the corpus striatum of rats following oral treatment with a 25-mg/kg bw dose of acrylamide (Agrawal et al., 1981).

Subacute and Chronic Effects

Subacute and chronic exposures to acrylamide have been demonstrated to produce symptoms of peripheral neuropathy in cats, rats, mice, guinea pigs, rabbits, and monkeys (McCollister et al., 1964). As with humans, intoxicated animals develop limb incoordination, which progresses to ataxia and weakness. This is most obvious in the hind limbs. Additional symptoms in acrylamide-intoxicated animals include weight loss, enlarged and distended bladders, and testicular atrophy. The enlarged bladders were attributed to "nervous retention"; however, the animals did continue to pass urine.

The duration of exposure to acrylamide required to produce neuropathy appears to be a direct function of the magnitude of the acrylamide dose. Kuperman (1958) demonstrated that cats treated with acrylamide (1 to 50 mg/kg/day) developed clinical signs of acrylamide intoxication after receiving an average total dose of approximately 102 mg/kg by a variety of routes, including intravenous and intraperitoneal administration, independent of whether the dose was administered over 2 days or 4 months. These data indicate that acrylamide is a cumulative neurotoxicant.

Fullerton and Barnes (1966) demonstrated that degeneration of the distal processes of large-diameter peripheral nerves was associated with acrylamide-induced neuropathy in rats. Exposure to acrylamide in the diet at daily doses of approximately 15 to 18 mg/kg bw for 10 weeks resulted

in severe axon loss and proliferation of Schwann's cells in distal peripheral nerves. Pathology was most evident in the longest fiber tracts containing large-diameter axons. Electron microscopic studies of acrylamide-treated rats conducted by Prineas (1969) revealed dramatic increases in the number of axoplasmic neurofilaments and organelles. Occasional axons demonstrated invaginations of finger-like Schwann's cell processes that may act to remove damaged or degenerating axonal constituents.

The dose of acrylamide required to produce neuropathy following chronic exposure has been investigated in several species. Structural or functional neurologic deficits have been noted after daily oral administration of acrylamide to rats (1 mg/kg bw for 93 days) (Burek et al., 1980), cats (0.7 mg/kg bw for 240 days) (McCollister et al., 1964), and monkeys (1 mg/kg bw for 18 months) (Schaumburg et al., 1982).

Mutagenicity

Acrylamide has been reported to be nonmutagenic in *Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100 at doses of 0.001 to 3 mg/plate with and without metabolic activation (Bull et al., 1984). Lack of acrylamide-induced genotoxicity was confirmed in the hepatocyte primary culture DNA repair test (Miller et al., 1984).

Carcinogenicity

Bullet al. (1984) investigated the carcinogenic effects of acrylamide by administering it to female Sencar mice six times over 2 weeks at oral and intraperitoneal doses of 12.5, 25, and 50 mg/kg. The shaved back of each animal was subsequently treated with 1 µg of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) three times a week for 20 weeks, and the animals were sacrificed after 52 weeks. Acrylamide was found to produce dose-dependent increases in incidence of squamous cell carcinoma. In addition, it produced dose-dependent decreases in the time to tumor appearances. No tumors were seen in animals not treated with TPA. In a separate experiment, Bull et al. (1984) found that oral or intraperitoneal administration of acrylamide to A/J mice at doses of 6.25, 12.5, and 25 mg/kg three times a week for 8 weeks resulted in dose-dependent increases in the incidence of lung adenomas when measured 4 months after the last dose of acrylamide was given.

K. A. Johnson et al. (1984) reported that male and female Fischer 344 rats developed tumors following a 2 year exposure to acrylamide in drinking water. Male rats exposed to acrylamide at 0.5 mg/kg bw a day for 2 years developed scrotal mesotheliomas. At 2.0 mg/kg/day, benign thyroid tumors were also observed in male rats, whereas females demonstrated benign and malignant thyroid tumors, glial tumors within the central nervous system, adenomas of the clitoral gland, squamous cell papillomas in the mouth, benign and malignant mammary tumors, and malignant uterine tumors.

TABLE 9-1 Tumor Incidence in Rats Fed Acrylamide-Contaminated Drinking Water[a]

Animal	Sex	Tumor Site	Dose (mg/kg/day)	Tumor Rates
Fischer 344 rat	Male	Scrotum	0	3/60
			0.01	0/60
			0.1	7/60
			0.5	11/60
			2.0	10/60

[a] Based on data from K. A. Johnson et al., 1984.

Carcinogenic Risk Estimate

In the drinking water study recently completed by K. A. Johnson et al. (1984), there was an increased incidence of scrotal mesothelioma in male Fischer 344 rats. In the study by Bull et al. (1984), there was an increase in lung tumors. The tumor incidences from the K. A. Johnson et al. (1984) study and the Bull et al. (1984) study are summarized in Table 9-1 and Table 9-2, respectively.

Using these data, the committee estimated the lifetime risk and upper 95% confidence estimate of lifetime risk in humans after a daily consumption of 1 liter of water containing acrylamide at a concentration of 1 µg/liter. The conversion of animal to human doses is based on body surface area, assuming the following weights: humans, 70 kg; rats, 400 g; and mice, 33 g. The conversion formula is: animal consumption = human consumption times (human weight/animal weight)$^{1/3}$. The risk estimates calculated with the generalized multistage model are shown in Table 9-3, and those based on the Weibull model are shown in Table 9-4. It is useful to compare the results obtained from the generalized multistage model with those of the Weibull model, which appeared to fit the data better. (See the discussion of risk-assessment models presented in Chapter 8.)

TABLE 9-2 Tumor Incidence in Mice Given Acrylamide Intraperitoneally[a]

	Tumor	Dose	Tumor Rates	
Animal	Site	(mg/kg/day)	Males	Females
A/J mouse	Lung	0	2/16	1/15
		1	8/16	6/17
		3	6/16	9/17
		10	10/17	11/14
		30	14/15	14/15

[a] Based on data from Bull et al., 1984.

TABLE 9-3 Carcinogenic Risk Estimates for Acrylamide from the Generalized Multistage Model[a]

Animal	Sex	Estimated Human Lifetime Risk[b]	Upper 95% Confidence Estimate of Lifetime Cancer Risk[b]
Fischer 344 rat[c]	Male	6.6×10^{-6}	1.2×10^{-5}
A/J mouse[d]	Male	3.8×10^{-6}	7.5×10^{-6}
A/J mouse[d]	Female	8.2×10^{-6}	1.4×10^{-5}

[a] From GLOBAL83, a software program developed in 1983 by R. B. Howe and K. S. Crump; modified for microcomputer compilation in 1985 by M. S. Cohn, U.S. Consumer Product Safety Commission, Washington, D.C.
[b] Assuming daily consumption of 1 liter of water containing the compound in a concentration of 1 µg/liter.
[c] Based on data from K. A. Johnson et al., 1984.
[d] Based on data from Bull et al., 1984.

In previous volumes of *Drinking Water and Health*, the risk estimates for male and female rats and mice were averaged to yield one composite number. If the data for the generalized multistage model in Table 9-3 are averaged, the estimated human lifetime risk is 1.2×10^{-5}; the upper 95% confidence estimate of lifetime cancer risk is 2.1×10^{-5}.

Developmental Effects

Treatment of rats with acrylamide at 200 or 400 ppm daily (20 to 40 mg/kg bw) during gestation has been shown to produce no gross or histologic evidence of teratogenicity (Edwards, 1976).

Conclusions And Recommendations

Acrylamide is a highly reactive molecule that produces peripheral neuropathy in animals and humans following repeated exposure. The mag

TABLE 9-4 Carcinogenic Risk Estimates for Acrylamide from the Weibull Model

Animal	Sex	Estimated Human Lifetime Risk[a]	Upper 95% Confidence Estimate of Lifetime Cancer Risk[a]
Fischer 344 rat[b]	Male	1.7×10^{-4}	9.7×10^{-4}
A/J mouse[c]	Male	4.0×10^{-5}	1.5×10^{-4}
A/J mouse[c]	Female	7.4×10^{-5}	2.7×10^{-4}

[a] Assuming daily consumption of 1 liter of water containing the compound in a concentration of 1 µg/liter.
[b] Based on data from K. A. Johnson et al., 1984.
[c] Based on data from Bull et al., 1984.

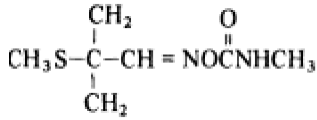
nitude of the dose of acrylamide required to produce neuropathy is inversely related to the duration of exposure. Thus, no-observed-effect levels determined from laboratory studies of relatively short duration (less than 2 years) may be of little value in determining human risk following lifetime exposure. For this reason and because acrylamide is a cumulative toxicant, no further risk assessment was attempted for neurotoxicity.

Recent data demonstrate that acrylamide is carcinogenic in laboratory animals. The estimated lifetime risk and the upper 95% confidence estimate of lifetime risk of cancer in humans presented above are based on both the multistage and Weibull low-dose extrapolation models.

CARBAMATE PESTICIDES (ALDICARB, DIALLATE, AND SULFALLATE)

ALDICARB

2-Methyl-2-(methylthio)propanal *O*-(methylamino)carbonyl]oxime
CAS No. 116-06-3
RTECS No. UE2275000



Since aldicarb was reviewed in Volumes 1 and 5 of *Drinking Water and Health* (NRC, 1977, pp. 635-643; 1983, pp. 10-12), the following section is primarily an examination of data not considered by the previous committees.

Health Aspects

Observations in Humans

The committee subjected to peer review a project report (Cope and Romine, 1973) on acute oral exposure of 12 healthy male volunteers (four males per group). This report indicated that aldicarb doses of 0.025 mg/ kg bw produced approximately 50% inhibition of blood cholinesterase, as measured by a radiometric technique. Cholinesterases are a family of enzymes responsible for hydrolyzing esters of choline such as acetylcholine or butyrylcholine. At the highest dose, 0.1 mg/kg bw, approximately 70% inhibition of blood cholinesterase occurred attended by signs and

symptoms of hypercholinergic action. The signs and symptoms of poisoning were for the most part gone within 4 hours after dosing, and the blood cholinesterase level was normal after 6 hours.

Observations in Other Species

Acute Effects

Cambon et al. (1979) gave corn oil solutions of aldicarb to pregnant female Sprague-Dawley rats by gastric intubation (eight rats per group fasted 24 hours). The doses were 0.1, 0.01, and 0.001 mg/kg bw. The effect of this treatment on fetuses was discussed in Volume 5 of *Drinking Water and Health* (NRC, 1983, pp. 10-12), but the significant inhibition of maternal erythrocyte and liver plasma cholinesterase in dams given 0.1 and 0.01 mg/kg bw was not noted.

Subacute Effects

Aldicarb sulfoxide is a more effective esterase inhibitor *in vitro* than the parent compound, whereas aldicarb sulfone is a poor esterase inhibitor. DePass et al. (1982) gave a 1:1 mixture of aldicarb sulfoxide/aldicarb sulfone to Wistar strain rats (10 rats of each sex per dose level range) in their drinking water for 29 days. The authors reported depressed body weight and food consumption 7 days into the exposure period in a group given 19.2-ppm concentrations of the mixture. These parameters remained depressed throughout the study in males (statistically analyzed on days 14, 21, and 29) but returned to normal in females. However, erythrocyte and plasma cholinesterase activity remained depressed after 8, 15, and 29 days in both males and females exposed to 19.2 ppm. Statistically significant reduction of plasma cholinesterase was seen in males exposed to 4.8 ppm for 8 days but not on later assay dates; erythrocyte cholinesterase was not depressed in this exposure group until day 29. Other exposure levels, where effects were not seen, were 1.2, 0.3, and 0.075 ppm. These data are difficult to interpret in that a mixture of aldicarb metabolites was used, and individual analyses of the two metabolites were not described. Chemical analysis revealed concentrations of the compound at only 80% of nominal values.

Weil and Carpenter (1968) fed Harlan-Wistar rats (15 animals of each sex per group) aldicarb sulfoxide in the diet in concentrations that were believed to provide daily doses of 1.0, 0.5, 0.25, and 0.125 mg/kg bw. It does not appear that true doses were established. Five rats of each sex were sacrificed after 3 months for assay of plasma, erythrocyte, and brain cholinesterase using a titrimetric method. The surviving animals were killed after 6 months on the diet, and the same measurements were taken (except that a colorimetric assay was used). It was found that plasma and erythrocyte cholinesterases were generally more sensitive than brain cholinesterases to aldicarb sulfoxide inhibition. The effect at the lowest dose

was significant inhibition of plasma cholinesterase in males given daily diets providing 0.125 mg/kg bw for 3 months. Six months after treatment ended, this effect was not detected. Erythrocyte cholinesterase was inhibited significantly in both sexes at doses of 0.25 mg/kg/day for 6 months, but effects of 0.125 mg/kg/day for this length of time are unclear. Brain cholinesterase was inhibited significantly by 1.0 mg/kg/day in both sexes at both exposure lengths; in females, it was inhibited by 0.5 mg/kg/day at 6 months.

In a similar study, Weil and Carpenter (1968) noted significant plasma cholinesterase inhibition in male rats (five per group) given diets providing aldicarb sulfoxide at 0.25 but not at 0.125 mg/kg bw a day for 3 months. In both males and females, erythrocyte cholinesterase was significantly inhibited after 3 months on diets providing 0.5 mg/kg/day, but no depression was observed at 0.25 mg/kg/day. In animals fed comparable diets but given a 1-day unadulterated diet prior to sacrifice, no inhibition of esterases was seen, suggesting that rapid reversal of inhibition occurs even after subacute exposures. Weil and Carpenter (1968) also fed beagle dogs aldicarb sulfoxide diets providing 0.5, 0.25, 0.125, or 0.0625 mg/kg bw each day for 3 months. The only significant depression was found in plasma cholinesterase after 1 month on 0.5 mg/kg/day. These animals lost weight in the first week of the study but then returned to normal for the remainder of the experiment.

Chronic Effects

No new data were found by the committee.

Mutagenicity

There have been no published evaluations of the genotoxicity of aldicarb. The National Toxicology Program has listed National Institute for Environmental Health Studies contracts to study the mutagenic and genotoxic effects of the compound in microbial systems and in *Drosophila* (NTP, 1984b).

Carcinogenicity

No new data were found by the committee.

Developmental Effects

No new data were found by the committee.

Conclusions and Recommendations

Signs of hypercholinergic activity, such as lacrimation, salivation, meiosis, and convulsions, are unequivocally toxic effects of aldicarb. These effects result from inhibition of acetylcholinesterase at specific neuroeffector sites, and it appears that a substantial inhibition of enzyme activity is required before an effect is observed. Studies with organophosphorus anticholinesterases suggest that greater than 50% inhibition of plasma acetylcholin

esterase would be required to produce overt hypercholinergic signs (Wills, 1972). Effects of low-level inhibition of acetylcholinesterase by aldicarb have not been encountered, although behavioral effects of long-term, low-level exposure to organophosphorus anticholinesterases have been suggested in the clinical literature (NRC, 1982a).

Research on the unconfirmed effects of low-level inhibition of acetylcholinesterase or other esterases is to be encouraged. Although aldicarb inhibits other esterases, the physiological functions for these enzymes are not known. For example, serum cholinesterase is aldicarb sensitive, but the genetically determined absence of this enzyme does not appear to affect one's health (Kalow, 1965). Serum cholinesterase inactivates the neuromuscular blocking agent succinylcholine (Taylor, 1980). Thus, inhibition of esterases by aldicarb might alter the metabolism of other xenobiotic substances.

Orally administered aldicarb inhibits esterases in both animals and humans at relatively low doses, compared to other carbamates. The onset of action and recovery from inhibition are rapid. In view of the transience of esterase inhibition, studies with drinking water or feed as a route of exposure probably lead to serious underestimations of the inhibition that would result from an equivalent dose given as a bolus. This may be part of the reason that doses considered NOELs in rodents (0.1-0.125 mg/kg bw over 24 hours) produced overt signs of hypercholinergic action when given to humans in a single, acute dose (0.1 mg/kg bw) (Cope and Romine, 1973). It appears that exposure through bolus administration is the worst-case circumstance that must be addressed when determining appropriate measures to avoid aldicarb toxicity.

Unfortunately, data on esterase inhibition after bolus administration are limited. Cambon et al. (1979) have relevant data, but the assays were done on tissue from pregnant female rats and the appropriateness of that model is highly debatable. Cope and Romine (1973) obtained data from a very small number of healthy human males but did not estimate a NOEL. However, there are several considerations in favor of using their data:

- The data pertain to healthy human male subjects, eliminating the uncertainty of interspecies extrapolation.
- The dose was administered in drinking water. Thus, the route of exposure is identical to accidental drinking water ingestions.
- Whole blood esterase was measured. This esterase activity is due primarily to red blood cell (RBC) acetylcholinesterase (Vandekar, 1980). Data from animal studies indicate that RBC acetylcholinesterase may be more sensitive to inhibition by oral aldicarb exposure than the acetylcholinesterase of the relevant neuroeffector sites. Although RBC acetylcholinesterase has no known physiological function, its use provides an additional

safety factor in determining daily intakes that would not be expected to produce toxicity. Wilkinson et al. (1983) have suggested, however, that the extreme dilution of central nervous system (CNS) tissue homogenates required for acetylcholinesterase assay may promote reversal of inhibition *in vitro*. This would result in underestimation of *in vivo* CNS acetylcholinesterase inhibition and provide an alternative explanation for the disparity in measurements between the CNS and erythrocytes.

In addition, this small study in humans produced a dose-effect curve that could be used to extrapolate to doses of aldicarb expected to cause minimal inhibition of whole blood esterase, which is unlikely to be associated with toxic effects. Because of the large variability in cholinesterase inhibition, a 20% inhibition of whole blood esterase was set as the point for extrapolation. This is a value that Gage (1967) suggested as the minimum to be considered abnormal, given the population variance in erythrocyte cholinesterase. Gage (1967) used a formula suggested by Callaway et al. (1951) and assumed that variation in erythrocyte cholinesterase activity in one person would be approximately 10%. Using these assumptions, he calculated that minimal significant inhibition would be 16.5% if derived from a mean of multiple preexposure activity determinations from one person, but would rise to 23% if based on a single preexposure determination.

A previous Safe Drinking Water Committee discussed the use of 20% inhibition in estimating the ADI for various pesticides (NRC, 1980, p. 31). Many organizations have set 30% inhibition of red cell cholinesterase as a level at which action to prevent further exposure to organophosphorus anticholinesterases should be taken (Permanent Commission and International Association of Occupational Health, Subcommittee on Pesticides, 1972; Vandekar, 1980). In their evaluation of aldicarb residues in Long Island groundwater, Wilkinson et al. (1983) expressed the opinion that up to 40% inhibition of whole blood cholinesterase would not be likely to produce overt toxic effects.

The maximum permissible cholinesterase inhibition in erythrocytes for repeated or continuous exposure before significant health effects occur cannot be determined from currently available data. The relationship between erythrocyte cholinesterase activity and signs of toxicity varies with different substances and also may vary among individuals, depending on factors such as rate of distribution and metabolism of inhibitors (Murphy, 1980). In this committee's view, repeated levels of 20% to 30% inhibition are important indicators of changes in biological status. Therefore, the committee decided to develop estimates for both these levels. The extrapolation was done on the upper 95% confidence interval of the dose. (The small number of subjects and the inherent variability in the population

were, of course, reflected in the size of the confidence interval.) The committee also used the maximum likelihood estimate dose (i.e., the dose above which approximately 50% of the population would be affected) with an added safety factor of 10, because the test population consisted exclusively of healthy males. Both the 20% and 30% cholinesterase inhibition in red blood cells were used to measure response against the logarithm of dose with the logit and the probit dose-response models. These mathematical models, more fully described in Chapter 8, both produce S-shaped dose-response curves and give essentially the same dose-response relationship for aldicarb, even though they represent different statistical treatments. The results of the curve-fitting are given in Table 9-5.

TABLE 9-5 Estimated Dose of Aldicarb for Cholinesterase Inhibition from the Probit and Logit Dose-Response Models

	Dose Estimated by Probit Model (mg/kg/day)		Dose Estimated by Logit Model (mg/kg/day)	
Percent Inhibition	MLE[a]	95% UCL[b]	MLE[a]	95% UCL[b]
20	0.0052	0.00011	0.0051	0.000105
30	0.0100	0.00052	0.0100	0.00051

[a] Maximum likelihood estimate.
[b] Upper confidence limit.

Because the percentages of inhibition data essentially reflect a continuous measure and are not the quantal response (yes-no) data that are usually fitted by tolerance (or individual sensitivity) distributions, the use of these models may be questionable for these data. However, the molecular interactions between chemical and enzyme may be considered quantal interactions for these types of models. Nonetheless, an alternative to the tolerance distribution models would be to fit a simple least-squares line to them, but such a dose-response curve has the inherent potential of estimating a less-than-zero or more-than-100% response at doses outside the observed range. Neither the probit model nor the logit model permits such antiintuitive estimates, and both reflect the S-shaped nature of the usual dose-response curve. These "reasonable" features of the models argue for their use in this context.

The results of long-term feeding studies in rats and dogs have been reviewed in previous volumes of *Drinking Water and Health* (NRC, 1977, pp. 635-693; 1983, pp. 10-12). These studies have established no-observed-effect levels of 0.1 mg/kg bw a day in both species. Using a safety factor of 100 for the animal study results, the committee obtained a value (0.001 mg/kg/day) identical to that obtained in human studies with

both the probit and logit dose-response models for 30% inhibition and a safety factor of 10. This value results in the calculation of a 0.007-mg/ liter ADI for aldicarb (NRC, 1983, p. 12).

TABLE 9-6 Estimated ADIs for Aldicarb Based on 20% to 30% Cholinesterase Inhibition for Adults and Children Using Both the MLE[a] and the 95% UCL[b]

Cholinesterase	Adult (μg/liter)		Child (μg/liter)	
Inhibition (%)	MLE[a]	95% UCL[b]	MLE[a]	95% UCL[b]
20	3.5	0.7	1	0.2
30	7	3.5	2	1

[a] Maximum likelihood estimate from the probit and logit models.
[b] Upper confidence limit.

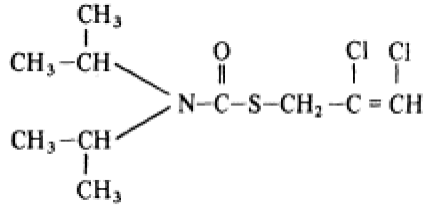
Using the values obtained in Table 9-5, the committee calculated ADIs for both adults and children. For these calculations, it is assumed that a 70-kg adult consumes 2 liters of water daily, that a 10-kg child consumes 1 liter of water daily, and that 20% of the intake is provided by water. The following is an example of the calculation for an adult using the maximum likelihood estimate (MLE) from either the probit or logit dose-response models for 30% inhibition and an uncertainty factor of 10:

$$\frac{0.01 \text{ mg/kg bw/day} \times 70 \text{ kg bw} \times 0.2}{2 \text{ liters} \times 10} = \frac{0.007 \text{ mg/liter,}}{\text{or } 7 \text{ μg/liter.}}$$

The results of these calculations are shown in Table 9-6.

DIALLATE

Diisopropylthiocarbamic acid *S*-(2,3-dichloroallyl) ester bis(1 methylethyl)carbamothioate
CAS No. 2303-16-4
RTECS No. EZ8225000



Diallate is an herbicide used for preemergence control of wild oats and other weeds in fields of sugar beets, flax, barley, corn, and a variety of

other crops. It has a molecular weight of 270.2 and is a liquid at temperatures greater than 25°C to 30°C but less than 150°C.

At 25°C, diallate has a vapor pressure of 0.00015 mm mercury and a density of 1.188 g/ml at 25°C. The herbicide is supplied in the United States as an emulsified concentrate containing 45% active ingredient and also as a powder consisting of 10% diallate. Diallate has a half-life in soil of approximately 30 days when applied at recommended levels of 1.5 to 3.5 pounds/acre (1.3 kg to 3.1 kg/ha). Estimated exposure to diallate during the application process is approximately 0.5 µg/kg bw as a result of inhalation and 980 µg/kg bw from dermal deposition (Dubelman et al., 1982).

Metabolism

Data are not available.

Health Aspects

Observations in Humans

Data are not available.

Observations in Other Species

Acute Effects

Oral administration of diallate to rats (500 to 1,600 mg/ kg bw) or guinea pigs (330 to 1,125 mg/kg bw) produced excitement and restlessness within 2 hours. There is increased sensitivity to pain and touch, ataxia, and hind limb paralysis. Animals died from respiratory failure within I to 4 days (Doloshitskii, 1969). The acute LD_{50} was approximately 1 g/kg bw in rats and 420 mg/kg bw in guinea pigs.

Subchronic Effects

Rats treated with oral diallate doses of 200 mg/ kg/day died within 18 days. Administration of 100 mg/kg/day resulted in the death of 76% of the animals tested. Prior to their death, white-blood-cell counts and neutrophils were significantly increased, lymphocytes were decreased, eosinophils were absent, coagulation time was increased, and weight loss was apparent (Doloshitskii, 1969). In another study (Palmer et al., 1972), sheep developed muscle spasms following three daily oral doses of diallate (25 mg/kg bw).

Chronic Effects

Oral administration of diallate to rats at daily doses of 5, 20, and 50 mg/kg bw for 8 months produced increased blood sugar levels, inability to maintain body temperature, decreased serum cholin

esterase activity, and alterations in central nervous system function (e.g., of rheobase and chronaxy). The apparent no-observed-effect level was 0.5 mg/kg bw a day (Doloshitskii, 1969).

TABLE 9-7 Hepatoma Incidence in Mice Exposed to Diallate (Avadex)[a]

	Tumor Rates			
	Controls		Treated	
Mouse Strain and Sex	All	Hepatic	All	Hepatic
(C57BL/6 x C3H/Anf)F_1	22/79	8/79	14/16	13/16
Males	8/87	0/87	5/16	3/16
Females				
(C57BL/6 x AKR)F_1	16/90	5/90	12/18	10/18
Males	7/82	1/82	2/15	1/15
Females				

[a] Based on data from Innes et al., 1969.

Neurotoxicity

In cats, rats, and mice, diallate produced central nervous system excitation that rapidly progressed to clonic convulsions (Pestova, 1966). Diallate has also been reported to produce delayed peripheral neuropathy in hens (EPA, 1977).

Mutagenicity

Diallate produced base-pair mutations in TA100 and TA1535 strains of *Salmonella typhimurium* at doses as low as 1 µg/plate (de Lorenzo et al., 1978; Sikka and Florczyk, 1978). Mutagenic activity was dependent upon metabolic activation by hepatic microsomes. Diallate appeared incapable of inducing frameshift mutations. The ultimate mutagen produced from diallate is believed to be 2-acrolein (Rosen et al., 1980).

Carcinogenicity

Diallate is carcinogenic in mice following oral administration (IARC, 1976). Daily administration of an oral 215-mg/kg bw dose for 4 weeks and then 560 ppm in the diet (approximately 215 mg/ kg bw) for an additional 74 weeks significantly increased the incidence of hepatomas in male and female (C57BL/6 x C3H/Anf)F_1 mice and male and female (C57BL/6 x AKR)F_1 (strain Y) mice (BRL, 1968a; Innes et al., 1969). The incidence of hepatomas is shown in Table 9-7. Administration of diallate by the subcutaneous route (1 g/ kg) for 74 weeks resulted in an increased incidence of systemic reticulum cell sarcomas only in male (C57BL/6 x C3H/Anf)F_1 mice (BRL, 1968a). Diallate was found to be noncarcinogenic when administered to rats in the diet at 150 and 300 ppm (15 and 30 mg/kg bw) for 78 weeks (Weisburger et al., 1981).

Developmental Effects

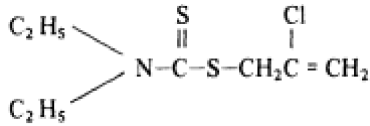
Data are not available.

Conclusions and Recommendations

Diallate is a mutagenic and carcinogenic herbicide. No risk estimate has been calculated, since the data on carcinogenicity of diallate are limited and the early study by Innes et al. (1969) involved a single-dose level using a limited protocol. Diallate should be reevaluated when further carcinogenicity data become available.

SULFALLATE

2-Chloro-2-propenyl diethylcarbamodithioate
CAS No. 95-06-7
RTECS No. EZ5075000



Sulfallate is a carbamate herbicide used for preemergent control of the growth of grasses and weeds in fruit and vegetable crops. Its molecular weight is 223.79. It is an amber liquid at 25°C, boils at 128-130°C, and has a density of 1.088 g/ml at 25°C. Sulfallate is soluble in most organic solvents and in water up to 100 ppm at 25°C.

Metabolism

Data are not available.

Health Aspects

Observations in Humans

Data are not available.

Observations in Other Species

Acute Effects

The oral acute LD_{50} for sulfallate in rats is 850 mg/kg bw (Gosselin et al., 1984). No acute effects were described.

Mutagenicity

Sulfallate produces base-pair substitutions in TA 100 and TA1535 strains of *Salmonella typhimurium* at doses as low as 10 µg/plate

in the presence of metabolic activation (de Lorenzo et al., 1978; Sikka and Florczyk, 1978). In *S. typhimurium* strains TA98 and TA1538, sulfallate is inactive, suggesting that it is incapable of inducing frameshift mutations even in the presence of metabolic activation (de Lorenzo et al., 1978; Sikka and Florczyk, 1978). The ultimate mutagen produced from sulfallate is believed to be 2-chloroacrolein (Rosen et al., 1980).

Chronic Effects

Chronic feeding studies have demonstrated that rats fed 250-ppm concentrations of sulfallate in feed for 6 months develop eye irritation, tubular nephropathy, and hyperkeratosis of the forestomach (NCI, 1978d).

Carcinogenicity

The carcinogenic potential of sulfallate has been studied in Osborne-Mendel rats and $B6C3F_1$ mice (NCI, 1978d). The compound was administered in the diet for 78 weeks. The high and low dietary concentrations (time-weighted averages) of sulfallate for each species were, respectively, 410 and 250 ppm for male rats, 404 and 250 ppm for female rats, 1,897 and 949 ppm for male mice, and 11,815 and 908 ppm for female mice. Rats were observed for 25 to 26 weeks after the last dose of sulfallate, whereas mice were observed for 12 to 13 weeks afterward.

Sulfallate-treated rats had statistically significant dose-dependent increases in incidence of mammary adenocarcinomas (females) and stomach neoplasms (males). In male mice there was an increased incidence of combined alveolar/bronchiolar carcinomas and adenomas, while the incidence of mammary adenocarcinomas was elevated in female mice. Female rats appeared to be the most sensitive species, so the mammary tumor data were used to estimate lifetime risk and an upper 95% confidence limit for human exposure.

Carcinogenic Risk Estimate

Table 9-8 shows the tumor incidence rates used to calculate the risk estimate by the generalized multistage model (Table 9-9), which is described earlier in this chapter.

TABLE 9-8 Tumor Incidence in Sulfallate-Exposed Rats[a]

Animal	Sex	Tumor Site	Dose, ppm (*mg/kg/day*)[b]	Tumor Rates
Osborne-Mendel rat	Female	Mammary gland[c]	0 250 (11) 404 (17.8)	0/50 7/50 11/48

[a] Based on data from NCI, 1978d.
[b] Converted from ppm in diet.
[c] Adenocarcinoma.

TABLE 9-9 Carcinogenic Risk for Sulfallate Estimated with the Generalized Multistage Model[a]

Animal	Sex	Estimated Human Lifetime Risk[b]	Upper 95% Confidence Estimate of Lifetime Cancer Risk[b]
Osborne-Mendel rat	Female	1.0×10^{-6}	1.6×10^{-6}

[a] Based on data from NCI, 1978d.
[b] Assuming daily consumption of 1 liter of water containing the compound in a concentration of 1 µg/liter.

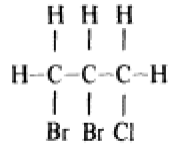
Developmental Effects
Data are not available.

Conclusions

Sulfallate is a mutagenic and carcinogenic herbicide. It appears to be a more potent carcinogen than diallate, discussed above. Sulfallate produced a broad range of tumors at multiple sites in several organs, whereas diallate produced a limited number of tumors at a single site. However, the carcinogenicity study of diallate was performed using a limited protocol.

DIBROMOCHLOROPROPANE

1,2-Dibromo-3-chloropropane
CAS No. 96-12-8
RTECS No. TX 8750000



1,2-Dibromo-3-chloropropane (DBCP) has a molecular weight of 236.4. It is an amber to dark-brown liquid with a boiling point of 196°C and a

melting point of 6°C. It is a short-chain aliphatic halogenated hydrocarbon. Technical DBCP, used as a soil fumigant and nematocide since the 1950s, typically contains several impurities such as epichlorohydrin and allyl chloride. Several reviews of the toxicity of DBCP have been published (Babich et al., 1981; IARC, 1979; NIOSH, 1978a; Whorton and Foliart, 1983). DBCP was evaluated in Volume 4 of *Drinking Water and Health* (NRC, 1982b, pp. 209-214). The following section is an examination of data that have become available to the committee since then.

Health Aspects

Observations in Humans

Observations regarding the health effects of DBCP in humans have been focused on workers employed in the manufacture and agricultural application of this nematocidal fumigant (Whorton and Foliart, 1983). Although produced and in use since the 1950s, the chemical's effect on human testicular function was not recognized until 1977. At that time, a study of male DBCP production workers at a California production plant confirmed workers' concern about apparent infertility by demonstrating azoospermia or severe oligospermia in 14 of the 25 men studied (Whorton et al., 1977). These findings were later reconfirmed by extended observations at the same plant (Whorton et al., 1979) and in studies of DBCP production workers elsewhere (Egnatz et al., 1980; Lipschultz et al., 1980; Márquez Mayaudón, 1978; Milby and Whorton, 1980; Potashnik et al., 1979). Similar sperm-count reductions were observed in workers exposed during agricultural application of DBCP (Glass et al., 1979; Sandifer et al., 1979). The degree to which spermatogenesis was reduced appears to have been directly related to the intensity and duration of chemical exposure.

Reduction in sperm production is accompanied endocrinologically by elevations in serum levels of follicle-stimulating and luteinizing hormones, and histologically by reduction or absence of spermatogenic cells in testicular seminiferous tubules, while Leydig's and Sertoli's cells remain normal (Biava et al., 1978; Potashnik et al., 1979). Egnatz et al. (1980) concluded that within a population of 232 workers who may have been exposed to DBCP, follicle-stimulating and luteinizing hormone levels were above normal, sperm counts were significantly lower, and testicular volume was less than that of a control population of 97 unexposed workers. From their findings of a significant correlation between the magnitude of DBCP-induced effects and the time since termination of DBCP exposure, the authors concluded that DBCP-induced effects may be reversible. The possibility that reduction of spermatogenesis may also involve disturbance of genetic material in sperm was suggested by Kapp et al. (1979), who

observed an increased frequency of Y-chromosome nondisjunction in the sperm of 18 DBCP-exposed workers (3.8% compared with 1.2% in 15 controls). An interview study of 62 agricultural workers in Israel suggests that spontaneous abortions may have been more frequent in wives of DBCP-exposed workers (19% of 121 pregnancies) than in wives of unexposed workers (6.6% of 76 pregnancies) (Kharrazi et al., 1980).

Follow-up studies of DBCP-exposed workers have demonstrated that recovery of sperm production either does not occur or is greatly impeded in men with a complete absence of spermatogenesis as a result of exposure. For men in whom spermatogenesis is only partially inhibited, complete recovery appears to take place within a few months after cessation of exposure (Lantz et al., 1981; Whorton and Milby, 1980; Whorton et al., 1979). Retrospective analysis of workers' reproductive histories suggests that if history-taking had been more thorough, the presence of impaired fertility in DBCP-exposed workers may have been detected as early as 1959, approximately 18 years before the effect was recognized (Levine et al., 1981, 1983).

The National Institute for Occupational Safety and Health (NIOSH, 1978a) described pineapple workers monitored for exposure to DBCP (0 to 1.8 ppm during the workday). Sperm counts were not found to vary among groups exposed to different doses in this range. Thus, NIOSH concluded that a 1-ppm exposure has no observable effects on male fertility.

Whorton and Foliart (1983) reviewed these epidemiological data and concluded that occupational exposure to DBCP at levels significantly above 1 ppm can lead to sperm and testicular abnormalities. However, they emphasized that the reversibility of those effects and their dose response have not been well defined.

No observations concerning possible toxic effects in women are recorded in the medical literature. Likewise, no studies have been published regarding possible long-term sequelae such as cancer in humans.

Observations in Other Species

Acute Effects

The acute lethality of ingested DBCP has been investigated in a number of species of laboratory animals. Oral LD_{50} values determined by Torkelson et al. (1961) ranged from 180 mg/kg bw in male rabbits to 410 mg/kg bw in female mice. The oral LD_{50} of 300 mg/kg bw in male rats was similar to an LD_{50} of 350 mg/kg bw for rats reported by Rakhamatullaev (1971), who also reported 316 mg/kg bw for guinea pigs and 440 mg/kg bw for rabbits. The dosed animals showed transient excitation followed by motor incoordination and impaired sensitivity to pain.

The kidney and testes are the primary target organs of DBCP following a single dose of the chemical. Kluwe (1981a) conducted a dose-response study in which male rats were given a single subcutaneous injection of 0, 10, 20, 40, 80, or 120 mg/kg bw and sacrificed 24 hours later. Although no adverse effects were seen in response to the 10- and 20-mg/kg doses, 40 mg/kg significantly altered several urinary indices of kidney injury and caused marked cytoplasmic vacuolization of the renal proximal tubular epithelium in the outer medulla. Forty mg/kg produced only mild hepatocellular swelling in the periportal region of liver lobules. The highest dose (120 mg/kg) was required to produce hepatic centrilobular necrosis. Examination of the testes and epididymis revealed relatively minor cellular injury 24 hours after the two highest doses (80 and 120 mg/kg).

In a subsequent study of the development and repair of DBCP-induced lesions, Kluwe (1981b) saw progressive seminiferous tubular desquamation and atrophy of the testes of rats given a single 100-mg/kg subcutaneous injection. Although there was some evidence of recovery, many seminiferous tubules were still devoid of germinal cells, and epididymal sperm density remained low 30 days after dosing. The kidneys contained large areas of fibrotic tissue and foci of interstitial nephritis at 30 days. Reznik and Sprinchan (1975) similarly observed prolonged effects in rats given a single oral dose of 100 mg/kg bw. Pronounced reductions in sperm count and sperm motility, as well as morphological changes of the testicular germinal epithelium, were noted 1 and 2 weeks after exposure.

Many studies in which DBCP was given in a single, large oral dose were conducted to investigate biochemical changes and mechanisms of toxicity. Kato et al. (1980b) found a dose-dependent reduction in levels of glutathione (known as GSH) in the livers of male rats given as little as 20 mg/kg bw orally. A sufficient dose of DBCP would be expected to reduce GSH levels, since GSH plays an important role in the detoxification of DBCP. Maximum covalent binding of ^{14}C-labeled DBCP to liver and kidney macromolecules in rats 6 hours after oral administration of a 20-mg/kg dose coincided with significant depletion of hepatic GSH (Kato et al., 1980a). This dose also resulted in incorporation of radioactivity into liver protein, RNA, and DNA 24 hours after dosing. The findings of Kato et al. (1980a,b) support the concept that GSH protects vital nucleophilic sites in cellular macromolecules of target organs from electrophilic attack by reactive DBCP metabolites.

Kluwe et al. (1981), however, discounted the protective role of GSH in DBCP toxicity. These investigators noted that the testes are a major target organ of DBCP, yet the chemical had little effect on testicular nonprotein sulfhydryl (NPS, largely GSH) in mice. They also observed that DBCP caused relatively minor liver injury, yet it markedly depleted hepatic GSH. Kluwe et al. (1981) pointed out that DBCP-induced GSH

depletion appears to correlate well with organ distribution of the chemical and with the organs' capacities for GSH conjugation.

In a subsequent study in Fischer 344 rats, Kluwe et al. (1982) found that subcutaneous injection of DBCP did not lower GSH levels in the kidneys or testes. The investigators did find that pretreatment with diethylmaleate lowered hepatic, renal, and caput epididymal NPS. Single subcutaneous injections of DBCP produced dose-dependent lesions in the liver, kidney, testes, and caput epididymis. Diethylmaleate treatment 90 minutes before DBCP treatment enhanced the nephrotoxic potency of DBCP. Seminiferous tubular degeneration, as determined 48 hours after DBCP treatment, was greater in rats pretreated with 600-mg/kg doses of diethylmaleate than in nonpretreated controls. These results indicate that DBCP is a depletor of hepatic and caput epididymal NPS in the acutely toxic dose range. Since NPS concentrations were not lowered in two of the major target organs, the kidney and testes, acute DBCP injury would not appear to be dependent on local GSH depletion. However, the greater susceptibility of kidney and testes to DBCP injury after pretreatment with diethylmaleate suggests an important role for NPS in modulating DBCP toxicity, especially in the liver.

Single, large oral doses of DBCP produce a number of effects in a variety of tissues. Moody et al. (1982a) found a significant reduction in the number of microsomes in the cytochrome P450 isolated from liver, kidney, testes, lung, and small intestine of male Sprague-Dawley rats 48 hours after the animals received 200 mg/kg orally. There was also a slight decrease in hepatic microsomal NADPH cytochrome*c* reductase activity and cytochrome-b_5 content, but no apparent change in dealkylation activities. Moody et al. (1982b) evaluated the influence of a 200-mg/kg bw oral dose on liver protein synthesis in male Sprague-Dawley rats. No effect was seen on total liver DNA content, on RNA, or on protein synthesis. Although DBCP did not cause a change in overall protein synthesis, the authors noted that their findings did not rule out the action of DBCP on the synthesis of specific proteins in the liver.

Suzuki and Lee (1981) gave male Sprague-Dawley rats a 125-mg/kg oral dose of DBCP and then measured aryl hydrocarbon hydroxylase (AHH) and epoxide hydrolase activities in selected tissues. AHH was not induced in the liver or prostate, but it was induced in the stomach, testes, and kidneys. Epoxide hydrolase was maximally increased 72 hours after dosing in the liver, kidney, testes, and prostate. Moody et al. (1981) assessed the influence of a 600-mg/kg bw oral dose on a series of hepatic microsomal parameters in male Sprague-Dawley rats in order to gain insight into the mechanism of DBCP-induced decrease in cytochrome P450. This very high dose did not alter the content of hepatic microsomal proteins, RNA, phospholipids, diene conjugates, NADPH cytochrome-*c*

reductase activity, or cytochrome-b_5, but it did significantly diminish cytochrome P450 levels. The decrease in cytochrome P450 was accompanied by changes in microsomal fatty acid composition, leading the authors to speculate that the decrease may have resulted from DBCP-induced perturbation of the lipid environment of cytochrome P450 in the microsomes.

Tofilon et al. (1980) offered another explanation for the drop in cytochrome P450. They observed a maximum decrease in testicular microsomal cytochrome P450 in male Sprague-Dawley rats 2 days after oral administration of 200 mg/kg bw and a return to normal cytochrome P450 levels after 6 days. Heme oxygenase and δ-aminolevulinic acid synthetase activities were not altered 24 hours after dosage, but heme synthesis was significantly inhibited. These results indicate that DBCP reduces cytochrome P450 levels by inhibiting heme synthesis through action at a site other than δ-aminolevulinic acid synthetase in the heme biosynthesis pathway.

The use of microsomal enzyme inducers and inhibitors to elucidate the role of metabolism in DBCP toxicity has yielded conflicting results. Kato et al. (1980b) found that phenobarbital pretreatment of rats exposed to ^{14}C-labeled DBCP resulted in increased elimination of radioactivity in urine, enhanced GSH depletion in liver and kidney, and increased binding of the radiolabeled compound in liver and kidney macromolecules, as well as greater liver damage and increased mortality. Pretreatment with SKF-525A had the opposite effect on each parameter. Kluwe et al. (1981) reported that pretreatment of male ICR mice with enzyme inducers—polybrominated biphenyls (PBBs)—or with the enzyme inhibitor piperonyl butoxide decreased the GSH-depleting action of DBCP in the liver and kidneys. SKF-525A was without demonstrable effect. Pretreatment with PBBs resulted in an increase in the LD_{50} of DBCP in mice, but piperonyl butoxide was without effect. In a subsequent paper, Kluwe (1983) reported that phenobarbital pretreatment reduced DBCP nephrotoxicity, hepatotoxicity, seminiferous tubular atrophy, and degeneration of the epithelium of the caput epididymis in rats. The influence of other microsomal enzyme inducers and inhibitors on DBCP toxicity varied from one organ to another, leading the author to point out the complexity of interpreting the effects of these agents on extrahepatic toxicity.

In another study of the role of metabolism in DBCP toxicity, Kluwe et al. (1983) examined the relative toxicity of DBCP, epichlorohydrin, α-chlorohydrin, and oxalic acid to the liver, kidney, testes, and epididymis. DBCP, epichlorohydrin, and α-chlorohydrin caused similar lesions in the epididymis and testes of rats. The morphological and functional effects of the four compounds on the kidney differed, suggesting that DBCP nephropathy does not result from the metabolism of DBCP to epichlo

rohydrin or α-chlorohydrin or from the subsequent metabolism of these two compounds to oxalic acid.

Subacute Effects

A number of studies on subacute effects have been conducted with oral doses of DBCP. Kluwe (1981a) gave 40 mg/kg of analytical-grade (>99% pure) DBCP daily for 4 days to male rats by gavage. This dosage regimen produced significant functional and histopathological changes of the kidneys as well as marked testicular and epididymal degeneration. Relatively little effect was apparent in the liver.

Technical-grade DBCP was given by gavage 5 times weekly for 6 weeks to male and female mice and rats in order to establish the maximally tolerated doses to be used in a subsequent carcinogenicity bioassay (NCI, 1978a). The mice and rats received doses of 100 to 631 and 25 to 160 mg/kg/day, respectively. Reduced body weight gain was seen in male and female rats at the lowest dosage level (25 mg/kg). Deaths occurred in groups of female rats receiving doses greater than 25 mg/kg as well as in male rats receiving more than 40 mg/kg. The mice were less sensitive to DBCP, in that a dose of 398 mg/kg was required to cause death of males and females.

Several investigators have conducted long-term oral dosing studies of DBCP. Torkelson et al. (1961) maintained male and female rats on powdered diets containing DBCP concentrations of 0, 5, 20, 50, 150, 450, or 1,350 ppm for 90 days. Decreased body weight gain was manifest in the females receiving 150 ppm and in animals of both sexes receiving 450 and 1,350 ppm. Female rats ingesting ≤20 ppm exhibited increased kidney-to-body-weight ratios. If the rats consumed a quantity of food equal to 10% of their body weight daily, the animals receiving 20 ppm would ingest 1 mg/kg bw daily. The lowest-observed-effect level (LOEL) in this study thus was 1 mg/kg bw, and the no-observed-effect level (NOEL) was 0.25 mg/kg bw. In another study, Reznik and Sprinchan (1975) gave male and female rats 10 mg/kg bw orally for 4 to 5 months. Decreases in sperm count and sperm motility were initially seen during the second month. These effects became more pronounced as the study progressed. Inhibition of spermatogenesis was observed upon examination of the testes. Progressive alterations of a number of biochemical parameters in testicular and ovarian tissue were reported as were increasingly severe disturbances of the menstrual cycle of the female rats.

Subacute inhalation studies have demonstrated that the kidneys, testes, and respiratory tract are particularly susceptible to injury from inhaled DBCP. Torkelson et al. (1961) exposed male and female rats, guinea pigs, and rabbits 7 hours daily to vapor containing 12-ppm concentrations of DBCP 50 to 66 times within 70 to 92 days. The male and female rats experienced 40% to 50% mortality. Cloudy swelling of the proximal

tubular epithelium and a slight increase in interstitial tissue were observed in the kidneys of male rats. The most striking changes seen at autopsy were severe atrophy and degeneration of the testes of all three species. In a follow-up experiment reported at the same time, male rats were exposed 7 hours daily to 5-ppm concentrations of DBCP 50 times in 70 days. Histopathological changes were focal: they were limited to the testicular epithelium, the collecting tubules of the kidneys, and the bronchioles.

Saegusa et al. (1982) also found that the testicles, kidneys, and lungs were severely injured in male rats subjected continuously for 14 days to vapor containing a 10-ppm DBCP concentration. Testes completely atrophied with irreversible aspermatogenesis. Reznik et al. (1980b) conducted a more definitive dose-response study in which emphasis was placed on respiratory pathology. Male and female rats and mice were exposed to 0-, 1-, 5-, or 25-ppm concentrations of DBCP by inhalation 6 hours/day, 5 days/week for 13 weeks. The severity and incidence of histopathological changes of the nasal cavity were dose related. Changes in all dosage groups in the region of the respiratory turbinates included cytomegaly of the basal cells, focal hyperplasia, squamous metaplasia and disorientation of basal and ciliated cells, and loss of cilia. Necrosis and squamous metaplasia of the olfactory, tracheal, and bronchial epithelium were present in the animals receiving 25 ppm.

Chronic Effects

Rakhamatullaev (1971) conducted a study in which male rats were given DBCP at 0, 0.005, 0.05, 0.5, and 5.0 mg/kg bw orally for 8 months. The doses of 0.5 and 5.0 mg/kg were reported to be gonadotoxic, to produce functional disturbances of the liver and kidneys, to alter the composition of the blood, and to cause disturbances in conditioned reflexes. The author concluded that 0.005 mg/kg was a NOEL and that 0.05 mg/kg was a LOEL. The latter dosage level was reported to impair conditioned reflexes and reduce the capacity of neutrophils from heat-stressed animals to digest bacteria. No data were presented for most parameters, so it is not possible to assess the magnitude, the statistical significance, or the toxicological significance of the effects reported by Rakhamatullaev.

The chronic oral toxicity of DBCP was assessed in conjunction with a National Cancer Institute (NCI, 1978a) carcinogenicity bioassay. Male and female Osborne-Mendel rats and B6C3F_1 mice were dosed orally with technical-grade DBCP (purity >90%) 5 times weekly for up to 78 weeks. It was necessary to terminate the study early due to excessive death rates. The time-weighted average doses were as follows: 0, 15, and 29 mg/kg bw for male and female rats; 0, 114, and 219 mg/kg bw for male mice; 0, 110, and 209 mg/kg bw for female mice. There were dose-related decreases in body weight gain and survival of male and female rats. Toxic

nephropathy was seen in virtually 100% of the male and female rats at both dosage levels, but not in controls. Microscopically, the nephropathy was characterized by cloudy swelling, fatty degeneration, and necrosis of the proximal convoluted tubular epithelium at the junction of the cortex and medulla. The male and female mice experienced a dose-dependent increase in mortality. The incidence of toxic nephropathy in the mice was also dose dependent.

Mutagenicity

Biles et al. (1978) investigated the mutagenicity of DBCP by using the Ames reverse mutation assay. They concluded that in the absence of S9 activation in rats pretreated with Aroclor, the mutagenic capability of standard DBCP preparations (0 to 1,600 µg/plate) was due solely to epichlorohydrin, which was included as a stabilizer. However, after the addition of S9, technical-grade and highly purified DBCP (20 to 200 µg/plate) were equally mutagenic. On the basis of those data, Biles et al. (1978) concluded that DBCP is a potent indirect mutagen in bacteria. That conclusion has been confirmed by recent work of Moriya et al. (1983), who found DBCP to be mutagenic to *Salmonella typhimurium* TA1535 and TA100 and to *Escherichia coli* WP2 hcr.

Zimmering (1983) fed 0.2-mg/ml concentrations of DBCP in 0.01% ethanol or ethanol alone to Canton-S male *Drosophila melanogaster* for 72 hours and then to individual males mated with *Basc* females. DBCP treatment produced sex-linked recessive mutations in 9.5% of the first brood. In *Drosophila*, it also caused loss of X or Y chromosomes and induced increases in heritable translocations.

DBCP has been shown to induce sister-chromatid exchange (SCE) and chromosome aberrations in cultured Chinese hamster ovary cells over a range of applied doses (Tezuka et al., 1980). The authors concluded that, compared with 10 other compounds in the same study, DBCP-induced SCE and chromosome aberrations correlate well with DBCP's relative potency in a bacterial assay for mutation.

Kapp et al. (1979) found that semen samples of 18 workers exposed to DBCP had higher numbers of sperms containing two Y-chromosomes than did nonexposed subjects. Based on an analysis of the aneuploid karyotypes detected, the authors suggested that environmental exposure to DBCP can produce irreversible genetic change in humans.

The mechanism by which DBCP is metabolically activated to a toxicant in mammalian cells has not been well characterized. Recently, however, Kluwe (1983) has shown that primary tissue damage in rats is enhanced by previous exposure to 3-methylcholanthrene, which is an inducer of AHH activity. Therefore, the author suggested, DBCP may be processed by the cytochrome's P450 enzyme pathway. Additional work is required to establish such a mechanism unambiguously.

Biochemical data suggest that DNA is a principal target for DBCP in mammalian cells. Lee and Suzuki (1979) showed that a single intraperitoneal 100-mg/kg injection of DBCP to prepubertal male mice induced significant unscheduled DNA synthesis (DNA repair) in premeiotic germ cells but not in spermatozoa. The authors concluded that in premeiotic germ cells (but not adult spermatozoa), metabolites of DBCP may attack DNA and then induce an excision-DNA repair mechanism. Work is required to verify those initial conclusions, but the data suggest that in mammalian cells, DBCP may be activated in a manner similar to other hydrocarbon carcinogens.

Carcinogenicity

In the NCI (1978a) bioassay, technical-grade DBCP (90% pure) containing 16 impurities was given by gavage in corn oil to male and female Osborne-Mendel rats and B6C3F_1 mice. Time-weighted average dosages for male and female rats were 0, 15, and 29 mg/kg bw. The time-weighted averages were 0, 114, and 219 mg/kg for male mice and 0, 110, and 209 mg/kg for female mice. In this bioassay, technical-grade DBCP induced a high incidence of squamous cell carcinomas of the forestomach and toxic nephropathy in male and female rats and mice and a high incidence of mammary adenocarcinomas in female rats.

Van Duuren et al. (1979) found that DBCP behaved as a strong initiator of carcinogenesis in an epidermal initiation-promotion assay with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mice. Applied to mouse skin alone, DBCP did not produce skin tumors, but it increased substantially the incidence of lung and stomach tumors. The authors concluded that DBCP is an efficient initiator of epidermal papillomas, but it is not a complete carcinogen in the skin (although it appears to be a complete carcinogen with respect to benign papillomas of the lung and squamous cell carcinomas of the stomach).

Reznik et al. (1980a) found that when mice were exposed to 0, 0.6, or 3.0 ppm DBCP for 6 hours/day, 5 days/week for 103 weeks by inhalation, they developed alveolar-bronchial adenomas and carcinomas. Females appeared to be more sensitive, but for both sexes, tumors appeared above background at doses as low as 0.6 ppm. Evidently, DBCP is a strong, complete carcinogen for the noses and lungs of mice. Tumors that could be attributed to DBCP exposure were not detected at distant sites. The National Toxicology Program (NTP, 1982a) conducted an inhalation carcinogenicity bioassay of technical-grade DBCP containing trace amounts of epichlorohydrin and 1,2-dibromoethane in male and female Fischer 344 rats and B6C3F_1 mice. The animals were exposed by inhalation to 0.6-or 3.0-ppm concentrations of DBCP 6 hours/day, 5 days/week for 76 to 104 weeks. DBCP induced significantly higher incidences of tumors of the nasal cavity and tongue in male and female rats and cortical adenomas

in the adrenal glands of female rats. In male and female mice, it induced significantly higher incidences of nasal cavity and lung tumors.

TABLE 9-10 Tumor Incidence in Rats Gavaged with Dibromochloropropane[a]

Animal	Sex	Tumor Site	Dose (mg/kg/day)	Tumor Rates
Osborne-Mendel rat	Male	Forestomach	0	0/39
			15	47/50
			29	47/50

[a] Based on data from NCI, 1978a.

Carcinogenic Risk Estimate

Results of the NCI (1978a) study, in which DBCP was found to elicit squamous cell carcinomas of the forestomach in rats, were used to estimate carcinogenic risk. The tumor incidence rates in Table 9-10 were used to make statistical estimates of both the lifetime risk and an upper 95% confidence limit for the lifetime risk. The risk estimates are expressed as a probability of cancer after a lifetime daily consumption of 1 liter of water containing the compound at a concentration of 1 µg/liter. By using the data in the NCI (1978a) study, the committee calculated the lifetime risk and upper 95% confidence bounds shown in Table 9-11 for males who daily consume 1 liter of drinking water containing 1 µg of DBCP per liter.

Developmental Effects

Ruddick and Newsome (1979) dosed pregnant rats with 0, 12.5, 25, or 50 mg/kg bw orally on days 6 through 15 of gestation. DBCP was not teratogenic, but it produced a dose-dependent reduction in maternal body weight gain.

Reproductive Effects

DBCP appears to alter male fertility in a significant way. Saegusa et al. (1982) found that the testicles were severely

TABLE 9-11 Carcinogenic Risk for Dibromochloropropane[a] Estimated with Generalized Multistage Model

Animal	Sex	Estimated Human Lifetime Risk[b]	Upper 95% Confidence Estimate of Lifetime Cancer Risk[b]
Osborne-Mendel rat	Male	7.8×10^{-6}	9.9×10^{-6}

[a] Based on data from NCI, 1978a.
[b] Assuming daily consumption of 1 liter of water containing the compound in a concentration of 1 µg/liter.

injured in a study of male rats subjected continuously for 14 days to vapor containing DBCP at 10 ppm. Testicles completely atrophied with irreversible aspermatogenesis. In a second experiment, Saegusa et al. (1982) exposed male rats continuously for 14 days to 0-, 0.3-, 1-, 3-, or 8-ppm concentrations of DBCP vapor. No testicular lesions were observed at 1 or 15 days after exposure in the 0.3-ppm or 1-ppm exposure groups. A slight decrease in germ cells and atrophy of a few seminiferous tubules were observed in the testes of one of two rats examined in the 3-ppm group. Severe testicular lesions were present in the rats receiving 8 ppm.

Rao et al. (1982) conducted a comprehensive inhalation dose-response study of gonadotoxic effects of DBCP. Male rabbits were exposed to DBCP in vapor at 0, 0.1, 1.0, or 10 ppm 6 hours/day, 5 days/week for up to 14 weeks. The time of onset and severity of decreases in sperm count and sperm viability were dose dependent. Under the conditions of this study, 0.1 ppm can be considered the NOEL and 1.0 ppm the LOEL, although the 0.1-ppm animals had an equivocal increase in abnormal sperm count at 14 weeks. The adverse effects on spermatogenesis were largely reversible in the rabbits exposed to DBCP at 1 ppm. Partial recovery from the severe effects of the 10-ppm exposure occurred by the end of a 38-week recovery period.

Kluwe et al. (1983) showed that a single subcutaneous treatment of Fischer 344 rats with a 100-mg/kg dose of DBCP decreased male fertility 2 to 7 days after exposure without affecting mating frequency. Infertility was found to be accompanied by a decrease in the rate of glucose oxidation in mature sperm. The authors concluded that among its other possible effects, exposure to DBCP may alter the overall metabolism of post-testicular sperm in rats.

Kluwe (1981b) has also presented data suggesting that among other tissue damage, single 100-mg/kg subcutaneous doses of DBCP induce generalized testicular damage: seminiferous tubular desquamation and atrophy and substantial cell death occurred after subcutaneous treatment was initiated in rats. On the other hand, Osterloh et al. (1983) exposed mice to increasing doses (5 to 300 mg/kg/day) of DBCP subcutaneously and found no evidence of DBCP-induced changes in sperm count, morphology, or overall testes weight. The authors concluded that DBCP-induced effects may be species specific.

Conclusions and Recommendations

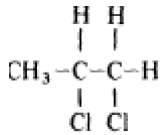
DBCP is mutagenic and carcinogenic to rats and mice in several organ systems. It also has adverse effects on human male fertility.

As there are no adequate feeding studies showing adverse reproductive effects with clear dose-response relationships, a risk assessment for reproductive effects would be inappropriate. Although good inhalation studies

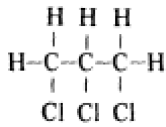
have been conducted, the committee decided not to use data from them to extrapolate to the oral route for this compound. Nevertheless, DBCP would be a good substance to use with the extrapolation model developed in Chapter 7 when additional pharmacokinetic values are experimentally determined.

CHLOROPROPANES AND CHLOROPROPENES

1,2-Dichloropropane
CAS No. 78-87-5
RTECS No. TX9625000



1,2,3-Trichloropropane
CAS No. 96-18-4
RTECS No. TZ9275000



1,3-Dichloropropene (*cis* and *trans*)
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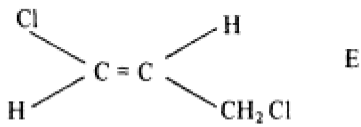
RTECS No. UC8310000



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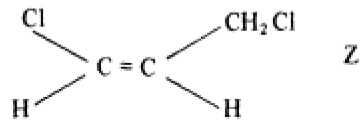


Z

The chloropropanes and chloropropenes (CPs) are generally found in mixtures used as soil fumigants and fungicides. Following the discovery

of the testicular toxicity and carcinogenicity of dibromochloropropane (DBCP) and the acute toxicity and carcinogenicity of ethylene dibromide (EDB) (see discussion later in this chapter), the toxicological literature for the CPs has generally concerned combinations of two or three of these compounds, or one of the compounds in combination with a brominated analog. Two combinations of 1,2-dichloropropane (1,2-DCP) (30%), *cis*-1,3-dichloropropene (28%), and *trans*-1,3-dichloropropene (27%) are commercially available as D-D (a broad-spectrum soil fumigant that consists primarily of *cis*-1 ,3-dichloropropene, *trans*-1,3-dichloropropene, and 1,2-DCP) and CBP-55 (primarily l-chloro-3-bromo-l-propene and 1,2-dichloropropane [40%]) (Hine et al., 1953; Hutson et al., 1971).

Metabolism

Following oral administration of 1,2-dichloro[1-^{14}C]propane or *cis* -or *trans*-1,3-dichloro[2-^{14}C]propene to rats, between 80% and 90% of the radioactivity was eliminated in the feces, urine, and expired air within 24 hours (Hutson et al., 1971). When rats were exposed to varying concentrations (30 to 900 ppm) of a vapor containing 91% 1,3-dichloropropene (both isomers), absorption was not observed to increase proportionately with increasing exposure level, partly because of a 40% to 50% depression in respiratory minute volume at the higher dose levels. Postexposure elimination curves for both isomers displayed an initial rapid and dose-dependent elimination phase (2- to 3-minute half-life), followed by a slower elimination phase (38- to 44-minute half-life) (Stott et al., 1985).

The metabolism of 1,2-DCP involves the formation of 1-chloro-2-hydroxypropane. This intermediate metabolite is detoxified via epoxidation to 1,2-epoxypropane, which can either conjugate with glutathione or be hydrolyzed to propane-1,2-diol. Conjugation would lead to the ultimate excretion of the major urinary metabolite, *N*-acetyl-*S*-(2-hydroxypropyl) cysteine. Its hydrolysis to propane-1,2-diol and subsequent oxidation to lactate would lead to its complete oxidation to carbon dioxide in the tricarboxylic acid cycle. Due to the volatility and lipophilic nature of 1,2-DCP, some is excreted via the lungs, but no unchanged compound is excreted in the urine (Jones and Gibson, 1980).

The *cis* isomer of 1,3-dichloropropene has been shown to be metabolized by conjugation with glutathione. Seventy-six percent of an oral dose appeared within 24 hours in the urine of rats as the mercapturic acid *N*-acetyl-*S*-(*cis*-3-chloroprop-2-enyl)cysteine (Climie et al., 1979). This compound is also the major urinary metabolite of 1,3-dichloropropene in mice (Dietz et al., 1984) and has been detected in the urine of humans exposed to cis-1,3-dichloropropene vapor during field applications (Osterloh et al., 1984).

Health Aspects

Observations in Humans

Published observations concerning humans exposed to 1,2-DCP consist of case reports of acute illness resulting from accidental exposure through inhalation, ingestion, or dermal contact. In Italy, four cases of acute liver damage (one fatal) were observed in persons ingesting unknown quantities of the commercial solvent "trilene," 70% to 100% of which is 1,2-DCP (Chiappino and Secchi, 1968; Secchi and Alessio, 1968; Secchi et al., 1968). Contact dermatitis on hands and feet (not described more fully) was found in two women —one who worked for 6 years and one for 4 years at two separate plastics production plants in Poland (Grzywa and Rudzki, 1981).

Dermatitis was found in workers using D-D as a soil fumigant (Bodansky, 1937). 1,2-DCP has also been reported to cause headache, vertigo, tearing, and irritation of the mucous membranes when used as a dry-cleaning agent. No concentrations were given for inhalation exposure from dry-cleaning or from use as a fumigant (Hine et al., 1953).

Venable et al. (1980) examined the fertility of male workers engaged in the manufacture of glycerine with potential exposure to allyl chloride, epichlorohydrin, and 1,3-dichloropropene. Sperm counts and normal sperm forms were determined for each worker and then compared with controls. The study included 79 volunteers out of a possible 123 workers, giving a volunteer rate of 64%. The authors reported that there was no association between decreased fertility in workers and workplace exposure to any of the three substances.

No other studies assessing potential delayed or chronic health effects associated with human exposure to 1,2-DCP are recorded in the literature. There are no published studies on human health effects from exposure to 1,2,3-TCP and 1,3-dichloropropene.

Observations in Other Species

Acute Effects

The acute lethality of 1,2-DCP has been studied in a number of animal species. Oral LD_{50} values of 860 mg/kg bw and 2,200 mg/kg bw have been reported, respectively, for mice (Windholz et al., 1976) and rats (Smyth et al., 1969). Smyth et al. (1969) also reported that three of six rats died from inhaling 2,000-ppm vapor concentrations of 1,2-DCP for 8 hours. Heppel et al. (1946) observed that 2,200-ppm vapor concentrations of 1,2-DCP caused marked irritation of ocular mucous membranes of guinea pigs as well as marked inebriation in rats and mice. The authors subjected guinea pigs, rats, mice, rabbits, and dogs to

a single 7-hour exposure to each of a series of vapor concentrations of 1,2-DCP. Mice were the most sensitive. All 26 mice exposed to a concentration of 1,000 ppm died within 4 hours after inhalation.

The liver, kidneys, and adrenals appear to be the primary target organs for acute injury by 1,2-DCP. In an early investigation of the anthelmintic properties of a series of chlorinated alkyl hydrocarbons, Wright and Schaffer (1932) observed gross and microscopic lesions in the livers of 11 dogs given a single oral dose of 230 to 580 mg/kg bw. Microscopic changes included parenchymatous degeneration, fatty vacuolation, and cloudy swelling or edema. The 1,2-DCP was said to have been obtained from a commercial source and purified by distillation.

Drew et al. (1978) exposed male CD rats for 4 hours by inhalation to 1,000-ppm concentrations of 1,2-DCP. Liver injury was evidenced at 24 and 48 hours after exposure by parallel increases in serum levels of glutamic-oxaloacetic transaminase (SGOT), glutamic-pyruvic transaminase (SGPT), and ornithine carbamyl transferase (OCT) activities. The activity of each of the three enzymes was higher at 48 hours than at 24 hours.

Highman and Heppel (1946) examined the development and regression of lesions in Sprague-Dawley rats and in guinea pigs exposed by inhalation to 2,200-ppm concentrations of commercial-grade 1,2-DCP for 7 hours. The results of fractional distillation studies led the authors to conclude that their 1,2-DCP probably contained a considerable percentage of other isomers. The authors found centrilobular necrosis and diffuse to midzonal fatty degeneration in the livers of rats 24 hours after exposure. Groups of rats sacrificed on subsequent days after the single exposure had progressive resolution of the hepatic lesions as well as diminished fatty vacuolation of the renal convoluted tubular epithelium. Fatty vacuolation of the liver and kidneys was also observed in the guinea pigs undergoing 7-hour inhalation exposures up to 2,200 ppm two to five times. In addition, these guinea pigs exhibited necrosis of the adrenal cortex affecting all but a narrow outer rim of the tissue. The adrenal cortical cells rapidly regenerated during a 16-day recovery period. Proliferation of fibroblasts and formation of a thin layer of connective tissue around the adrenal medulla were also noted during this time. Heppel et al. (1946) found histopathological lesions in mice that died after being exposed for 2 hours to 1,500 ppm or for 4 hours to 1,000 ppm 1,2-DCP by inhalation. The lesions included hepatic centrilobular necrosis and fatty degeneration of the liver and kidneys.

No data pertaining to the acute toxicity of 1,3-dichloropropene were located in the literature. However, Hine et al. (1953) did evaluate the acute toxicity of technical-grade D-D, which is a broad-spectrum soil fumigant that consists primarily of *cis*-1,3-dichloropropene, *trans*-1,3-dichloropropene, and 1,2-DCP. They reported that the oral LD_{50} for

D-D was 300 mg/kg bw in inbred male Swiss mice and 140 mg/kg bw in Long-Evans rats (sex not reported). The animals exhibited hyperexcitability followed by tremors, incoordination, depression, and breathing difficulties. Fatty degeneration of the liver and hemorrhage of the lungs were occasionally found in animals that died several days after oral dosing. The 4-hour inhalation LC_{50} for D-D in rats was calculated by the authors to be 1,000 ppm. Rats that died from inhaling the vapors exhibited severe pulmonary edema with varying degrees of interstitial and alveolar hemorrhage. Undiluted D-D was also found to be a severe skin and eye irritant and to cause erosion and occasional hemorrhage of the gastrointestinal mucosa when administered intragastrically.

Little information on the acute toxicity of 1,2,3-TCP was found in the scientific literature. In an early paper, Wright and Schaffer (1932) reported that 1,2,3-TCP was the most toxic of a series of 18 chlorinated alkyl hydrocarbons that they tested in dogs. The 1,2,3-TCP was synthesized by the investigators and purified by distillation. Three dogs (sex and strain unspecified) were fasted for 12 hours before intragastric administration of the chemical. One dog received 0.2 ml/kg, one received 0.3 ml/kg, and one received 0.5 ml/kg. All three were completely narcotized within 1 to 2 hours and died within 1 to 2 days of dosing. Microscopic examination of the livers revealed fatty vacuolation and centrilobular necrosis. Cloudy swelling, desquamation of the tubular epithelium, and nuclear changes indicative of beginning necrosis were observed in the kidneys. In a more recent study, Drew et al. (1978) found that spectrograde (99% pure) 1,2,3-TCP produced liver damage in male CD1 rats. Rats that inhaled 500-ppm concentrations of the chemical for 4 hours exhibited increased levels of SGOT, SGPT, and OCT 24 and 48 hours after exposure. Concurrent 4-hour inhalation exposure to 1,2,3-TCP at 500 ppm and 1,2-DCP at 1,000 ppm resulted in additive increases in SGOT and OCT. By 48 hours after exposure, the increases were significantly less than additive.

Subacute Effects

It appears that the only subacute studies in which 1,2-DCP has been given orally were conducted as part of an NTP (1983a) carcinogenesis bioassay. In a preliminary range-finding study, Fischer 344/N rats and $B6C3F_1$ mice of each sex were given reagent-grade (99.4% pure) 1,2-DCP in corn oil by gavage in doses of 0, 125, 250, 500, 1,000, or 2,000 mg/kg daily for 14 consecutive days. All rats receiving 2,000 mg/kg died. The two highest doses were lethal to all but one of the male and female mice receiving the 2,000- and 1,000-mg/kg doses. Three of five male mice receiving 500 mg/kg died.

A follow-up study was conducted to determine the doses of 1,2-DCP to be used in a chronic carcinogenicity bioassay. $B6C3F_1$ mice of each sex were given reagent-grade 1,2-DCP in corn oil by gavage in doses of

0, 30, 60, 125, 250, or 500 mg/kg, 5 days/week for 13 weeks. Male and female Fischer 344/N rats similarly received doses of 0, 60, 125, 250, 500, or 1,000 mg/kg. All male and female rats given 1,000 mg/kg died, as did 5 of 10 males in the 500-mg/kg group. Histopathological examination revealed fatty changes, centrilobular congestion, and centrilobular necrosis in the livers of some of the rats receiving 1,000 mg/kg. There were no apparent increases in mortality or histopathological changes in mice at any dosage level.

Heppel et al. (1946) conducted a series of experiments in which guinea pigs, rats, rabbits, and dogs were exposed to commercial-grade 1,2-DCP by repeated daily inhalation exposures. The investigators noted that their test materials probably contained a considerable percentage of other isomers. The animals were subjected to 1,000-, 1,600-, or 2,200-ppm vapor concentrations of 1,2-DCP for 7 hours/day, 5 days/week for as many as 128 exposures. The lowest concentration (1,000 ppm) caused some deaths among all animal species except rabbits during the course of the exposure regimens. Liver, kidney, and adrenal injuries were most pronounced during the initial days of the study. Animals that did not succumb to the chemical became resistant to its injurious effects. Guinea pigs that survived 26 exposures to 1,000-ppm concentrations developed no significant histological changes other than a subcortical layer of fibrous tissue in the adrenals. Rats sacrificed after as many as 97 exposures to 1,000 ppm exhibited few changes other than splenic hemosiderosis.

In a follow-up histopathological study, Highman and Heppel (1946) confirmed that surviving rats and guinea pigs become resistant upon repeated exposure to concentrations of 1,2-DCP. Sprague-Dawley rats and guinea pigs inhaled 2,200-ppm concentrations of 1,2-DCP 7 hours daily for 5 consecutive days. Daily sacrifices revealed that lesions in the primary target organs (liver, kidneys, and adrenals) in each species were most severe at 3 days. Resolution was seen thereafter despite continuation of the daily inhalation exposures.

The results of subsequent studies indicate that inhalation of relatively low concentrations of 1,2-DCP can produce detrimental effects in some species. Heppel et al. (1948) exposed male and female rats and guinea pigs and female dogs 7 hours/day, 5 days/week for approximately 6 months to 400 ppm of commercial-grade 1,2-DCP. Groups of animals were sacrificed at intervals during the study and examined for histopathological changes. No ill effects were attributed to the exposures other than decreased body weight gain in the male and female rats. The authors also subjected C57-strain mice to daily 7-hour inhalation exposures to 400-ppm concentrations of 1,2-DCP. Most of the mice died during the course of the study. Thus, the mouse appears to be particularly sensitive to inhaled 1,2-DCP.

Sidorenko et al. (1976) exposed male rats by inhalation continuously for 7 days to 217- to 434-ppm concentrations of 1,2-DCP. The investigators stated that "verifiable" changes in blood catalases and acetylcholinesterase were first observed after 4 hours in the 434-ppm group and after 24 hours in the 217-ppm group. Because no data were presented, the magnitude and significance of these changes cannot be ascertained. The authors also reported histological and functional changes in the liver and kidneys. Unfortunately, they did not provide detailed data on these changes, nor did they indicate which vapor level(s) produced the injury or note when the changes occurred following exposure.

There is very little information on the subacute toxicity of 1,2,3-TCP. Sidorenko et al. (1976) exposed male rats by inhalation continuously for 7 days to 350-mg/m^3 (58-ppm) or 800-mg/m^3 (133-ppm) concentrations of 1,2,3-TCP. The exposures were reported to produce changes in blood catalases and acetylcholinesterase as well as "histostructural" evidence of liver and kidney injury. Unfortunately, as for 1,2-DCP, the researchers did not provide specific data or give an adequate description of their findings.

Torkelson and Oyen (1977) investigated the effects resulting from repeated inhalation of 1,3-dichloropropene. The sample they used in their study contained 46% *cis*-1,3-dichloropropene, 53% *trans*-1,3-dichloropropene, and 1% other chemicals, including epichlorohydrin. In a preliminary experiment, repeated inhalation exposure of male and female rats and guinea pigs to 11 or 50 ppm produced gross evidence of liver and kidney damage. Male rats were then exposed to 3 ppm for 0.5, 1, 2, or 4 hours/day, 5 days/week for 6 months. The only adverse effect seen was very slight cloudy swelling of the renal tubular epithelium in the 4-hour exposure group. In a subsequent experiment, 24 male and 24 female rats, 12 male and 12 female guinea pigs, 3 male and 3 female rabbits, and 2 dogs were exposed 125 to 130 times over 185 days to 1 or 3 ppm. Male rats exposed to 3 ppm exhibited cloudy swelling of the renal tubular epithelium, whereas female rats exposed to 3 ppm had a slight but statistically significant increase in liver-to-body-weight ratio. No changes were observed in animals inhaling 1 ppm. On the basis of the study results of Torkelson and Oyen (1977), 1 ppm appears to be a NOEL, whereas 3 ppm appears to be a LOEL in rats exposed to 1,3-dichloropropene by inhalation.

Parker et al. (1982) investigated the subacute toxic potential of D-D. The sample used contained 25% *cis*-1,3-dichloropropene, 27% *trans* -1,3-dichloropropene, 29% 1,2-DCP, and lesser amounts of 3,3-dichloropropene, 2,3-dichloropropene, and related chlorinated hydrocarbons. Male and female Fischer 344 rats and CD1 mice were exposed by inhalation 6 hours/day, 5 days/week for 6 or 12 weeks to 0-, 5-, 15-, or 50-ppm

concentrations of D-D. Significant decreases in body weight gain in comparison to controls were seen in male rats at all three exposure levels during the first 6 weeks, but not thereafter. Hematological evaluations revealed statistically significant decreases in white-blood-cell counts at 12 weeks in the 15-ppm males and the 50-ppm females, although the authors did not consider these changes to be of toxicological significance. No exposure-related effects on urinalysis or clinical chemistry parameters were observed. At 6 and 12 weeks, increases were noted in liver-to-body-weight ratios of male rats and in kidney-to-body-weight ratios of female rats at the 50-ppm exposure level. No histopathological changes were seen in rats, but diffuse hepatocytic enlargement was observed in the 50-ppm male and female mice at 12 weeks.

Chronic Effects

Chronic toxicity data on 1,2-DCP were obtained in an NTP (1983a) carcinogenicity bioassay. Female Fischer 344/N rats and B6C3F_1 mice of each sex were given reagent-grade 1,2-DCP in corn oil by gavage 5 days/week for 103 weeks in doses of 0, 125, or 250 mg/kg. Male Fischer 344/N rats similarly received doses of 0, 62, or 125 mg/kg. A dose-related decrease in body weight gain was observed in both male and female rats throughout most of the study. There was a significant reduction in survival of the high-dose female rats. The high-dose female rats also experienced an increased incidence of focal and centrilobular necrosis of the liver. Hepatocytomegaly, as well as focal and centrilobular hepatic necrosis, were seen in male mice receiving 1,2-DCP. The incidence of these lesions appeared to be dose dependent, although it was not clear whether their occurrence in the low-dose group was frequent enough to be statistically significant.

The chronic oral toxicity of 1,3-dichloropropene was evaluated during the course of an NTP (1985a) carcinogenicity bioassay of Telone II, a soil fumigant containing approximately 42% *cis*-1,3-dichloropropene, 46% *trans*-1,3-dichloropropene, 2.5% 1,2-dichloropropane, 1.5% of a trichloropropane isomer, 1% epichlorohydrin (a known carcinogen), and the remainder other compounds. Male and female Fischer 344/N rats and B6C3F_1 mice were given the formulation in corn oil by gavage 3 times weekly for 104 weeks. The rats received doses of 0, 25, or 50 mg/kg; the mice received 0, 50, or 100 mg/kg. Blood samples were taken from designated rats every 4 weeks up to 39 weeks for hematological studies and up to 69 weeks for assessment of clinical chemistry parameters. No toxicologically important changes were observed in the hematology or the clinical chemistry studies. Rats of each sex were sacrificed after 9, 16, 21, 24, and 27 months to assess the development of histopathological changes over time. Basal cell hyperplasia of the forestomach was found in male and female rats 9 to 16 months after dosing began. No other

lesions were observed in any tissue examined during the time-course study. The following histopathological findings were reported at the terminal sacrifice: dose-related increase in basal cell hyperplasia of the forestomach of male and female rats as well as high-dose female mice; edema of the submucosa of the urinary bladder in high-dose male and female mice; dose-related increase in the incidence of epithelial hyperplasia of the urinary bladder in male and female mice; and increased incidences of nephropathy in female rats and hydronephrosis in female mice.

No chronic toxicity studies of 1,2,3-TCP were found in the literature.

Neurotoxicity

There is no evidence that these compounds, alone or in combination, induce permanent neurological deficits (structural or functional). However, the CPs are similar to other short-chain aliphatic, halogenated hydrocarbons in that they can cross the blood-brain barrier and induce anesthesia or other transient effects on the central nervous system (Sidorenko et al., 1976). There is a time-dose relationship with respect to the onset of ataxia and loss of righting reflex that is additive for the three compounds at all doses and combinations tested. There was no indication of synergism or antagonism from the results seen in a multifactorial experiment.

Mutagenicity

Principe et al. (1981) and Carere and Morpurgo (1981) reported that 1,2-DCP induced forward mutations (8-azaguanine resistance) in *Aspergillus nidulans* but did not induce forward mutation (streptomycin resistance) in *Streptomyces coelicolor*. Principe et al. (1981) found that 1,2-DCP at 11 mg/plate was marginally mutagenic to *Salmonella typhimurium* TA1535 and TA100. De Lorenzo et al. (1977) found that 1,2-DCP at 10, 20, or 50 mg/plate was mutagenic in the Ames *Salmonella* test with and without mammalian enzyme activation mixture (S9). Stolzenberg and Hine (1980) detected no mutagenic activities in *Salmonella typhimurium* TA100 with 1,2-DCP up to 10 mg/plate. Several genotoxicity studies with 1,2-DCP were conducted by NTP (1983a). The compound was not a mutagen in the Ames mutagenesis bioassay but did induce sister-chromatid exchange at levels of approximately 1 mg/ml and chromosome aberrations (unspecified) at similar levels without S9. With S9, the compound induced these changes at approximately half this level.

1,3-Dichloropropene (with 1% epichlorohydrin added as a stabilizer) induced mutations in the Ames *Salmonella* test strains TA1535, TA100, and TA1978 (de Lorenzo et al., 1977) and sex-linked recessive mutations in *Drosophila* (Climie et al., 1979). Removal of the polar metabolites from 1,3-dichloropropene eliminated the direct-acting mutagenic activity (Talcott, 1981; Talcott and King, 1984).

Commercial 1,3-dichloropropene preparations were mutagenic in the Ames assay without microsomal extract S9 (Stolzenberg and Hine, 1980). However, Talcott and King (1984) found that pure 1,3-dichloropropene isomers were not mutagenic in an Ames test in the absence of S9. They showed that oxygenated and chlorinated degradation products of 1,3-dichloropropene are the direct-acting mutagens in an Ames mutation assay.

Stolzenberg and Hine (1980) reported that 1,2,3-TCP is mutagenic to Ames *Salmonella typhimurium* TA100 at a concentration of 0.1 µmol/ plate but only in the presence of S9 microsomal extract. They concluded that 1,2,3-TCP is mutagenic after metabolic activation.

1,2,3-TCP was not tested for its ability to include the SOS (error-prone DNA repair) response in Ames *Salmonella* tester strains. However, a chemically similar compound (1,2-dibromo-3-chloropropane) was tested and was found to be very mutagenic by that assay (Ohta et al., 1984).

Carcinogenicity

Heppel et al. (1948) exposed C3H mice to 400-ppm concentrations of 1,2-DCP 37 times for 4 to 7 hours each exposure. Most of the mice died during the course of the experiment. Nine that died after having received 14 to 28 exposures had moderate to marked congestion and fatty degeneration of the liver, seven had extensive centrilobular necrosis of the liver, and six of eight had slight to moderate fatty degeneration of the kidney. Only three mice survived both the exposures and a subsequent observation period of 7 months, by which time they were 13 months of age. These animals had developed multiple hepatomas.

NTP (1983a) conducted a carcinogenesis bioassay for 1,2-DCP (>99% pure). The chemical was administered in corn oil by gavage to female Fischer 344/N rats at doses of 125 or 250 mg/kg bw, to male and female $B6C3F_1$ mice at doses of 125 or 250 mg/kg bw, and to male Fischer 344/ N rats at doses of 62 or 125 mg/kg bw. Doses were administered 5 days/ week for 103 weeks. There was no evidence of carcinogenicity for male Fischer 344/N rats. For female rats there was equivocal evidence of carcinogenicity in that 250-mg/kg doses of 1,2-DCP caused a marginally increased incidence of adenocarcinomas in the mammary gland. These borderline malignant lesions occurred concurrently with decreased survival and reduced body weight gain. The increased incidence of hepatocellular adenomas provided some evidence that 1,2-DCP was carcinogenic for male and female $B6C3F_1$ mice. No studies have been conducted with the compound added to drinking water.

Telone II was given in corn oil by gavage 3 days/week for 104 weeks at doses of 0.25 or 50 mg/kg to 52 male and 52 female Fischer 344/N rats and at doses of 0.50 or 100 mg/kg to 50 male and 50 female $B6C3F_1$ mice. These exposures induced time-and dose-dependent tumors of the forestomach and liver nodules in male Fischer 344/N rats (NTP, 1985a).

TABLE 9-12 Tumor Incidence in Mice Gavaged with Technical-Grade 1,3-Dichloropropene (Telone II)[a,b]

Animal	Sex	Tumor Site	Dose (mg/kg/day)	Tumor Rates
$B6C3F_1$ mouse	Female	Urinary bladder	0	0/50
			50	8/50
			100	21/48

[a] Based on data from NTP, 1985a.
[b] Contains 1% epichlorohydrin, 2.5% 1,2-DCP, and 1.5% trichloropropene.

There was equivocal evidence for tumor induction in the forestomach of female Fischer 344/N rats. In female $B6C3F_1$ mice, the compound induced tumors at several sites, including the urinary bladder, forestomach, and the lung (adenoma). Based on the evidence presented by NTP (1985a), Telone II is a carcinogen in Fischer 344/N rats and $B6C3F_1$ mice.

Van Duuren et al. (1979) reported that subcutaneous injection of *the cis* isomer of 1,3-dichloropropene produces sarcomas in Swiss mice but that tumors were not induced by repeated skin applications. *cis*-1,3-Dichloropropene failed to elicit a response in Swiss mice in an initiation-promotion assay using phorbol myristate acetate (PMA) as a tumor promoter (van Duuren et al., 1979).

These data suggest that 1,3-dichloropropene may be carcinogenic in mice. Because the data are incomplete, however, and because there is evidence that contaminants of the preparations may be more mutagenic than 1,3-dichloropropene itself (Talcott and King, 1984), it is not possible to determine the organ specificity or dose response of the effects.

No information was found pertaining to the carcinogenicity of 1,2,3-TCP. However, because of its structural similarities to certain chemicals that have been found to be carcinogenic in animals (e.g., 1,2-DCP, 1,3-dichloropropene, ethylene dibromide, and 1,2-dibromo-3-chloropropane), it would be prudent to limit exposure to it. There are insufficient data on the acute, subacute, and chronic noncarcinogenic toxicity of 1,2,3-TCP to conduct a toxicity risk assessment. The committee recommends that short-and long-term oral toxicity studies and a carcinogenicity bioassay be conducted with 1,2,3-TCP.

Carcinogenic Risk Estimate

The results of the NTP (1985a) study in which Telone II was found to induce tumors in rats and mice are used to estimate carcinogenic risk. The tumor incidence rates in Table 9-12 were used to make statistical estimates of lifetime risk. An upper 95% confidence bound for human exposure is shown in Table 9-13.

TABLE 9-13 Carcinogenic Risk Estimates for Technical-Grade 1,3-Dichloropropene (Telone II)[a] from Generalized Multistage Model[b]

Animal	Sex	Estimated Human Lifetime Risk[c]	Upper 95% Confidence Estimate of Lifetime Cancer Risk[c]
$B6C3F_1$ mouse	Female	0.5×10^{-6}	1.1×10^{-6}

[a] Contains 1% epichlorohydrin, 2.5% 1,2-DCP, and 1.5% trichloropropene.
[b] Based on data from NTP, 1985a.
[c] Assuming daily consumption of 1 liter of water containing the compound in a concentration of 1 µg/liter.

Developmental Effects

Data are not available.

Reproductive Effects

Osterloh et al. (1983) determined that 1,3-dichloropropene administered by intraperitoneal injections of 0 to 75 mg/ kg/day for 5 days had no effect on sperm count, sperm morphology, or testicular weights in mice. However, several substances that are known to induce sperm abnormalities in humans have not been observed to produce positive results in the mouse assay because of species differences or because observations were made at different times. In the study by Osterloh and colleagues, observations were made on the 35th day after exposure; thus, earlier or later effects may have been missed. The authors noted, therefore, that care must be taken when interpreting this negative response with 1,3-dichloropropene.

Hardin et al. (1981) administered intraperitoneal 37-mg/kg bw doses of 1,2,3-TCP in corn oil to Sprague-Dawley rats. This dose caused maternal toxicity but did not produce fetal toxicity or teratogenesis.

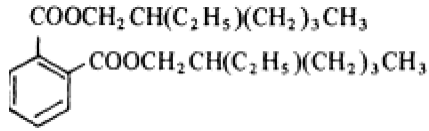
Conclusions and Recommendations

The toxicity of the chloropropanes and chloropropenes has been examined in only a few studies. Most of the studies reported in the literature have focused on the toxicity of two or more of these compounds in mixtures.

1,2-DCP causes injury in the liver, kidneys, and adrenals following acute, subacute, and chronic exposure. 1,2,3-TCP produces the same target organ toxicity under similar exposure conditions. Following long-term gavage studies, 1,3-dichloropropene (Telone II) was found to be carcinogenic in rats and mice. The toxicity of these substances should be reassessed when additional information becomes available.

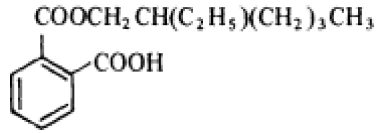
DI(2-ETHYLHEXYL) PHTHALATE (DEHP)

***bis*(2-Ethylhexyl) 1,2-benzenedicarboxylate**
CAS No. 117-81-7
RTECS No. T10350000



MONO(2-ETHYLHEXYL) PHTHALATE (MEHP)

Mono(2-ethylhexyl) 1,2-benzenedicarboxylate
CAS No. 4376-20-9
RTECS No. T12500000



Di(2-ethylhexyl) phthalate (DEHP) can be hydrolyzed in the environment to produce mono(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol (2-EH). Pharmacokinetic studies indicate rapid conversion of DEHP in the body to MEHP and 2-EH so that plasma levels of MEHP are much higher than those of DEHP. Thus, DEHP and MEHP are considered together in this analysis.

DEHP is widely used as a plasticizer to impart flexibility in many consumer plastic products and medical devices. Some formulations of polyvinyl chloride pipe used to carry drinking water contain as much as 30% of the compound. An estimated 2 billion pounds of DEHP are produced annually worldwide, making DEHP one of the most widespread synthetic environmental chemicals (Thomas and Thomas, 1984).

DEHP has been found in air over ocean water at levels ranging from 0.25 ppt to 1.8 ppt (IARC, 1982). Concentrations in water have varied widely: 5 ng/liter in the North Atlantic, 80 ng/liter in the Gulf of Mexico, and 130 ng/liter near the Gulf Coast. Ocean sediments in the Gulf of Mexico contained 2 ng/g, compared with 69 ng/g in the Mississippi delta. In surface water near chemical plants, DEHP levels of 1 to 50 µg/liter have been detected. In a study of the environmental transport of DEHP, there was an initial concentration of 200 µg/liter in a particular industrial

effluent, decreasing concentrations through various water treatment steps, and a final level of 0.6 µg/liter in finished drinking water in Philadelphia (Sheldon and Hites, 1979).

DEHP is found in animals and animal products in the human food chain. The highest levels have been detected in milk (31.4 mg/kg, fat basis) and cheese (35 mg/kg, fat basis). These data indicate that DEHP can accumulate in animal tissue. This is further established by an 886-fold concentration found in fathead minnows exposed to 4.6-µg/liter concentrations of ^{14}C-labeled DEHP (Mehrle and Mayer, 1976).

DEHP is metabolized by several species of bacteria (Engelhardt et al., 1975). Because of the wide distribution of DEHP in the environment, the rates of DEHP degradation by enzymatic and nonenzymatic reactions in different environmental compartments may vary widely. Thus, an overall environmental half-life may not be useful in estimating the residue levels in particular locations.

Occasional acute exposures to very high levels may result when DEHP leaches from plastics used in medical apparatus such as storage bags and tubing for blood transfusions. Blood plasma stored in polyvinyl chloride bags has had DEHP levels of up to 250 mg/liter (Pik et al., 1979).

Metabolism

DEHP appears to be efficiently absorbed from the gut of the rat, as indicated by urinary excretion of more than 90% of the activity in an oral 2,000-mg/kg dose of ^{14}C-labeled DEHP (Williams and Blanchfield, 1974). Very little of the diester is absorbed intact: DEHP is hydrolyzed by intestinal esterases, and the product MEHP is the primary form absorbed (IARC, 1982; Oishi and Hiraga, 1982; White et al., 1980). Comparative studies with rats, dogs, and miniature pigs have shown major differences in absorption rates and excretion patterns (Ikeda et al., 1980). Absorption, metabolism, and excretion of ^{14}C-labeled DEHP were determined in animals that had been given 50-mg/kg bw oral doses of DEHP for 21 to 28 days. Approximately 84% of the radioactivity was excreted in the urine and feces of rats during the first 24 hours; during this time, excretion in dogs and pigs was 67% and 37%, respectively. Fecal excretion predominated in dogs, whereas urine was the major route of excretion in rats and pigs. Excretion was virtually complete in all species within 4 days.

Blood concentrations of DEHP and MEHP in rats reached maximum levels within 6 to 24 hours of administration. In the heart and lungs, however, the highest levels were reached within 1 hour (Oishi and Hiraga, 1982). At 6 hours after administration, the highest ratios of MEHP to DEHP (mol%) were found in the testes (210%); other tissues contained less than 100%. MEHP disappeared exponentially with half-life values

ranging from 23 to 68 hours for the different tissues; the half-life of DEHP ranged from 8 to 156 hours. Values for several tissues were slightly longer for MEHP than for DEHP; e.g., the values for MEHP and DEHP in blood were 23.8 and 18.6 hours, respectively. There is little or no accumulation of radioactivity in rats following repeated dietary treatment. The highest concentrations were found in liver (110 to 165 mg/kg) and adipose tissue (60 to 80 mg/kg) after subchronic administration of 5,000 mg/kg in the diet. Estimated half-lives for DEHP and its metabolites in rats are 3 to 5 days for fat and 1 to 2 days for other tissues (Daniel and Bratt, 1974).

Rats have been reported to metabolize DEHP to 5-keto-2-ethylhexyl phthalate, 5-carboxyl-2-ethylpentyl phthalate, 5-hydroxy-2-ethylhexyl phthalate, and 2-carboxymethylbutyl phthalate after initial hydrolysis to MEHP (Albro and Moore, 1974; Albro et al., 1982; Daniel and Bratt, 1974; IARC, 1982). Only a small amount (less than 5%) is completely hydrolyzed to phthalic acid. In contrast to rats, African green monkeys (Albro et al., 1981) and ferrets (Lake et al., 1976) excrete DEHP metabolites in urine as glucuronide derivatives of MEHP. Apparent species differences in DEHP metabolism could be due to differences in the intestinal microflora.

Health Aspects

Observations in Humans

Ganning et al. (1984) examined dialysis patients for liver changes that may result from their treatment regimen. They estimated that these patients were receiving approximately 150 mg of DEHP per week intravenously during their treatment. At 1 month, no morphological liver changes were observed by liver biopsy. At 1 year, peroxisomes were described as being "significantly higher in number." No other observations were made. It was assumed by these investigators that the production of liver peroxisomes was the result of the patients' exposure to DEHP. However, the livers in dialysis patients compared with those of healthy individuals would be exposed to a greater number of blood contaminants at higher levels because of their impaired clearance abilities.

There were no other data describing the effects of DEHP in humans other than those proposed as possible effects in persons receiving intravenous solutions.

Observations in Other Species

Acute Effects

Several studies, beginning as early as 1943 (Hodge, 1943), have been conducted to examine the acute toxic effects of DEHP.

In these early studies and in subsequent investigations (Thomas et al., 1978), DEHP was found to have low acute toxicity. The single oral LD_{50}s for DEHP vary for other routes of exposure, partly because of its poor solubility in biological solutions. The principal acute effects reported were mild irritation of the gastrointestinal tract or of other sites of administration.

Chronic Effects

Exposures of Sherman rats to 4,000-ppm (300-mg/ kg bw) dietary concentrations of DEHP for 2 years induced no tumors (Carpenter et al., 1953). Nor were any tumors observed in Wistar rats given DEHP at 5,000 ppm (375 mg/kg bw) in the diet for 2 years (Harris et al., 1956). The information from these studies was limited because of the small sample size or limited extent of pathological examination. No tumors were reported in rats treated for 2 years with 20,000 ppm DEHP in the diet (Ganning et al., 1984). A bioassay more in keeping with current standards was conducted by the National Toxicology Program (NTP, 1983b). In this study, Fischer 344 rats of both sexes were exposed to 6,000 and 12,000 ppm in the diet and $B6C3F_1$ mice of both sexes were exposed to 3,000 and 6,000 ppm in the diet, all for 2 years. An increased yield of hepatocellular tumors was observed in both sexes of both species. There was a trend toward increasing yield with increasing dose.

Mutagenicity

The committee has relied primarily on published, peer-reviewed reports of studies on mutagenicity. The International Program for Chemical Safety (IPCS) of the World Health Organization provided additional information through a large study designed to evaluate the performance of a variety of short-term *in vitro* assays with a series of chemicals of interest in terms of their genotoxicity as it relates to carcinogenic activity. Its summary of some 90 individual sets of experiments yielded a large data base on the activity of DEHP in a variety of short-term genotoxicity tests. The primary research reports reviewed by IPCS have not yet been published. Thus, they have not undergone peer review.

The IPCS compared the number of positive and negative assays, presented the results of each assay, and discussed the results (Ashby et al., 1985). The committee recognizes that these data should be reevaluated when the results are published in the scientific literature.

Studies conducted in several laboratories to examine DEHP and its principal metabolites, MEHP and 2-EH, have failed to show mutagenic activity in a variety of bacterial mutagenesis assays (CMA, 1982; Kirby et al., 1983; Kozumbo et al., 1982; Seed, 1982; Yoshikawa et al., 1983; Zeiger et al., 1982, 1985). In the IPCS study, all five assays for mutagenesis in *Salmonella* were negative (Ashby et al., 1985).

There is one report that DEHP and MEHP were mutagenic in the Ames *Salmonella* test and in *Escherichia coli* (Tomita et al., 1982). The same investigators described a positive response in the *rec*-assay with *Bacillus subtilis*. This report is in conflict with other published observations. Furthermore, in the *Salmonella* test, MEHP was reported to be mutagenic without exogenous metabolic activation, despite the fact that MEHP almost certainly will not react with DNA. In the IPCS study, DEHP was reported to be negative in five of six assays for yeast gene mutation (Ashby et al., 1985).

DEHP, MEHP, and 2-EH were not mutagenic in the L5178Y mouse lymphoma mutagenesis assay (CMA, 1982; Kirby et al., 1983) or in the Chinese hamster ovary (CHO) mutagenesis assay (Phillips et al., 1982). In the IPCS study, DEHP was negative in five of six mammalian-cell gene mutation assays (Ashby et al., 1985). In contrast, Tomita et al. (1982) showed MEHP to be mutagenic in V79 cells and both DEHP and MEHP to induce point mutations in embryonic Syrian hamster embryo cells exposed transplacentally.

No activity was seen in a mouse bone-marrow micronucleus assay with DEHP, MEHP, or 2-EH (CMA, 1982) or in a rat bone-marrow cytogenetics assay (Putman et al., 1983). In the IPCS study, all three assays for chromosome aberrations in mammalian-cell cultures were negative (Ashby et al., 1985). Abe and Sasaki (1977) reported increased sister-chromatid exchange (SCE) induction in CHO cells after 24-hour exposure to DEHP with a dose-related increase from 8.8 to 11.0 SCE per cell over a dose range of about 4 to 400 μg/ml. They did not observe chromosome aberrations in either CHO or human cells. However, Tomita et al. (1982) reported that DEHP and MEHP also induced chromosome aberrations and SCEs in Chinese hamster V79 cells. No cytotoxicity information was provided. DEHP did not produce chromosome damage in human lymphocytes or in human fetal lung cells (Stenchever et al., 1976).

There is one report that MEHP but not DEHP was clastogenic at high concentrations in CHO cells (Phillips et al., 1982). The same study reported both compounds as negative in the CHO sister-chromatid exchange assay. These results are curious because the SCE assay is usually the more sensitive cytogenetic end point. In the Phillips et al. study, however, cells were exposed for only 2 hours, and the data reported for MEHP showed a positive trend (not statistically significant). SCEs per cell increased from 12.8 in the controls to 14.5 at the highest dose. Further studies by the same group suggest that induction of chromosome aberrations in the CHO cells was the result of severe cytotoxicity (Phillips et al., 1986). The authors found that CHO cultures were not viable in the presence of 0.5 mM MEHP as measured by Trypan Blue exclusion, whereas the chromosome effects were only seen above 1.0 mM. MEHP is a detergent

strong enough to lyse cells, and this may be occurring at the higher concentrations. More recently, NTP-sponsored studies of DEHP in CHO cells have demonstrated a modest, statistically significant, reproducible increase in SCE after a 24-hour exposure to high doses of DEHP in the absence of S9 (Ashby et al., 1985). In the IPCS study, all three of the assays for mutagenesis in somatic cells in *Drosophila* were negative (Ashby et al., 1985).

DEHP, MEHP, and 2-EH did not induce DNA repair in metabolically competent primary rat hepatocyte cultures (Butterworth et al., 1984; CMA, 1982; Hodgson et al., 1982; Kornbrust et al., 1984). Similarly, no activity was seen with DEHP or MEHP in the primary human hepatocyte DNA repair assay (Butterworth et al., 1984). In the IPCS study, DEHP was negative in all three of the DNA repair assays done by autoradiography (Ashby et al., 1985).

In the IPCS study, a positive result was obtained in a primary hepatocyte DNA repair assay with scintillation counting (Ashby et al., 1985). Scintillation counting cannot distinguish a general DNA repair response from a stimulation of cells in S-phase (Doolittle et al., 1984). Other hypolipidemic agents have been shown to stimulate S-phase DNA synthesis in primary hepatocyte cultures (Bieri et al., 1984).

It is currently believed that cell transformation assays are capable of measuring some of the stages in the progress of a cell from the normal to the malignant state. The C3H 10T1/2 assay measures the conversion of cells from a preneoplastic to a tumorigenic state. The assay measures both initiating and promoting events (Frazelle et al., 1984). Both DEHP and MEHP were negative as complete transforming agents, initiators, or promoters in the 10T1/2 cell transformation assay (J. Sanchez, D. Abernethy, and C. Boreiko, Chemical Industry Institute of Toxicology, Research Triangle Park, N.C., personal communication, 1985). DEHP, MEHP, and 2-EH were also negative in the BALB/3T3 cell transformation assay (CMA, 1982).

The Syrian hamster embryo cell transformation assay measures transformation from the normal to the preneoplastic state. This assay is sensitive not only to chemicals that affect the primary DNA sequence but also to agents that cause aneuploidy. Several carcinogens, such as asbestos and diethylstilbestrol (DES), that are negative in traditional mutagenicity tests are positive in the Syrian hamster embryo cell transformation assay (Barrett et al., 1981; Hesterberg and Barrett, 1984). The action of these agents appears to lie in the induction of aneuploidy (Barrett et al., 1984). DEHP has been shown to be positive in the Syrian hamster embryo cell transformation assay at concentrations ranging from 1.0 to 100 mg/ml (0.0026 to 0.26 mM) with reasonable cell survival (Barrett and Lamb, 1985). In the IPCS study, four of five assays for cell transformation were positive

and three of the five IPCS assays for aneuploidy were positive (Ashby et al., 1985).

There is a report that DEHP was weakly active in a hamster transplacental mutagenesis and transformation assay (Tomita et al., 1982). In this experiment, very high doses were administered, and the control values were much higher than expected for an assay of this type.

Kornbrust et al. (1984) reported that DEHP was negative in the *in vitro* Chinese hamster V79 metabolic cooperation assay for tumor promoters (upper concentration of 0.12 µg/ml). This is in contrast to the findings of Malcolm et al. (1983), who reported that DEHP inhibited metabolic cooperation in the same assay at concentrations of 1 to 30 µg/ml.

Conclusions on Mutagenicity

DEHP/MEHP have been subjected to extensive testing in short-term tests. Despite two positive *in vitro* tests for bacterial and mammalian cell mutagenicity, the committee concluded from the weight of the evidence that DEHP and MEHP were not mutagenic for bacterial or mammalian cell systems, with or without metabolic activation. DEHP is not mutagenic in *Drosophila*, does not induce DNA repair in metabolically competent cultures of human or rat hepatocytes, and is largely negative for the induction of chromosome aberrations. However, it has been reported to be a weak inducer of SCEs.

DEHP is able to induce aneuploidy in cells in culture and is capable of inducing cell transformation as shown in the Syrian hamster embryo. However, there are reasons to be cautious about concluding that the sole mechanism of action of DEHP is the induction of aneuploidy or other chromosomal events, such as sister-chromatid exchanges. First, other biological effects exerted by DEHP in the whole animal may be as important as or more important than its carcinogenic activity. Second, although reported to be a weak inducer of SCEs, DEHP does not induce other chromosome abnormalities associated with agents such as benzene or DES, which induce other genetic activities such as clastogenicity and polyploidy (Ashby et al., 1985).

Possible Mechanisms of Carcinogenicity

According to several researchers, peroxisome proliferators such as DEHP produce secondary genetic toxicity by stimulating biosynthesis of liver-cell peroxisomes, increasing peroxisomal hydrogen peroxide-generating oxidases (Cerutti, 1985; Warren et al., 1982), and modifying the pattern of enzyme activities. This results in an excessive production of hydrogen peroxide (paralleled by a decrease in catalase) and, by further reactions, of hydroxy (·OH) radicals. Hydrogen peroxide, hydroxy radicals, and other forms of active oxygen can cause reactions that result in mutations, sister-chromatid exchanges, chromosome aberrations, and cancer (Cerutti, 1985). Active oxygen

appears to play a role mostly in the promotion phase but also in the progression phase of carcinogenesis (e.g., by inducing chromosome aberrations). According to Cerutti (1985), the metabolite MEHP may also produce a prooxidant state by inhibiting the electron transport chain.

DEHP induces several functional changes in the liver, including hepatomegaly and peroxisomal proliferation (Miyazawa et al., 1980) in a manner similar to that of hypolipidemic drugs such as clofibrate. Livers from rats fed a diet containing 2% DEHP had an increased number of peroxisomes after a few days. Liver biopsies of patients undergoing dialysis for one year have an increase in peroxisomes, possibly related to DEHP exposure (Ganning et al., 1984).

There are conflicting data on the role of peroxisome proliferation in DEHP's carcinogenicity. Reddy and Lalwani (1983) hypothesized that peroxisomal proliferating agents may constitute a novel class of carcinogens. Warren et al. (1982) proposed that following peroxisomal proliferation, genotoxic activity results from an increase in the production of DNA-damaging reactive oxygen species. Accordingly, the ability of DEHP to induce DNA damage or repair has been examined in the *in vivo* hepatocyte DNA repair assay (Mirsalis and Butterworth, 1980). DNA damage has also been measured by alkaline elution of cellular DNA from the same cultures (Bermudez et al., 1982). Female rats were treated with 12,000-ppm concentrations of DEHP in the diet for 30 days or for 30 days followed by a 500-mg/kg dose of DEHP by gavage for 14 days. Male rats were treated by gavage with 500-mg/kg doses of DEHP 2, 12, 24, or 48 hours before sacrifice or with 150 mg/kg by gavage for 14 days. No chemically induced DNA damage or repair was seen under any of the conditions of the study (Butterworth et al., 1984).

In a similar study, male Sprague-Dawley rats were given a single oral DEHP dose of 5,000 mg/kg bw or fed a diet containing 20,000-ppm concentrations of DEHP for up to 8 weeks. Genotoxicity was evaluated in the *in vivo* hepatocyte DNA repair assay. No chemically induced DNA repair was observed, even in animals pretreated with 3-amino-1 *H*-1,2,4,-triazole to inhibit catalase activity, thus maintaining any elevated peroxide levels that might have existed (Kornbrust et al., 1984). These studies indicated that neither the parent compound nor its metabolites bound to the DNA to elicit the DNA repair response. Similarly, Lutz (1986) observed no covalent binding to the DNA in female Fischer 344 rats given DEHP by oral gavage in olive oil (the covalent binding index was estimated to be less than 0.05). From these studies, the committee concluded that neither DEHP nor its metabolites bind to the DNA in the whole animal.

More recent evidence challenges the hypothesis that peroxisomal proliferation is the basis of the carcinogenic activity of DEHP. With the alkaline elution assay, no significant DNA strand breaks were detected in

the cells from the treated animals (Butterworth et al., 1984). Administration of DEHP in concentrations ranging from 500 to 5,000 ppm in the diet of male Sprague-Dawley rats caused large increases in carnitine palmitoyltransferase, carnitine acetyltransferase, and β-oxidation capacity (Morton, 1979). Similar increases have been observed in Fischer 344 rats (Butterworth et al., 1984). Therefore, it is likely that the animals fed 6,000-ppm concentrations of DEHP in the long-term bioassay must have had significant peroxisomal proliferation. Neither the female nor the male rats developed a statistically significant increase in hepatocellular carcinomas at that dose, although a nonsignificant increase was seen in female rats.

Similarly, di(2-ethylhexyl) adipate (DEHA) is a peroxisome proliferator in rodents (Reddy and Lalwani, 1983) but produced no tumors in Fischer 344 rats given 25,000-ppm concentrations of DEHA in the diet for 2 years (NTP, 1982c). However, DEHA did produce tumors in mice at the same dose. Furthermore, DEHP is more effective in inducing peroxisomal enzymes in male than in female Sprague-Dawley (Osumi and Hashimoto, 1978) or Fischer 344 rats (CMA, 1982; F. E. Mitchell et al., 1984). In contrast, the female rats had a greater increase in the rate of liver tumors than did the male rats (NTP, 1983b). Lipid peroxidation measured in animals given 20,000-ppm concentrations of DEHP in the diet for 6 weeks plus a single 5-g/kg bw DEHP dose by gavage did not differ from controls (Kornbrust et al., 1984).

Thus, although a role for peroxisomal proliferation in carcinogenesis cannot be ruled out, genotoxic activity does not appear to be associated with DEHP-induced peroxisomal proliferation at the level of sensitivity that has been measured, suggesting that peroxisomal proliferation alone is not sufficient to produce tumors. More research should be done to test this hypothesis.

Carcinogenicity

Promotional activity is a plausible hypothesis for the mechanism of action of DEHP in light of its lack of mutagenic activity in most cell culture and animal models. There are several models for initiation and promotion in the rodent liver in which the animals are treated with a single dose of initiator followed by an extended period of treatment with promoter. The end points generally observed are focal hepatocellular proliferative lesions (FHPLs) such as foci positive for γ-glutamyl transpeptidase (GGT). Although it has never rigorously been shown that GGT-positive foci are actual precursors of liver tumors, the evidence indicates that these assays reflect initiating and promoting events. Popp et al. (1985) reported no increase in enzyme-altered foci, preneoplastic nodules, or liver tumors in female Fischer 344 rats under either of two regimens: DEHP given as an initiator followed by a growth selection regimen to

express initiated sites or diethylnitrosamine (DEN) given as the initiator followed by 12,000-ppm dietary concentrations of DEHP for 6 months as a promoter. Similarly, no promoting activity of DEHP was seen in female Fischer 344/NCr rats initiated with DEN followed by 14 weeks of promotion with 12,000-ppm concentrations of DEHP (Ward et al., 1986).

As with the rats, DEHP was found to have no initiating activity in $B6C3F_1$ mice (Ward et al., 1983). In contrast to the results found in the rats, DEHP given in the diet in concentrations ranging from 3,000 to 12,000 ppm was shown to promote FHPL in mouse liver (Ward et al., 1983, 1984a). Phenobarbital is a standard positive control with known promoting activity used in these assays. Whereas phenobarbital induced an increase in both the number and size of the FHPLs, the effect of DEHP was to increase FHPL size but not number (Ward et al., 1983). Phenobarbital required 168 days of continuous exposure for a promoting effect to be evident. DEHP was an effective promoter after 28 days (Ward et al., 1984a).

In skin-painting studies, DEHP displayed weak complete promoting activity but did show second-stage promoting activity in Sencar mouse skin. In contrast, the compound was inactive in CD1 mouse skin-painting experiments (Ward et al., 1986). Thus, DEHP does appear to have species-specific promoting activity in the mouse. This is consistent with the cancer studies, which yielded a response in the mouse liver at lower doses than in the rat liver (NTP, 1983b).

Stott et al. (1981) suggested that some carcinogens are not directly genotoxic but may exert their effects through cell turnover activity, either by producing cytotoxicity, thus resulting in increased regenerative DNA synthesis, or by inducing hyperplasia directly (Stott et al., 1981). Continual excessive cell turnover could result in an increased frequency of mutations and in promotional effects. One observed action of DEHP on the DNA of Fischer 344 rats was a small increase in the number of hepatocytes in the S-phase (Butterworth et al., 1984). This is consistent with the increased liver-to-body-weight ratios observed upon prolonged administration of DEHP (F. E. Mitchell et al., 1984; Miyazawa et al., 1980; Morton, 1979). Similar hepatomegaly and mitogenic stimulation has resulted from the carcinogenic hypolipidemic peroxisomal proliferators (Moody et al., 1977; Reddy et al., 1976, 1978). The spontaneous and DEHP-induced tumor incidences in the mice were greater than those in the rats (NTP, 1983b). These responses are similar to those of a variety of other compounds that induce tumors more easily in mice than in rats and that appear to be without mutagenic activity (Doull et al., 1983; Ward et al., 1979).

If the carcinogenic activity of DEHP were due to effects of cell turnover, one might expect mice to be more susceptible to induction of cell repli

cation. Studies show that DEHP induces an S-phase response in mouse hepatocytes that is approximately an order of magnitude greater than that seen in rats following a single dose or following the administration of diets containing 6,000 ppm for 2 weeks (Smith-Oliver et al., 1985). More work should be done in both the rat and mouse to learn how long the increased S-phase response is maintained in response to DEHP feeding. At this time, the carcinogenic effects of DEHP appear to be correlated with cell replication (NTP, 1983b). More studies should be conducted to examine the duration and shape of the dose-response curve for induced cell turnover. This is not to suggest that induction of cell replication is likely to be the sole explanation of the carcinogenic effect of DEHP, but it may be an important contributing factor.

TABLE 9-14 Hepatocellular Carcinoma Incidence in Rats and Mice Fed DEHP for 2 Years[a]

		Concentration in Feed (ppm)			
Animal	Sex	Controls	3,000	6,000	12,000
Fischer 344 rat	Male	1/50		1/49	5/49
	Female	0/50		2/49	8/50
B6C3F$_1$ mouse	Male	9/50	14/48	19/50	
	Female	0/50	7/50	17/50	

[a] Based on data from NTP, 1983b.

Statistically significant increases in hepatocellular carcinomas were observed in the NTP bioassay described above (NTP, 1983b) for female rats receiving 12,000-ppm, male mice receiving 6,000-ppm, and female mice receiving 6,000- and 3,000-ppm concentrations of DEHP in feed. The incidence of hepatocellular carcinomas in this bioassay is given in Table 9-14. Statistically significant decreases in the incidence of pituitary tumors, thyroid C-cell carcinomas, and testicular interstitial tumors were found among the male rats. DEHP also produced a statistically significant increased incidence of either hepatocellular carcinomas or neoplastic nodules in male rats (controls, 3/50; 6,000 ppm, 6/49; 12,000 ppm, 12/49).

Carcinogenic Risk Estimate

In a study by the National Toxicology Program, an increase in hepatocellular tumors was observed in both sexes of Fischer 344 rats and B6C3F$_1$ mice (NTP, 1983b). There was a trend toward increases in the number of tumors with increasing doses. The tumor incidences for the mice, the more sensitive species, are summarized in Table 9-15.

The data for each sex were used to estimate a lifetime risk and an upper 95% confidence estimate of lifetime risk in humans weighing 70 kg fol

lowing a daily consumption of 2 liters of water containing the compound in a concentration of 1 µg/liter. No estimates were calculated for children weighing 10 kg and consuming 1 liter of water; however, the risk estimates for children would be higher than those shown below due to increased consumption per body weight. The estimate of lifetime risk is based on the generalized multistage model for carcinogenesis described earlier in Chapter 8 (see Table 9-16). Calculations based on the generalized multistage model indicate that a DEHP dose of 0.032 mg/kg/day presents an estimated lifetime risk to humans of 1×10^{-6}.

TABLE 9-15 Tumor Incidence in Mice Fed Di(2-ethylhexyl) Phthalate[a]

Animal	Sex	Tumor Site	Dose,[b] ppm (mg/kg/day)	Tumor[c] Rates
$B6C3F_1$ mouse	Male	Liver	0	14/50
			3,000 (312)	25/48
			6,000 (615)	29/50

[a] Based on data from NTP, 1983b.
[b] Of the DEHP metabolite mono(2-ethylhexyl) phthalate.
[c] Hepatocellular carcinomas or adenomas.

Developmental Effects

Because of the apparent ubiquity of the phthalate esters in the environment, their teratogenicity and reproductive toxicity, particularly of the most commonly used ester DEHP, have been investigated in numerous laboratories. Examination of these effects of DEHP and of its principal metabolite MEHP (Albro et al., 1973; Schulz and Rubin, 1973) have centered on the use of DEHP as a plasticizer for polyvinyl chloride polymers used in the manufacture of various medical products and blood storage devices.

DEHP has induced malformations in both rats and mice at very high dosages; more equivocal results were obtained at lower concentrations.

TABLE 9-16 Carcinogenic Risk for DEHP[a] Estimated with the Generalized Multistage Model[b]

Animal	Sex	Estimated Human Lifetime Risk[c]	Upper 95% Confidence Estimate of Lifetime Cancer Risk[c]
$B6C3F_1$ mouse	Male	1.2×10^{-7}	2.1×10^{-7}

[a] Based on data from NTP, 1983b.
[b] The Weibull model produces similar results.
[c] Assuming daily consumption of 1 liter of water containing the compound in a concentration of 1 µg/liter.

Singh et al. (1972) administered DEHP intraperitoneally to pregnant rats at doses of 0.3 to 10.0 ml/kg on days 5, 10, and 15 of gestation. They reported increases in gross abnormalities (primarily hemangiomas) and fetal resorptions and decreased fetal weight at only the high dose tested (10.0 ml/kg, equivalent to approximately 9,810 mg/kg). Lower dosages (up to 4,905 mg/kg) had no effect. Subsequently, Singh et al. (1974) described both mutagenic and antifertility effects in male mice that received a single intraperitoneal injection of 12,800-, 19,100-, or 25,600-mg/kg doses of DEHP prior to mating with untreated females. At the highest dose, females mated with treated males over a 12-week period exhibited a decrease in the incidence of pregnancy and in numbers of implantations and offspring as well as an increase in the incidence of early fetal death. No gross or skeletal abnormalities were described. There were no effects at lower dosages. The significance of these studies (Singh et al., 1972, 1974) is reduced by the very high dosages used and the use of the relatively inappropriate intraperitoneal route of administration. In a similar study (Domon et al., 1984), males of two different strains of mouse received subcutaneous injections of DEHP (approximately 625, 2,000, and 4,900 mg/kg/day) on 3 days during the first 2 weeks of the study and were then mated to females of the same strain over an 8 week period. DEHP did not induce significant dominant lethality in either strain and produced little, if any, infertility at the dosages examined.

Nikonorow et al. (1973) treated female rats orally with 340 or 1,700 mg/kg/day throughout gestation and recorded a decrease in fetal body weight and an increased number of resorptions, but no gross abnormalities. Yagi et al. (1980) examined the teratogenic potential of DEHP and MEHP in mice by giving them oral doses of these compounds. Treatment with 5.0-ml/kg (4,905-mg/kg) or 10.0-ml/kg (9,810-mg/kg) concentrations of DEHP on day 7 of gestation resulted in 100% fetal death. Survival rates of fetuses exceeded 90% when the treatment was given on day 9 or 10, however. These investigators recorded both gross and skeletal abnormalities in live fetuses after oral treatment with 2.5 or 7.5 ml/kg (2,450 or 7,360 mg/kg) on day 7 or 8 of gestation.

Administration of 70, 190, 400, 830, and 2,200 mg/kg/day in the diet of female mice resulted in decreased maternal weight gain (probably due to reduced numbers of viable fetuses) and increased resorption rate (Shiota et al., 1980). In addition, there were no viable fetuses in the two highest dose groups and a dose-related decrease in fetal weight in the 400- and 830-mg/kg groups. In this latter group, there was also a slight but significant increase in fetal malformations, primarily neural tube defects (exencephaly and spina bifida). Recent whole-body autoradiography studies of the distribution of ^{14}C-labeled DEHP (Lindgren et al., 1982) have shown a pronounced concentration of DEHP in the neuroepithelium of

the developing embryo, which may correlate with the DEHP-induced neural tube defects. Shiota et al. (1980) have determined that the maximum no-effect level is 70 mg/kg/day, perhaps as much as 2,000 times higher than the estimated current intake by humans from environmental sources (Nakamura et al., 1979).

Ruddick et al. (1981) administered 225-, 450-, and 900-mg/kg doses of MEHP to female rats by intubation on days 6 to 15 of gestation. They reported decreases in the number of litters and in the mean litter weight in the 450-mg/kg group but no skeletal or visceral abnormalities in living fetuses. Maternal toxicity at the higher dosage prevented analysis (11 of 15 treated females died). No fetal effects were recorded at the lower dosage tested, and the no-effect dosage for dams was determined to be 50 mg/kg.

Tomita et al. (1982) administered a single oral dose of DEHP (0.2% to 100% of the acute LD_{50} of 30 mg/kg) to pregnant female mice on day 6, 7, 8, 9, or 10 of gestation. Decreases in both number and body weight of fetuses and an increased incidence of gross and skeletal fetal malformations were observed in dams treated with the higher dosages. The fetal LD_{50} of DEHP was found to be 592 mg/kg, and the maximum nonlethal dose for the fetuses was calculated to be about 64 mg/kg. Tomita et al. (1982) also estimated that the ineffective maximum dosages (no-observed-effect levels) for gross and skeletal abnormalities were probably less than 789 and 670 mg/kg, respectively. In humans, the maximum intake of DEHP by blood transfusion or hemodialysis is approximately 4 mg/kg bw (Jaeger and Rubin, 1972).

In an interesting comparative study, Wolkowski-Tyl et al. (1983a) reported teratogenic effects in mice at high doses of DEHP (0.10% and 0.15% in the feed during the entire gestation period), which induced maternal toxicity, but no such effects at doses not toxic to the dam (0.05%). In contrast, DEHP was not teratogenic in rats at any concentration tested with the identical protocol (Wolkowski-Tyl et al., 1983b).

These studies demonstrate that the effects of DEHP on fetal development and viability appear only at dosages much higher than those possible in humans (see review by Thomas et al., 1978). Several studies have been conducted using more clinically relevant dosage regimens. Garvin et al. (1976) examined the teratogenic potential of plasma-soluble extracts of polyvinyl chloride containing approximately 1- and 3.7-mg/kg doses of DEHP administered intravenously on gestation days 6 to 15. No embryotoxic or teratogenic effects were detected. Lewandowski et al. (1980) administered mean daily intravenous 1.3- to 5.3-mg/kg doses of similar plasma-soluble extracts of DEHP-plasticized polyvinyl chloride to rats on gestation days 6 to 15. The high doses are similar to those predicted to be received by a 60-kg human undergoing an exchange transfusion with

21-day-old blood. There were no significant differences in fetal weights, in the number of live and resorbed fetuses, or in the incidence of gross external, skeletal, and visceral defects between control and treated groups. The authors concluded that the tested plasma extracts were not teratogenic when administered intravenously to pregnant rats. Studies of pregnant rabbits (Thomas et al., 1979) confirmed that exposure to more clinically relevant concentrations of MEHP (1.14, 5.69, and 11.38 mg/kg daily on gestation days 6 to 19) were neither teratogenic nor embryotoxic.

It is clear that only very high dosages of DEHP (or MEHP) are embryotoxic or teratogenic in the species tested. Levels that produce such effects generally are far greater than those anticipated for human exposure (Shiota et al., 1980).

Reproductive Effects

Gonadal toxicity of DEHP was first reported in the mid-1940s, when Shaffer et al. (1945) described the results of a 90-day feeding study in which male rats received dietary concentrations of 0.075%, 0.75%, 1.5%, and 5.0% DEHP in feed. These investigators reported atrophy of the seminiferous tubules and testicular degeneration resembling "senile changes" at the two highest dosages. In a two-generation feeding study (Carpenter et al., 1953) in which rats were maintained on diets containing 0.04% to 0.4% DEHP, a decrease in the number of litters per female was reported in F_1 rats in the highest dose group. Subsequent metabolic studies with the phthalate diesters have revealed that the testicular effects are mediated via the corresponding monoesters (MEHP in the case of DEHP) that are formed by partial hydrolysis of the parent compound in the gastrointestinal tract (Albro et al., 1973; Rowland et al., 1977). The alcohols also formed by this hydrolysis (2-ethylhexanol in the case of DEHP) have more recently been shown to have no activity in induction of the testicular lesions produced by the phthalate diesters (Gray and Beamand, 1984; Rhodes et al., 1984).

Other investigators have described the morphology of the testicular degeneration and examined the mechanism(s) responsible for the gonadal toxicity and fertility effects. Singh et al. (1974) treated groups of young (8 to 10 weeks old) 25- to 30-g male mice with a single intraperitoneal injection of undiluted DEHP representing one-third, one-half, and two-thirds of the acute LD_{50} (12,800, 19,200, and 25,600 mg/kg, respectively), and they subsequently bred these males to untreated females over a 12-week period. There was a reduction in the number of implantations, in litter size, and in the incidence of pregnancy at the high dose tested, particularly in the first few weeks after injection.

Gray et al. (1977) reported significant decreases in the testicular weight of rats fed a 2.0% DEHP diet for 6 or 17 weeks and histological changes that included severe seminiferous tubular atrophy and cessation of sper

matogenesis. Lower dosage groups (0.2% and 1.0%) also exhibited histological changes in the testes, the incidence and severity of which correlated with the dietary concentration of DEHP. Testicular weights from rats that received a 0.2% diet were not reduced, but they did show histological evidence of reduced spermatogenesis with a reduction in late-stage spermatids and mature sperm. The interstitial cells of the testes appeared undamaged histologically, but the authors described the appearance of "castration cells" (enlarged basophilic cells) in the pars distalis of the pituitary gland. The presence of castration cells in the pituitary is considered to be a sensitive indication of gonadal deficiency (Russfield, 1967) and suggests that the testosterone-producing Leydig's cells of the testicular interstitium may have been damaged by exposure to dietary DEHP.

In a later study, Gray and Butterworth (1980) reported that other phthalate esters produced testicular injury similar to that produced by DEHP: loss of the advanced germinal cells with only spermatogonia, Sertoli's cells, and occasional primary spermatocytes remaining. In this study, in which 4- and 10-week-old male rats received DEHP doses of 2,800 mg/ kg/day by intubation for 10 days, the testicular atrophy was not prevented by concurrent treatment with testosterone (200 µg/kg/day) or follicle-stimulating hormone (FSH; 100 units subcutaneously), suggesting that DEHP does not interfere directly with hormone production or release. The testicular effects in 4-week-old rats that received dietary DEHP (2%, or approximately 1,200 mg/kg/day) were reversible, whether the treatment was stopped before or after the rats normally reached sexual maturity. The authors suggested that the histological appearance of the lesion and the kinetics of its reversibility make it unlikely that DEHP acted by direct cytotoxic action on the germ cells.

Agarwal et al. (1985) fed male rats DEHP in dietary concentrations of 0, 320, 1,250, 5,000, or 20,000 ppm for 60 consecutive days. They reported a dose-dependent decrease in total body weight, testis weight, epididymal weight, and prostate weight at 5,000 and 20,000 ppm. In addition, degenerative changes in the testis, decreased epididymal sperm number and motility, and increased frequency of abnormal sperm forms were reported in the 20,000-ppm dose group. Reductions in fertility parameters were correlated with gonadal effects, but they were not as severe. (Only average litter size was reduced in the 20,000-ppm group. No reduction in pup weights or growth rate and no increase in abnormalities were observed.) There were no fertility effects at lower doses. These data suggest that DEHP may also affect epididymal function, but not at dosages below those that produce minor seminiferous tubule degeneration.

In a recent study, male rats received repeated intravenous infusions of a DEHP emulsion in doses of (5, 50, or 500 mg/kg) during a 3-hour period every other day for 12 days (Sjöberg et al., 1984). The route of

administration and dose concentrations (at the low dose, at least) used in this study are relevant to situations in which humans are exposed (i.e., generally by tranfusion with DEHP-contaminated blood or plasma). At the high dose, some degeneration of primary spermatocytes occurred, and ultrastructural changes were observed in the nuclei of late-stage spermatids. No effects were found in the two lower dose groups. The author concluded that DEHP-infused intravenous solutions produced only very minor changes when low doses were used (and these were in the liver, not the testes), whereas the high doses resulted in distinct but not very severe changes.

Since the germinal cells affected by DEHP (postmitotic spermatocytes and spermatids) are inside the Sertoli's cell barrier (Steinberger and Steinberger, 1977), Gray and Butterworth (1980) proposed that phthalate esters may act as a result of injury to the Sertoli's cells. This suggestion is supported by the work of Foster et al. (1982), who described the histological changes in the rat testes after oral administration of a single dose of di-*n*-pentyl phthalate (DPP), which produces testicular lesions in young male rats similar to those produced by DEHP. These changes, which occurred within 6 hours after dosing, included a severe vacuolation of Sertoli's cell cytoplasm and extended to degradative changes in the mitochondria of Sertoli's cells, spermatocytes, and spermatids within 24 hours.

Recently, Gray and Beamand (1984) tested the effects of several phthalate esters, including DEHP, on primary cultures of testicular cells. Cultures consisted of a monolayer of Sertoli's cells to which clusters of spermatogonia and spermatocytes were attached. The addition of MEHP to the culture medium resulted in a concentration-dependent increase in germ cell detachment over the range of 10^{-7} to 10^{-4} M. No effect was produced by the addition of DEHP or 2-EH. These observations suggested that the germ cell cultures lack the capability to metabolize DEHP to MEHP and 2-EH, and that 2-EH itself is without toxic effect. Testing with other phthalate monoesters revealed that only those esters that produce testicular injury *in vivo* caused detachment of germ cells in culture. The authors suggested that the effects produced by the phthalate monoesters in culture may reflect damage to the Sertoli's cells.

Gray and Gangolli (1986) reported that a single oral dose of DPP (2,200 mg/kg) or MEHP (1,000 mg/kg) administered in corn oil to 4to 5-week-old male Sprague-Dawley rats markedly inhibited both the secretion of seminiferous tubular fluid and androgen-binding protein indicative of compromised Sertoli's cell function. As in the earlier study, phthalate esters that did not cause testicular injury also did not affect Sertoli's cell function *in vivo*.

The sensitivity of the testis to the toxic effects of DEHP appears to be age dependent. Gray and Butterworth (1980) observed that treatment with DEHP (2% of the diet, or approximately 1,200 mg/kg/day) produced testicular lesions in 4- and 10-week-old rats but not in mature 15-week-old male rats, demonstrating that DEHP interferes with the normal development of rat testes but not with the mature testis. Curto and Thomas (1982) reported that mature male mice (35 to 40 g) were resistant to the effects of DEHP or its principal metabolite MEHP. Single intraperitoneal or subcutaneous doses of MEHP or DEHP up to 100 mg/kg failed to induce alterations in testicular or accessory sex gland weight in these animals. In a recent study (Seth and Mushtaq, 1984), DEHP was administered orally (2,000 mg/kg) to male rats ranging in age from 4 to 12 weeks. Rats up to 8 weeks old had a 60% to 70% reduction in testis weight as well as changes in several biochemical parameters indicative of testicular damage. Rats older than 8 weeks exhibited no testicular weight reduction and no changes in any biochemical parameter, suggesting that developing males are more susceptible to the damaging effects of DEHP than are mature males.

Several investigators have studied the possible role of testicular zinc concentrations in mediating the effects of the phthalate esters on the testes. In rats, dietary zinc deficiency is known to cause testicular atrophy which appears to be due to inhibited pituitary testosterone output (Millar et al., 1960). In rats, phthalate esters causing testicular damage enhance urinary zinc excretion and lower testicular zinc concentrations (Foster et al., 1980, 1982). A 1 week dietary administration of 2% DEHP (Oishi and Hiraga, 1980a) or 2% MEHP (Oishi and Hiraga, 1980b) lowered testicular zinc concentrations in the rat. Foster et al. (1982) demonstrated that the loss of zinc following oral administration of DPP (2,200 mg/kg bw) occurred in the Golgi region of spermatids and was seen before there was any evidence of morphological damage in these cells. Curto and Thomas (1982) failed to detect any depletion of zinc in the testes of mature mice after intraperitoneal or subcutaneous treatment with up to 100-mg/kg doses of MEHP or DEHP, but they did see a reduction in gonadal zinc in the testes of mature rats after intraperitoneal administration of 100-mg/kg doses of DEHP.

Cater et al. (1977) reported that coadministration of zinc by subcutaneous injection substantially protected against the testicular damage produced by a DPP dose of 2,000 mg/kg in rats, whereas Oishi and Hiraga (1983) did not observe any measure of protection from DEHP-induced damage (2,000-mg/kg doses of DEHP administered orally) when zinc was administered to male rats by diet or intraperitoneally. However, although liver and serum zinc concentrations were elevated in this study, testicular

zinc concentrations were unchanged even in the control animals, leading the authors to conclude that the toxic effect of DEHP on the testis does not result from interference with gastrointestinal absorption of zinc. In subsequent studies, young rats fed dietary DEHP (2% for 1 week) developed changes in testicular cholesterol and free fatty acid content as well as changes in fatty acid composition in phospholipids and triglycerides that were similar to animals fed a zinc-deficient diet (Oishi, 1984a). This testicular injury was not attributed to the vitamin A deficiency associated with lowered zinc concentrations in the testes (Oishi, 1984b).

T. J. B. Gray et al. (1982b) and Gangolli (1982) reported the species specificity of testicular toxicity of the phthalate esters in young (4- to 6-week-old) males. Rats and guinea pigs were severely affected by oral administration of 2,000-mg/kg bw doses of DEHP for 10 days, mice were much less affected, and hamsters showed no effect. Curto and Thomas (1982) described similar differences in the sensitivity of mature mouse and rat testes to the effects of DEHP or MEHP treatment.

Little attention has been paid to the gonadal toxicity of the phthalate esters in the nonpregnant female. Gray et al. (1977) reported that absolute ovary weights in female rats were decreased by a 17-week exposure to 2.0% dietary DEHP, but that there were no histological abnormalities in either the ovary or the pituitary. The authors suggested that DEHP may produce gonadal damage only in the male and that the decreased ovary weights were primarily a reflection of the reduced body weight of the treated females. Lower dosages (0.2% and 1.0%) had no effect. In an 18-week dietary exposure to 0.3% DEHP, Reel et al. (1985) found significant decreases in the reproductive tract (uterus and ovary) weight of female mice and complete suppression of fertility in these females when bred to unexposed males immediately following the 18-week exposure. These data demonstrate that DEHP is toxic to the reproductive tract of female and male rodents, but only at very high doses.

Conclusions and Recommendations

The committee believes that any chemically induced, measurable alteration in normal biochemistry or physiology should be viewed with concern, even if the resulting health effects are not immediately appreciated. Because DEHP has known effects on the liver, a risk assessment is presented for alterations in liver function.

As noted earlier, Ganning et al. (1984) found that dialysis patients who were receiving approximately 150 mg of DEHP intravenously each week as a consequence of their treatment experienced no reported morphological changes in the liver, but at one year, peroxisomes were "significantly

higher in number." For a 70-kg person, the exposure would be approximately 2.1 mg/kg/week, or 0.3 mg/kg/day. This study population includes persons who are not in peak condition and who have an undue burden of toxicants. If a 10-fold safety factor were applied to the dose level in addition to a 10-fold safety factor for person-to-person variability, a value of 0.003 mg/kg/day would be obtained.

TABLE 9-17 Increases in Liver Function in Male Sprague-Dawley Rats after Exposure to DEHP[a]

	Dietary Concentrations (ppm)					
Change	50	100	500	1,000	2,500	5,000
Liver wet weight	NC[b]	NC	NC	+[c]	+	+
Peroxisomes	NR[d]	NR	NC	NR	NR	+
Hepatic catalase	NR	NC	NR	NC	NR	+
Hepatic carnitine acetyltransferase (CAT)	NR	NC	+	+	+	+
Hepatic carnitine palmitoyltransferase (CPT)	NR	NC	+	+	+	+
Hepatic 2-oxidation capacity	NC	NC	+	+	NR	+
Hepatic triglycerides	NC	NC	NC	NC	NC	NC

[a] Adapted from Morton, 1979.
[b] NC = no change compared with controls.
[c] + = statistically significant, $p \leq 0.05$.
[d] NR = not reported.

In a study by Morton (1979), male Sprague-Dawley rats were fed diets containing 0- to 5,000-ppm concentrations of DEHP for 7 days. The resultant increases in liver enzymes are listed in Table 9-17.

When all indices of altered liver function are considered as a whole, the no-observed-effect level (NOEL) in this series of studies appears to be approximately 50 ppm (3.30 mg/kg bw a day) in the diet. If a safety factor of 1,000 is applied because this was only a short-term animal study, the acceptable daily intake would be 0.003 mg/kg.

In comparison, Ganning et al. (1984) fed rats DEHP for 2 years in the diet and found alterations in palmitoyl-coenzyme A dehydrogenase and dolichol in the liver. The lowest-observed-effect level (LOEL) for this study was 0.02% (200 ppm or 13 mg/kg/day). If a safety factor of 100 is applied in addition to a safety factor of 10 because a LOEL is being used, the acceptable daily intake would be 0.01 mg/kg.

The committee noted that the areas of concern for possible toxic effects induced by DEHP are changes in liver function, reproductive and fertility effects, developmental effects, and cancer. In accordance with the guide

lines in Chapter 8, it suggests that the LOEL or NOEL and a judgmental safety factor approach should be used for the first three end points. A linearized multistage model should be applied in cancer risk assessment.

TABLE 9-18 Summary of Estimated Acceptable Daily Intakes (ADIs) for DEHP

Type of Study	Estimated ADIs[a] (mg/kg/day)	Reference
Human, dialysis patients	0.003	Ganning et al., 1984
Rat, liver function	0.003	Morton, 1979
Rat, liver enzymes	0.01	Ganning et al., 1984
Rat, teratogenicity study	0.50	Ruddick et al., 1981

[a] The committee noted that in the NTP report (1983b) a 0.032-mg/kg/day dose produced a 1 x 10^{-6} risk of cancer in a bioassay for carcinogenesis in mice.

The estimated acceptable daily intakes shown in Table 9-18 were derived by the different procedures and from the different studies noted above. The most sensitive responses from the estimates appear to be liver peroxisomal proliferation, function, and tumor production. The teratologic effects are the least sensitive of the responses examined.

The mechanism of action of DEHP carcinogenicity is not known, and the contribution of genetic toxicity to its carcinogenicity is unclear. The only genotoxic activities observed in cell culture models were the induction of aneuploidy, Syrian hamster embryo-cell transformation, and some small but significant increase in SCEs. Short-term *in vivo* tests for genetic toxicity have thus far been negative. Activities alone or in combination that may contribute to carcinogenicity include promotional activity, forced cell proliferation, and genetic effects resulting from peroxisomal proliferation.

Several properties of DEHP, e.g., induction of peroxisomal proliferation, merit further investigation to determine whether there should be concern about this and related compounds. Experiments that show lack of genotoxic activity in peroxisomally proliferated animals and examples of noncarcinogenic peroxisome proliferators (DEHA in the rat) indicate that peroxisomal proliferation is not a sufficient event for complete carcinogenesis. Nonetheless, since a number of hypolipidemic agents induce peroxisomal proliferation in rodents, further work should be done to evaluate the role of this process in carcinogenesis and its risk to humans. Differences between species in the rates of peroxisomal proliferation and its extent have been observed (Rhodes et al., 1986; Reddy and Lalwani, 1983).

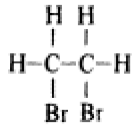
Microbodies (peroxisomes) were observed in humans following long-term administration of the hypolipidemic agent clofibrate, but the effect was less pronounced than that observed in rodents (Reddy and Lalwani, 1983). Kidney patients receiving DEHP in the form of a contaminant

during the process of dialysis had an increase in hepatic peroxisomal proliferation after a year (Ganning et al., 1984). Results of studies with primary hepatocyte cultures show that DEHP and the metabolites responsible for peroxisomal proliferation induce peroxisomal enzymes in rat hepatocytes but not in human hepatocytes (T. J. B. Gray et al., 1982a; A. M. Mitchell et al., 1984; Rhodes et al., 1986). The ability of hypolipidemic agents to induce dramatic changes in hepatocytes, including cell proliferation, indicates receptor-mediated regulation of many genes (Reddy and Lalwani, 1983). This could possibly play a role in carcinogenesis. The extent to which this occurs in human cells should be examined further.

The ability of DEHP to transform Syrian hamster embryo cells and induce aneuploidy and possibly SCEs is reminiscent of other human carcinogens that do not show mutagenic activity. Surfactants as a whole appear to be without genotoxic activity in cell culture assays (Yam et al., 1984). The structure-activity relationships of DEHP, MEHP, and similar surfactants with membrane-altering properties should be examined in cell transformation bioassays and correlated with the ability to induce aneuploidy and, if possible, carcinogenic activity.

ETHYLENE DIBROMIDE

1,2-Dibromoethane
CAS No. 106-93-4
RTECS No. KH9275000



Since ethylene dibromide (EDB) was reviewed in Volume 3 of *Drinking Water and Health* (NRC, 1980, pp. 98-101), the following discussion is mainly an examination of data that have since become available to the committee.

Health Aspects

Observations in Humans

Information concerning the health effects in humans resulting from exposure to EDB are limited to descriptions of acute symptoms observed after episodes of relatively high-dose exposure (NIOSH, 1977a); an epidemiological study of limited size regarding cancer incidence among workers engaged in EDB production (Ott et al., 1980); and several evaluations of reproductive performance of such workers (Ter Haar, 1980; Wong et

al., 1979). Acute dermal exposure produces painful local inflammation, swelling, and blistering. Acute systemic exposure by respiratory or oral routes causes vomiting, diarrhea, abdominal pain, and in some cases delayed lung damage and depression of the central nervous system. In one instance, a woman who ingested 4.5 ml of EDB died from massive centrilobular necrosis of the liver and focal damage to renal proximal tubular epithelium (Olmstead, 1960). In addition, two fatalities were reported after an acute occupational exposure of workers cleaning a storage tank. Both workers had metabolic acidosis, hepatic damage, and acute renal failure (Letz et al., 1984).

In an epidemiological study, Ott et al. (1980) examined the mortality experience of 161 white males employed in various phases of EDB synthesis at two production plants from 1940 through 1976. Numbers were limited, and the reported excess mortality from cancer was not statistically significant (7 cases observed, 5.8 expected). Average levels of workplace exposure to EDB were estimated to have ranged from 0.4 ppm in air for these two cohorts of workers. The power of this study to find a risk of twofold or more is roughly 63%, which is below the usually minimum power of 90%.

In a study of reproductive performance, Wong et al. (1979) evaluated the reproductive experience, from 1958 to 1977, of 297 EDB-exposed men employed in four EDB manufacturing facilities and their wives. Subjects were divided into two exposure groups: less than 0.5 ppm and between 0.5 and 5.0 ppm. The number of live births was determined for each group and compared to the national average. The authors concluded that overall, the standardized birth ratio was not significantly reduced among EDB-exposed workers and their wives, and no evidence of a dose-response relationship was seen. Ter Haar (1980) reviewed a number of similar studies, also concluding that there was no evidence of EDB-related reproductive hazard.

Observations in Other Species

Acute Effects

There is information on the acute lethality of EDB in a number of species. Oral LD_{50} values reported by Rowe et al. (1952) range from 55 mg/kg bw in female rabbits to 420 mg/kg bw in female mice. The LD_{50} values of Rowe et al. (1952) for male and female rats are 146 and 117 mg/kg, respectively.

Although animal studies have confirmed that EDB is acutely toxic to the liver and kidneys, near-lethal doses are apparently required to cause observable organ damage. Storer and Conolly (1983) saw little evidence of liver or kidney damage in male B6C3F_1 mice given a single intraper

itoneal injection of 94 or 141 mg/kg. An intraperitoneal dose of 188 mg/ kg was required to produce significant increases in liver and kidney weight as well as increases in blood urea nitrogen and serum enzyme levels. Nachtomi and Alumot (1972) observed an increase in total liver lipids and triglycerides in fasted male rats given EDB doses of 110 mg/kg bw orally and a modest increase in lipid peroxidation of microsomal liver lipids. In a subsequent study, the 110-mg/kg dose was found to produce central and midzonal hepatic necrosis in fasted male rats (Broda et al., 1976). Acute oral no-observed-effect levels (NOELs) and lowest-observed-effect levels (LOELs) for injury of the liver, kidneys, or any other organ have not been identified.

There have been a number of investigations of biochemical changes in the livers of EDB-dosed rats. Moody et al. (1981) found that a large oral dose (220 mg/kg bw) of EDB produced a drop in hepatic microsomal cytochrome P450 levels but no significant change in fatty acids. Moody et al. (1982a) extended their findings by demonstrating significant decreases in the cytochrome P450 content of microsomes isolated from the liver, kidney, testes, and lungs of fasted male rats given a single oral dose of 159 mg/kg. Little or no effect was observed for other microsomal parameters, including dealkylations catalyzed by mixed-function oxidases.

Botti et al. (1982) administered 75-mg/kg bw doses of EDB orally to fasted male rats and examined the time course of changes in glutathione levels. Liver glutathione levels diminished within 15 minutes and remained low for up to 4 hours after dosing. Cytosolic glutathione-S-transferase activities did not diminish until 2 hours after dosing and returned to normal by 4 hours. The glutathione levels decreased due to metabolism of EDB by glutathione-*S*-transferases. When glutathione levels fell too low, reactive EDB-glutathione conjugates and other EDB metabolites were apparently free to interact with (and inhibit) the transferase enzymes as well as other proteins, RNA, and DNA.

Nachtomi and Sarma (1977) found that 4 hours after oral dosing of rats with ^{14}C-labeled EDB, the radioactivity was incorporated in decreasing amounts as follows: cytoplasmic protein > nuclear protein > microsomal protein ≤ RNA > nuclear DNA. The peak occurrence of single-strand DNA breaks coincided with a substantial decrease in hepatic glutathione levels. Kowalski et al. (1985), using single, varying intravenous or intraperitoneal doses of EDB with rats and mice, recently observed that several tissues, mainly the target tissues for the EDB-induced carcinogenic effects, can metabolize EDB to reactive products that become irreversibly bound to tissue constituents. White et al. (1981) reported a dose-dependent increase in single-strand DNA breaks in hepatic cells of mice given 25-, 50-, or 75-mg/kg doses of EDB by intraperitoneal injection. The extent

of DNA damage was statistically significant at the two highest dosage levels. White et al. (1981) found no evidence of EDB-induced DNA cross-links or DNA-protein cross-links.

Subacute Effects

Few subacute toxicity studies have been conducted with oral administration of EDB. In one study, EDB was given in corn oil by gavage 5 times weekly for 6 weeks to male and female mice and rats in order to establish the maximally tolerated doses of EDB for use in a subsequent carcinogenesis bioassay (NCI, 1978b). A dosage level of 100 mg/kg/day caused a reduction in body weight gain and 20% mortality in male and female rats. Male mice receiving 63 and 159 mg/kg/day exhibited a dose-dependent reduction in body weight gain. Nachtomi (1980) administered fumigated feed containing 100 and 200 ppm EDB to male rats for 18 days. Consumption of these diets was said to be equivalent to a daily intake of 10 to 20 mg/kg. The dietary regimen produced no change in liver weight or DNA concentration, compared to controls, but DNA synthesis was slightly increased. This increase was not dose dependent, and it was not large enough to be statistically significant.

The subacute toxic potential of inhaled EDB has been investigated in several species. Rowe et al. (1952) subjected male and female rats, rabbits, guinea pigs, and monkeys to as many as 156 7-hour exposures to 25-ppm concentrations of EDB for periods as long as 220 days. There was no evidence of adverse effects in any species. Repeated 7-hour exposures to 50 ppm for up to 91 days, however, were not well tolerated. Guinea pigs were the most sensitive, in that they had depressed body weight gain; increased lung, liver, and kidney weights; slight central fatty degeneration of the liver; and slight degeneration of the renal tubular epithelium.

Reznik et al. (1980b) exposed male and female Fischer 344 rats and $B6C3F_1$ mice to 0-, 3-, 15-, or 75-ppm concentrations of EDB vapor 6 hours/day, 5 days/week for 13 weeks. Histopathological changes were limited to the respiratory tract in animals sacrificed at the end of 13 weeks. Most of the male and female mice and rats subjected to 75-ppm concentrations of EDB exhibited nasal cavity changes, including loss of cilia, cytomegaly, focal hyperplasia, and squamous metaplasia. No mice, and only one or two rats, developed these changes in the 15-ppm groups. Morphological alterations were not seen in the control or the 3-ppm groups.

Nitschke et al. (1981) also conducted a 13-week inhalation study of EDB. Male and female Fischer 344 rats were exposed to 0-, 3-, 10-, or 40-ppm concentrations of EDB 6 hours/day, 5 days/week for 13 weeks. Groups of animals were sacrificed after 1, 6, and 13 weeks of exposure as well as after a recovery period of 88 to 89 days. Male rats exposed to 40-ppm concentrations of EDB experienced decreased body weight gain and increased liver and kidney weight. There were no changes in clinical

chemistry parameters and only slight, transient alterations in some hematology and urinalysis indices in female rats. The most sensitive index of EDB inhalation exposure was a morphologic change of the nasal respiratory epithelium, which was observed as early as 1 week in the 10-ppm-dosed animals. The changes progressed in the 40-ppm group from scattered to diffuse hyperplasia at 1 week to diffuse or focal nonkeratinizing squamous metaplasia and hyperplasia with focal cell necrosis at 13 weeks. There was a reversion to normal within the 88- to 89-day recovery period in both the 10- and 40-ppm groups.

Chronic Effects

The chronic toxicity of EDB has been assessed in conjunction with two carcinogenicity studies. NCI (1978b) reported the results of a cancer bioassay in which male and female Osborne-Mendel rats and $B6C3F_1$ mice were given EDB in corn oil by gavage 5 times weekly. Although the study was originally scheduled to last approximately 2 years, it was terminated early due to excessive death rates. The doses were reported as time-weighted averages (TWAs), since both dosage levels and dosage schedules were changed during the course of the investigations. The TWA for the low-and high-dose male and female rats was approximately 40 mg/kg. The TWA for the low-dose male and female mice was 62 mg/kg; for the high-dose mice, it was 107 mg/kg. The low- and high-dose mice and rats had decreased body weight gain and decreased survival, relative to controls. Approximately 40% of the high-dose male and female rats died in week 15, ostensibly due to "acute toxic reactions." Eleven of the 18 high-dose males dying at the time exhibited acanthosis and hyperkeratosis of the forestomach. Pathological changes observed in other organs of low-and high-dose male and female rats included degenerative changes in the liver and adrenal cortex. Both male mice and rats exhibited testicular atrophy. Unfortunately, the excessive mortality and alterations in the dosage regimen preclude identifying a NOEL or a LOEL in the NCI (1978b) study.

In the second chronic-effects study, Wong et al. (1982) exposed male and female Sprague-Dawley rats to 20-ppm concentrations of EDB vapor 7 hours/day, 5 days/week for 18 months. Body weights of five EDB-exposed rats were slightly lower than those of the controls throughout the study period, but the decreases were not always sufficient to be statistically significant. Increased mortality in both males and females inhaling EDB was manifest at 15 and 18 months. Hematological studies performed on the moribund animals yielded normal results. No mention was made of histopathological evidence of toxicity, other than the finding of a higher tumor incidence in EDB-exposed rats than in controls. Apparently, the increased tumor incidence was responsible for the increased mortality.

EDB has been found to interact synergistically with disulfiram (Antabuse) to produce greatly increased toxicity (NIOSH, 1978b). In laboratory rats exposed simultaneously to both EDB by inhalation (20 ppm) and disulfiram in the diet (0.05% by weight in the feed), there was exceptionally high mortality after approximately 13 months of exposure when compared to animals exposed to either EDB or disulfiram alone. Furthermore, in a chronic carcinogenicity study (NIOSH, 1981) of rats exposed to both substances, EDB by inhalation and disulfiram in the feed, there was an approximately 10-fold increase in the incidence of hepatocellular carcinomas compared to the rate in animals exposed to either substance alone.

Mutagenicity

Moriya et al. (1983) monitored the dose response of EDB-induced mutation in the *Salmonella typhimurium* bacterial reverse mutation assay by using several tester strains as well as *Escherichia coli* WP2 her. They found that EDB is mutagenic at a very high dose and that the mutation rate is not enhanced by the addition of rat liver microsomal extract. Unfortunately, the results were expressed as the number of revertants per plate, not revertants per survivors, so toxicity was not assessed. The authors concluded that EDB can be a direct-acting mutagen in bacteria. That conclusion is consistent with an earlier, less detailed study by Buselmaier et al. (1972), which was reviewed in Volume 3 of *Drinking Water and Health* (NRC, 1980, p. 99). Ohta et al. (1984) demonstrated that EDB induces the SOS (error-prone DNA repair) response in *E. coli* tester strains and that this response does not require microsomal activation. The authors concluded from those data that EDB behaves as a direct-acting mutagen with respect to the error-prone DNA repair response.

In contrast, van Bladeren et al. (1980) found that the addition of the 100,000 x g microsomal supernatant fraction considerably enhanced the mutagenicity of EDB in the *S. typhimurium* TA100 strain. The authors concluded that the primary glutathione adduct is responsible for the mutagenic effect.

When treated with gaseous EDB, *Drosophila* develop sex-linked lethal mutations (Kale and Baum, 1983) that appear at acute and chronic exposure levels as low as 2.3 ppm/hr. The authors concluded that EDB can be mutagenic in a eucaryote and that such a mutation may be a general property of the EDB interaction.

Although some bacterial assay systems suggest that EDB can be a direct-acting mutagen, EDB may be activated chemically or enzymatically in eucaryotic cells. DiRenzo et al. (1982) showed that in an *in vitro* assay EDB is activated by hepatic microsomal extracts to form a species that can bind covalently to DNA. Similarly, van Bladeren et al. (1981) showed

that EDB may be conjugated with glutathione in microsomes to form thiiranium ions, which might then bind covalently to DNA or other target macromolecules.

Carcinogenicity

The NCI bioassay (1978b) of EDB showed that, when administered intragastrically, EDB produces squamous cell carcinomas of the forestomach in Osborne-Mendel rats and, to a lesser extent, in $B6C3F_1$ mice. For this experiment, EDB doses began at 80 and 40 mg/kg/day for rats, but after 16 weeks the higher dose level was discontinued for 14 weeks due to toxicity. At week 30, the high-dose group was placed on a 40-mg/kg/day regimen. Administration of EDB to the lower-dose group was continued without interruption. For mice, the original dose levels were 120 and 60 mg/kg/day. After 13 weeks, however, a 2 week trial of 200 and 100 mg/kg/day was begun. At week 15, the original dose levels were given, but after 42 weeks all mice were changed to one level of 60 mg/kg/day. Doses were ultimately reported as time-weighted averages of 0, 37, or 39 mg/kg/day for female rats; 0, 38, or 41 mg/kg/day for male rats; and 0, 62, or 107 mg/kg/day for male and female mice.

The tumor rates for male rats were 33/50 and 45/50, whereas the rates for female rats were 29/50 and 40/50 for the high-and low-dose groups, respectively. Tumors invaded the forestomach locally, eventually metastasizing throughout the abdominal cavity. The authors believed that because so many animals died early due to the extensive toxicity of the compound, the incidence of tumor development was reduced. No forestomach tumors were observed in control groups. An increased incidence of liver cancer and hepatic nodules was also observed in rats, especially among the high-dose females. Hemangiosarcoma was observed among male rats.

In mice, squamous cell adenocarcinomas of the forestomach occurred in 29 of the 49 high-dose males, 28 of the 50 high-dose females, 45 of the 50 low-dose males, and 46 of the 49 low-dose females. None was found in control groups. Lung cancers were observed in 10 of the 47 high-dose males, 6 of the 46 high-dose females, 4 of the 45 low-dose males, and 10 of the 43 low-dose females. No lung tumors were seen in control mice.

In another carcinogenesis bioassay (NTP, 1982b), Fischer 344 rats and $B6C3F_1$ mice were chronically exposed to 10 or 40 ppm EDB by inhalation for 6 hours/day, 5 days/week. Increased mortality was observed for the high-dose rats and for mice at both doses. Male rats developed tumors of the nasal cavity with the following incidences: 20 adenocarcinomas and 3 squamous cell carcinomas in the 50 low-dose animals, and 28 adenocarcinomas, 21 carcinomas, and 3 squamous cell carcinomas in the 50 high-dose animals. In the nasal cavity of female rats, there were 20 ad

enocarcinomas and 1 squamous cell carcinoma in the 50-animal low-dose group, and 29 adenocarcinomas, 25 carcinomas, and 5 squamous cell carcinomas in the 50-animal high-dose group. One female control rat developed a squamous cell carcinoma of the nasal cavity. Hemangiosarcomas were also associated with EDB exposure, occurring in 1 of 50 low-dose males, 15 of 50 high-dose males, and 5 of 50 high-dose females. The incidence of testicular mesotheliomas among males and mammary fibroadenomas among females was also increased by EDB exposure.

In mice exposed to EDB by inhalation, only 6 of 50 high-dose females exhibited an incidence of nasal cavity carcinoma. However, alveolar/bronchiolar carcinomas were found in 3 of 48 low-dose males, 5 of 49 low-dose females, 19 of 46 high-dose males, and 37 of 50 high-dose females. One control female developed this type of tumor. Occurrences of hemangiosarcomas, fibrosarcomas, and malignant mammary neoplasms were also associated with EDB exposure.

Van Duuren et al. (1979) showed that EDB applied three times weekly to the dorsal skin of male and female Swiss mice at doses of 25 or 50 mg per application yielded significant incidences of skin papillomas, skin carcinomas, and lung tumors. In the 30-animal low-dose group, 2 had skin papillomas, 2 had skin carcinomas, and 24 had benign papillomas of the lung. In the 30-animal high-dose group, 8 had skin papillomas, 3 had skin carcinomas, and 26 had lung papillomas. These investigators also found that a single 75-mg dermal application of EDB followed by repeated applications of the promoter phorbol myristate acetate did not cause tumors.

Wong et al. (1982) conducted an inhalation study of EDB exposure with Sprague-Dawley rats. (This study was described above in the chronic effects section.) As compared to controls, there were significant ($p < 0.05$) incidences of spleen hemangiosarcomas (10 of 46 males, 6 of 48 females), adrenal tumors (11 of 46 males, 6 of 48 females), subcutaneous mesenchymal tumors (11 of 46 males), and mammary tumors (25 of 48 females).

A carcinogenic risk estimate was not attempted for EDB for several reasons. The results of the carcinogenicity study in which EDB was administered by gavage to rats and shown to cause squamous cell carcinomas of the forestomach are quantitatively equivocal. Following the extensive toxicity caused by the higher dose of EDB early in the experiment, treatment was discontinued for 14 weeks and then reinstituted at a lower dose. The total dose is thus expressed as a time-weighted average and as such is not representative of a situation in which humans are chronically exposed to a low dose of the chemical. When expressed as time-weighted averages, the doses given to the low-and high-dose groups are virtually identical.

The inversion in tumor yield can be accounted for only by early toxicity and does not represent a reliable dose response. The inhalation study also had some of the same shortcomings as the gavage study, such as increased early mortality. In addition, inhalation was considered less relevant to the estimation of risk for drinking water than the oral route.

Reproductive Effects

Early studies in hens demonstrated that EDB causes decreased egg size, impaired uptake of labeled proteins by ovarian follicles, egg infertility, and cessation of egg laying (Alumot and Harduf, 1971; Alumot et al., 1968; Bondi et al., 1955). Amir et al. (1977) reported that oral administration of 10 EDB doses of 4 mg/kg bw produced a large but transient decrease of sperm count and a large but transient increase in the number of abnormal sperm in bulls. More recently, in the NTP (1982b) inhalation bioassay described in the carcinogenicity section, testicular degeneration was found in 10 of 50 low-dose and 18 of 49 high-dose rats, and testicular atrophy was observed in 2 of 50 low-dose and 5 of 49 high-dose rats. (EDB was administered at doses of 10 or 40 ppm for 6 hours/day, 5 days/week.) Among the 50 control rats, testicular degeneration occurred in one animal and atrophy was observed in another. Testicular changes were not reported for EDB-exposed mice (NTP, 1982b).

In the NCI carcinogenesis bioassay of EDB by gavage, early development of testicular atrophy was observed in dosed rats and high-dose mice (NCI, 1978b). Administration of three doses of EDB by inhalation (Short et al., 1979) to both male and female rats for 10 weeks resulted in adverse effects on reproduction in both sexes exposed to the highest dose (89 ppm for males and 80 ppm for females). Mortality and morbidity were observed as well, however, so it was not possible to attribute reproductive effects directly to EDB. When EDB was administered by inhalation (20 ppm) in the presence of dietary disulfiram (0.05%), Wong et al. (1982) found that 90% of Sprague-Dawley rats developed testicular atrophy. Edwards et al. (1970) found that intraperitoneal administration of five daily 10-mg/kg doses of EDB impaired spermatogenesis in Wistar rats. Motor reflexes and motor coordination were assessed in the F_1 progeny of male rats treated intraperitoneally for 5 days with EDB doses of 1.25, 2.5, 5.0, or 10.0 mg/kg. Although litter size remained unchanged, several indices of behavior were affected. Doses of EDB that produced these behavioral effects were much less than those (close to the LD_{50}) required to produce abnormal sperm morphology (Fanini et al., 1984).

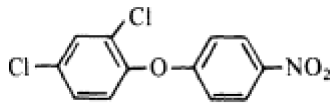
Together these data suggest that like haloalkanes, EDB appears to induce testicular damage at low doses in experimental animals. Although evidence suggests that EDB may also induce sperm death and sperm abnormalities, those data are incomplete.

Conclusions and Recommendations

Ethylene dibromide is mutagenic, carcinogenic, and toxic to the reproductive system of laboratory animals. In some bacterial *in vitro* assay systems, it appears to be a direct-acting mutagen. Nonetheless, it can be activated by hepatic microsomal extracts to form glutathione adducts and bind with DNA. EDB produced squamous cell carcinomas of the forestomach, which metastasized throughout the abdominal cavity in rats and mice. In addition, lung tumors were observed in mice gavaged with EDB. Inhalation studies with EDB produced nasal cavity tumors and tumors at various other sites. A carcinogenicity risk estimate was not calculated for EDB because of the lack of reliable dose-response data from the single oral exposure study available.

NITROFEN

2,4-Dichloro-1 -(4-nitrophenoxy)benzene
CAS No. 1836-75-5
RTECS NO. KN8400000



Nitrofen is a selective contact herbicide used on a variety of food crops to control annual grasses and weeds both before and after the crops begin to grow. The Rohm and Haas Company obtained the first registration for vegetable crop use in 1966, and the first European authorizations followed the next year. This compound is a member of the chlorophenoxy class of herbicides and is also called nitrophene, TOK, TOK E-25, and Nip (Burke Hurt et al., 1983).

Before 1981, nitrofen was registered in the United States for the control of annual bluegrass, crabgrass, goosefoot, lamb's-quarters, malva, nightshade, nettle, pigweed, purslane, shepherd's purse, and spergularia on broccoli, Brussels sprouts, cabbage, cauliflower, carrots, celery, garlic, horseradish, onions, parsley, sugar beets, carnations, chrysanthemums, roses, stocks, ground covers, and noncropped lands. Its principal use has been as a herbicide to control malva or cheeseweed in California cole crops and purslane in the onion fields of New York and the upper Midwest (Burke Hurt et al., 1983). Food tolerance levels of nitrofen ranging from 0.02 to 0.75 ppm have been established for 24 commodities, including vegetables, milk, rice, poultry, and meats (EPA, 1984a). The estimated

annual production was between 227,000 and 454,000 kg in 1974 (Kimbrough et al., 1974).

Nitrofen is used to control weeds in peas, beans, and spring wheat and other cereal grains in Northern Europe and for a variety of vegetables in the Mediterranean regions. In China, Japan, and the Far East, it is used principally on rice. Effective applications are generally between 2 and 6 kg/ha applied as a thin layer to the soil surface or to actively growing weeds at the 2- to 4-leaf stage (Burke Hurt et al., 1983).

Technical-grade nitrofen contains approximately 95% active ingredient and impurities consisting of approximately 3% *p*-chloronitrobenzene, 1% dichlorophenol, and 1% unknowns. Woolson et al. (1972) specifically determined that technical-grade nitrofen lacked 2,3,7,8-tetrachlorodibenzo*p*-dioxin contaminants. The technical-grade material is a dark brown solid with a slight aroma, a melting point of 64°C to 71°C, and a vapor pressure of 8×10^{-6} mm mercury at 40°C to 4×10^{-4} mm mercury at 70°C. It is relatively insoluble in water (0.7 to 1.2 mg/liter at 22°C) but readily soluble in most organic solvents (Ambrose et al., 1971). The odor threshold in water was determined to be 0.58 to 1.38 mg/liter, and was found to be highly stable (Shardina, 1972). Nitrofen was detected at levels of less than 0.2 mg/m^3 in samples of natural running water around areas of commercial use in Belgium (Deleu and Copin, 1979).

Metabolism

Nitrofen spread on soil surfaces is adsorbed into weeds as they emerge. The compound is activated by sunlight and kills weeds by inhibiting photosynthesis. Activation for herbicidal activity appears to involve, but not be exclusively related to, cleavage at the ether linkage to form dichlorophenols and nitrophenol (Hawton and Stobbe, 1971). The biochemical mechanism of herbicidal activity is based upon interference with both oxidative and photosynthetic phosphorylation in mitochondria and chloroplasts of plants. Nitrofen and other diphenyl ether herbicides have been shown to inhibit chloroplast noncyclic electron transport by removing or inactivating an electron carrier associated with photosystem II of photosynthesis, or the Hill reaction. Mitochondrial respiration appears to be disrupted at several sites along the electron transport chain (Moreland et al., 1970).

Health Aspects

Observations in Humans

No data are available.

Observations in Other Species

Acute Effects

The acute toxicity of nitrofen in laboratory animals has been summarized by Burke Hurt et al. (1983), who reported oral LD_{50}s from intragastric administration ranging from 2.4 to 3.6 g/kg in rats and mice. Lower values of 740 mg/kg for rats and 450 mg/kg for mice were cited by Ivanova (1967). The oral LD_{50} by the dietary route has been reported to range from 410 to 3,580 mg/kg bw in rats (Ambrose et al., 1971; Kimbrough et al., 1974; Shardina, 1972; Wiswesser, 1976) and from 780 to 1,620 mg/kg in rabbits (Shardina, 1972; Wiswesser, 1976). Burke Hurt et al. (1983) reported the dermal LD_{50}s for rats and rabbits to be 5 and 3.27 g/kg, respectively, whereas Ivanova (1967) found the percutaneous LD_{50} for rabbits to be greater than 5 g/kg. A 1-hour inhalation exposure of rats to 271 mg/m^3 decreased body weight gain but did not cause signs of toxicity. On the basis of inhalation studies with cats, Ivanova (1967) recommended that the maximum permissible concentration of nitrofen in the air of occupational settings should not exceed 1 mg/m^3. Clinical manifestations of poisoning with acute exposure are characterized by decreased activity, a rise in the respiratory rate and disturbances in respiratory rhythm, and hyperthermia, proceeding to tremors and convulsions. Death from acute exposure generally occurs within 2 to 8 days (Burke Hurt et al., 1983; Ivanova, 1967).

Subchronic Effects

The subchronic toxicity of nitrofen in laboratory animals has been summarized by Burke Hurt et al. (1983). Wistar rats were fed diets containing 0-, 100-, 500-, 2,500-, 12,500-, or 50,000-ppm concentrations of nitrofen in a 13-week study by Ambrose et al. (1971). In the 50,000-ppm group, none of the rats survived the first week, and survival and body weight gain were affected in the 12,500- and 2,500-ppm groups. At 500 ppm (25 mg/kg) and lower, there were no treatment effects on growth, food consumption, or mortality. Dose-related increases in relative liver weights were observed at 100 ppm (5 mg/kg) and higher. In another study with dietary doses ranging from 0 to 2,500 ppm for 13 weeks, dose-related increases in liver, testes, and kidney weights occurred at 500 ppm (25 mg/kg) and higher (O'Hara et al., 1983). Elevated liver weights as well as induction of cytochrome P450 activity levels have been consistent, early signs of low-dose exposure of rodents (Burke Hurt et al., 1983).

Chronic Effects

The only chronic effect studies found were limited to carcinogenicity.

Carcinogenicity

A bioassay of technical-grade nitrofen for possible carcinogenicity was conducted using Osborne-Mendel rats and B6C3F$_1$ mice (NCI, 1978c). Nitrofen was administered in the feed at two different concentrations to groups of 50 male and 50 female animals of each species. Two of the 50 female rats fed 1,300 ppm developed a metastatic and locally invasive ductal carcinoma of the pancreas as did 7 of the 50 rats fed 2,600 ppm (which was statistically significant). This rare tumor was not observed in the simultaneous or pooled control groups from more than 200 bioassays (Milman et al., 1978). Poor survival precluded the evaluation of carcinogenicity in male rats (NCI, 1978c).

In mice of both sexes, the incidence of hepatocellular carcinoma was statistically elevated at both treatment levels. This lesion occurred in 36 of 49 (73%) males in the 2,348-ppm group and in 46 of 48 (96%) males in the 4,696-ppm group, compared with 4 of 20 (20%) in simultaneous controls and 9 of 72 (12%) in pooled controls. In females, hepatocellular carcinomas occurred in 36 of 41 (88%) low-dose and 43 of 44 (98%) high-dose animals, whereas no such carcinomas were observed in simultaneous (19) or pooled (80) controls. There was a significant positive association between dose and elevated incidence of hemangiosarcomas for male and female mice compared with pooled controls.

The results of this study (NCI, 1978c) in Osborne-Mendel rats and B6C3F$_1$ mice indicate that orally administered technical-grade nitrofen is carcinogenic in the livers of mice of both sexes and in the pancreas of female rats.

Another bioassay to assess the carcinogenicity of nitrofen was conducted using Fischer 344 rats and B6C3F$_1$ mice (NCI, 1979). The same design was used as in the previous study, except the high and low dietary concentrations of nitrofen were 6,000 and 3,000 ppm for both species. A dose-related depression in body weight gain was observed in rats of both sexes, but the exposures had no effect on survival. Furthermore, there were no positive associations between treatment and tumor incidences in rats of either sex.

In mice, dose-related decreases in body weight gain were observed for both sexes, but treatment had no effect on survival. Although a variety of tumors was noted, only the incidence of liver tumors was related to nitrofen exposure. Hepatocellular adenomas were seen in 1 of 20 (5%) control males, in 18 of 49 (37%) low-dose males, and in 20 of 48 (42%) high-dose males; hepatocellular carcinomas were found in none of the 20 controls, in 13 of 49 (26%) low-dose males, and in 20 of 48 (42%) high-dose animals. The tumor incidence in male mice from this study is summarized in Table 9-19.

Of the females, none of the 18 controls, 9 of 48 (19%) low-dose mice, and 17 of 50 (34%) high-dose mice had hepatocellular adenomas; hepa

tocellular carcinomas were observed in none of the 18 controls, in 5 of 48 (10%) low-dose females, and in 13 of 50 (26%) high-dose females. The incidences of hepatocellular adenomas and carcinomas in male and female mice were statistically elevated for the low-dose and high-dose groups (NCI, 1979).

TABLE 9-19 Tumor Incidence in Male Mice Fed Nitrofen in the Diet[a]

Animal	Sex	Tumor Site	Dose, ppm (mg/kg/day)	Tumor Rates
$B6C3F_1$ mouse	Male	Liver	0	1/20
			3,000 (132)	31/49
			6,000 (264)	40/48

[a] Based on data from NCI, 1979.

Groups of 6-month-old beagle dogs, two of each sex per group, received diets containing 0-, 20-, 200-, or 2,000-ppm concentrations of nitrofen for 2 years. In the 2,000-ppm group, the liver-to-body-weight ratio was significantly increased at termination. Otherwise, there were no observed effects on survival, general health, body weight gain, food consumption, hematologic values, or urine tests conducted at 3-month intervals throughout the study. Furthermore, no histopathological lesions were found in any treatment group. The no-observed-effect level (NOEL) was 200 ppm (5 mg/kg/day) (Ambrose et al., 1971).

On the basis of results from the two NCI studies (1978c, 1979) and the absence of data on humans, IARC (1983, pp. 271-282) concluded that there was sufficient evidence to classify technical-grade nitrofen as a carcinogen in experimental animals.

Carcinogenic Risk Estimate

In the NCI (1979) study, dose-related increases in the incidence of liver tumors were seen in mice of both sexes, but to a greater extent in males (Table 9-19). These tumor incidence rates were then used to make statistical estimates of both the lifetime risk and an upper 95% confidence limit for lifetime risk for adults weighing 70 kg (see Table 9-20). The risk estimates are expressed as a probability of developing cancer after a lifetime daily consumption of 1 liter of water containing nitrofen at a concentration of 1 µg/liter and are based on the generalized multistage model for carcinogenesis described in Chapter 8. No risk estimates were calculated for children. If children weighed 10 kg and consumed 1 liter of water per day, their risks would be higher than those of adults because of their higher ratio of water consumption to body weight.

TABLE 9-20 Carcinogenic Risk for Nitrofen[a] Estimated with the Generalized Multistage Model

Animal	Sex	Estimated Human Lifetime Risk[b]	Upper 95% Confidence Estimate of Lifetime Cancer Risk[b]
$B6C3F_1$ mouse	Male	4.4×10^{-5}	5.6×10^{-5}

[a] Based on data from NCI, 1979.
[b] Assuming daily consumption of 1 liter of water containing the compound in a concentration of 1 μg/liter.

Developmental Effects

The teratogenic properties of nitrofen have been examined in an extensive series of studies published over the last 10 years. Those conducted up to 1983 have been reviewed by Burke Hurt et al. (1983). The majority were undertaken with the intent of examining the underlying mechanism of teratogenic activity. Insofar as the exposure levels used in these studies were usually high, they are not well suited for use in risk estimation. These investigative studies are briefly summarized here to identify those processes in development most susceptible to disruption by nitrofen. The studies conducted at low, environmentally relevant exposure levels are then examined in detail for application in the risk-assessment process.

In a three-generation reproductive toxicity study, Ambrose et al. (1971) first found that nitrofen caused neonatal mortality at dietary levels of 100 ppm and higher. Kimbrough et al. (1974) administered the compound to rats on days 7 through 15 of gestation at 10, 20, or 50 mg/kg/day by gavage and observed that survival of offspring to weaning was reduced at 20 mg/kg and that all animals died at 50 mg/kg/day. Affected pups developed signs of respiratory distress immediately after birth, became cyanotic, and died within an hour. Examination of fetal lungs by electron microscopy revealed an abnormal appearance of Type II cells. Incomplete expansion of alveoli, fibrosis, and immaturity of the alveolar epithelium were observed in treated fetal and neonatal lungs.

Stone and Manson (1981) tested whether prenatal exposure to nitrofen accelerated the catabolism of glucocorticoids via induction of cytochrome P450 levels, leading to a depression in glucocorticoid levels and a delay in fetal lung surfactant synthesis. They found that nitrofen exposure of Sprague-Dawley rats to 20 to 50 mg/kg/day orally on days 8 to 18 of gestation did not induce maternal hepatic mixed-function oxidase activity, unlike findings at identical exposure levels in male and nonpregnant female rats. Cortisone and corticosterone levels in maternal and fetal plasma were unaffected. In addition, fetal lung surfactant synthesis was not altered, as indicated by measurements of total lung phospholipids, ^{14}C-labeled choline

uptake in fetal lung lipids, choline phosphotransferase activity, and surface tension values in saline extracts of fetal lungs. They concluded that nitrofen has an inhibitory effect on lung growth but that it does not specifically influence lung surfactant synthesis.

Costlow and Manson (1981) found that day 11 of gestation was the most sensitive time for nitrofen-induced neonatal mortality in Long-Evans hooded rats. A range of oral exposures from 75 to 250 mg/kg was given on day 11, and the neonatal LD_{50} was found to be 116 mg/kg. Studies of maternal toxicity, embryo lethality, and teratogenicity were carried out by exposing the dams to neonatal LD_{15} to LD_{99} doses (exposures of 70 to 400 mg/kg) on day 11. No significant treatment effects on maternal weight gain, live fetuses per litter, or major malformations of the fetal skeleton were observed at term throughout the dose range. Fetal body weight was depressed in all treatment groups. Soft tissue examination revealed dose-related incidences of diaphragmatic hernias, hydronephrosis, and heart anomalies.

Kang et al. (1985) compared the response of different strains of rats—Long-Evans hooded, Sprague-Dawley, and virus-antibody negative (VAN) Sprague-Dawley rats—exposed orally to nitrofen at 0, 6.25, 12.50, or 25 mg/kg/day on days 6 to 15 of gestation. Abnormalities of the lung (hypoplasia), kidneys (hydronephrosis), diaphragm (hernia), and heart (aortic arch anomalies, ventricular septal defects, transpositions of great vessels) were observed in fetuses at term. The mean percentage of malformed fetuses per litter was nearly identical across dose levels for each strain of rat. There were, however, substantial differences in the pattern of malformations identified within each strain. The Sprague-Dawley and VAN Sprague-Dawley rats responded similarly for all malformations but had significantly higher incidences of diaphragm and lung anomalies than did the Long-Evans hooded rats. Conversely, the Long-Evans hooded rats had significantly elevated levels of kidney anomalies compared with Sprague-Dawley and VAN Sprague-Dawley rats. The frequency of heart malformations was generally low across strains at the dose levels used; significantly elevated incidences occurred at exposure to 25 mg/kg/day and above. These results suggest that although potency (total percentage of fetuses malformed) of nitrofen is similar in different strains of rats, there are marked strain differences in the embryonic target organs affected.

A series of studies have indicated that nitrofen alters thyroid function in adult mice and, after prenatal exposure, in offspring. L. E. Gray, Jr., et al. (1982) found that maternal exposure of mice to nitrofen at 6.25 to 200 mg/kg/day on days 7 to 17 of gestation resulted in altered development of the reproductive system and the Harderian glands of offspring—effects that were attributed to alterations in the hypothalamic-pituitary-thyroid axis during the perinatal period. Gray and Kavlock (1983) determined that

serum thyroxine (T_4) levels were significantly reduced after intraperitoneal exposure of nonpregnant mice to nitrofen, whereas triiodothyronine (T_3) levels were unaffected. Manson et al. (1984) examined the influence of nitrofen exposure on pituitary-thyroid function in nonpregnant, pregnant, and fetal rats and attempted to relate alterations in thyroid hormone status to induction of birth defects. In adult thyroparathyroidectomized (TPTX) female rats, nitrofen exposure at 15 and 30 mg/kg/day for 2 weeks resulted in significant suppression of thyroid-stimulating hormone (TSH) levels. When a single dose of 250 mg/kg was administered to euthyroid rats, a depression was observed in the release of TSH after a thyrotropin-releasing hormone (TRH) challenge. Pregnant euthyroid rats given a single dose of 250 mg/kg on day 11 had significantly depressed TSH and T_4 levels, and fetal T_4 levels were markedly depressed at term. Coadministration of T_4 and nitrofen to TPTX pregnant rats resulted in a 70% reduction in the frequency of malformed fetuses compared with nitrofen exposure alone. Heart malformations, in particular, were prevented by coadministration of nitrofen with T_4. Competitive displacement studies in radioimmunoassays for T_4 and T_3 indicated that a nitrofen metabolite (4-aminophenyl 2,5-dichloro-4-hydroxyphenyl ether) competed with ^{125}I-labeled T_3 for antibody binding, whereas the parent compound and six isolated metabolites failed to compete with ^{125}I-labeled T_4 or T_3 for antibody binding. These results were interpreted to indicate that the teratogenicity of nitrofen is mediated, at least in part, by alterations in maternal or fetal thyroid hormone status, and that it may be due to a premature and pharmacologic exposure of the embryo to a nitrofen-derived, T_3-active metabolite.

The studies described thus far involved oral exposure of rodents during pregnancy and examination of the fetuses at term for malformations. The lowest dose found to cause a significant elevation in major malformations in term fetuses was 6.25 mg/kg/day given on days 6 to 15 of pregnancy (Kang et al., 1985). Investigators at the Environmental Protection Agency's Health Effects Research Laboratories have conducted an extensive series of studies on the postnatal sequelae of prenatal exposure to nitrofen. L. E. Gray, Jr., et al. (1982) administered 100 mg/kg/day by gavage to mice on days 7 to 17 of gestation. Dams delivered spontaneously, and offspring were monitored up to 100 days of age. The age of eye opening was significantly delayed in the offspring of treated mice. The height of the palpebral fissures and the weight of the Harderian glands were significantly depressed at 100 days of age. The absence or reduction of the Harderian glands were only apparent after day 14 of neonatal life, at which time they undergo rapid growth. In the rat, growth of the Harderian glands after eye opening is correlated with an increase in thyroxine secretion.

A more extensive examination of the Harderian gland effect was carried out by L. E. Gray, Jr., et al. (1982). These investigators monitored

postnatal development in CD1 mice following oral exposure to doses of 0, 6.25, 12.5, 25, 50, 100, 150, and 200 mg/kg/day on days 7 to 17 of gestation. Major malformations and neonatal deaths occurred at 150 and 200 mg/kg/day; growth rates were substantially retarded at 12.5, 25, 50, and 100 mg/kg/day. The Harderian glands were reduced or absent in 97% of offspring dosed at 100 mg/kg/day, in 65% at 50 mg/kg/day, and in 4% at 25 mg/kg/day. Gland weights were reduced at all dosages, including the 6.25-mg/kg/day level. Lung and liver weights were also significantly depressed at 110 days of age in offspring exposed prenatally to 6.25 mg/ kg/day.

Kavlock and J. A. Gray (1983) examined the effects of prenatal nitrofen exposure on morphometric, biochemical, and physiological aspects of adult renal function. Sprague-Dawley rats were given oral doses of 0, 4.17, 12.5, and 25 mg/kg/day on days 8 to 16 of pregnancy. Nitrofen reduced neonatal survival, primarily because of diaphragmatic hernias, at 12.5 and 25 mg/kg/day. Decreases in body weight, kidney weight, renal protein content, and glomerular number were also observed at these exposure levels. Total urine production was suppressed by 12.5 and 25 mg/ kg/day. Pups in the 4.17- and 12.5-mg/kg/day groups failed to concentrate their urine to the same extent as control pups, as evidenced by significantly lower hydropenic urinary osmolalities. The results at 4.17 mg/kg/day were questionable, however, given the range of variability in control animals.

There have been additional studies exploring biochemical end points of organ differentiation (Kavlock et al., 1982), morphological and functional defects in the rat kidney and Harderian gland (Kavlock and L. E. Gray, Jr., 1983), and postnatal lung function in the rat (Raub et al., 1983). In these studies, however, the postnatal function deficits were obtained with prenatal exposure levels higher than 6.25 mg/kg/day.

Developmental Risk Estimate

In a study by Weatherholtz et al. (1979), dermal exposure was found to be more effective than oral exposure in reducing neonatal survival. Sprague-Dawley rats were treated percutaneously with 0-, 0.3-, 3.0-, or 30-mg/kg/day doses of nitrofen (formulated as the emulsifiable concentrate of TOK E-25 containing 25% active ingredient, xylene, and surfactants) on days 6 through 15 of pregnancy. Similarly treated groups of pregnant females were allowed to deliver their litters naturally, and pups were observed for 4 days postpartum. Major malformations of the diaphragm, accompanied by displacement of lungs and heart and ectopic testes, were observed in the 30-mg/kg/day treatment groups. Neonatal survival to day 4 was significantly reduced in litters treated prenatally with 3 or 30 mg/kg/day. The 0.3-mg/kg/day dose was the NOEL.

Costlow et al. (1983) conducted a teratology study to demonstrate dose-response relationships and to establish a NOEL with dermal exposure. Nitrofen was administered dermally to Sprague-Dawley rats on days 6 through 15 of gestation. The compound was prepared as an aqueous dilution of the emulsifiable concentrate and was administered at 0- (water), 0- (solvent), 0.3-, 0.6-, 1.2-, and 12.0-mg/kg/day doses of active ingredient. The dams were allowed to deliver their litters naturally, and the offspring were observed for up to 149 days postnatally. Necropsies were performed on dead or moribund animals. Randomly selected offspring were necropsied on postnatal day 42 and the remaining offspring on days 146 to 149.

At 12 mg/kg/day, diaphragmatic hernias were found in 95% of the pups found dead at birth, in 17% of the offspring sacrificed on day 42, and in 21% of those sacrificed on days 146 to 149. Survivors in this group also had increased incidences of missing or reduced Harderian glands and slight to severe dilation of the kidneys. In addition, the weights of the thyroid gland, liver, lung, and gonads were decreased in animals surviving prenatal maternal exposure to 12 mg/kg/day. Slight to moderate dilation of the kidneys was observed in all dose groups, including the 0.3-mg/kg/day group, and the severity and incidence increased with dose at day 42 and at days 146 to 149.

A NOEL was not demonstrated experimentally but was calculated using the kidney dilation data. The proportion of fetuses within a litter with any degree of renal pelvic dilation was determined for each sex. These data were transformed using an arcsin transformation and plotted against log dose using regression analysis. A weighting factor was used to control for litter size, and the best fit of the model arcsin $(Y) = B_0 + B_1$ log (dose) was used to predict the NOEL, where Y was the proportion of affected pups in a litter and B_0 and B_1 were constants. For male offspring, the NOEL was estimated to be 0.28 mg/kg/day for exposure on days 6 to 15 of gestation with a 95% confidence interval of 0.12 to 0.34. For female offspring, the NOEL was calculated to be 0.17 mg/kg/day with a confidence interval of 0.05 to 0.27 (Costlow et al., 1983).

Unger et al. (1983) administered 0, 0.15, 0.46, 1.39, 4.17, 12.50, and 25 mg/kg/day orally to Sprague-Dawley rats from day 7 of pregnancy through day 15 of lactation to identify dose-response relationships based on pup survival and the incidence of diaphragmatic hernias. Any cyanotic, moribund, or dead pups were placed in fixative and examined for internal malformations by the Wilson technique. On day 29 postpartum, one male and one female per surviving litter were randomly selected, sacrificed, and examined for gross internal abnormalities.

At the dose levels used in this study, nitrofen exerted no adverse maternal effects other than nonsignificant reduction in weight gain during

pregnancy and significantly decreased body weights of lactating dams on days 5 and 8 post partum at the two highest dose levels. The percentage of viable implantation sites, the number of live pups per litter, birth weights, and pup survival were decreased in animals receiving the two highest dose levels. Organ weights of pups selected randomly at weaning were lowered at dose levels ≤ 4.17 mg/kg/day, the major effect occurring in weights of the Harderian gland. Hydronephrosis was sporadically observed in offspring from treated groups and did not appear to be dose related. The incidence of diaphragmatic hernias was significantly different from controls at doses of 1.39 mg/kg/day and higher. The mean litter frequencies of diaphragmatic hernias in pups that died or were sacrificed are given in Table 9-21.

TABLE 9-21 Mean Litter Frequencies in Diaphragmatic Hernias in Rats Gavaged with Nitrofen[a]

Dose	Affected Pups		Affected Litters		
(mg/kg/day)	N	%	N	%	Mean Litter Frequency
0	0/31	0	0/19	0	0
0.15	2/34	5.88[b]	1/17	5.88	2.9
0.46	0/21	0	0/14	0	0
1.39	4/32	12.50[b]	2/16	12.50[b]	10.9
4.17	5/37	13.51[b]	4/30	20.00[b]	17.5
12.50	19/35	54.29[b]	11/14	78.56[b]	51.2[b]
25.00	32/42	76.19[b]	6/6	100b	71.0[b]

[a] Based on data from Unger et al., 1983.
[b] Significantly different ($p \leq 0.05$) from concurrent control.

This study is limited in its usefulness in identifying a NOEL or a LOEL because only dead or dying pups and two offspring per litter were examined for malformations. This would most likely lead to an underestimation of the true malformation rate insofar as examination was weighted toward identification of lethal malformations. Many of the anomalies associated with prenatal exposure to nitrofen are not lethal. Hydronephrosis, isolated ventricular septal defects, aortic arch anomalies, and even small diaphragmatic hernias, especially if they are right-sided, have been observed in surviving offspring after prenatal exposure to nitrofen. The decision to perform visceral examinations on dead and dying pups alone probably increased the detection of lethal malformations, e.g., diaphragmatic hernias, and the decision to examine just two offspring from surviving litters probably led to an underreporting of nonlethal malformations. Consequently, the 0.15-mg/kg/day dose should be selected as the LOEL, despite the fact that some outcomes at this level were not found to be significantly

elevated when different methods of data presentation and analysis were used.

In addition, Unger et al. (1983) did not summarize the mean frequency of malformed fetuses among the litters; rather, the mean percentage of fetuses with individual anomalies (diaphragmatic hernias) was presented. Consequently, the impact of malformations at sites other than the diaphragm, i.e., the kidneys, lungs, and heart, was not evaluated. It is not possible to reconstruct the mean litter frequency of malformed fetuses from these data because an individual fetus can be represented more than once when data are presented on a malformation basis rather than on a fetus basis. This lends further weight to using 0.15 mg/kg/day as the LOEL insofar as there appeared to be elevations in sites other than the diaphragm at this dose.

The Unger et al. (1983) study appears to be the most appropriate for use in cross-species extrapolation, since the authors used oral doses, which are most relevant to drinking water contamination. Two different approaches were considered by the committee in making the cross-species extrapolation. In the first, a LOEL of 0.15 mg/kg/day was identified in the Unger et al. (1983) study. The committee felt that a conservative safety factor of 1,000 was appropriate because a LOEL rather than a NOEL was used and also because nitrofen induces a spectrum of major malformations, some of which are lethal to the newborn while others persist and cause irreversible impairment of the offspring. In addition, these malformations occur at nonmaternally toxic exposure levels (see Chapter 2). Assuming that a 70-kg human consumes 2 liters of water daily and that 20% of the intake of nitrofen is derived from water, one may calculate the suggested acceptable daily intake (ADI), as

$$\frac{0.15 \text{ mg/kg bw/day} \times 70 \text{ kg} \times 0.2}{2 \text{ liters/day} \times 1{,}000} = \begin{array}{l}0.0011 \text{ mg/liter,} \\ \text{or } 1.1 \text{ } \mu\text{g/liter.}\end{array}$$

In the second, for purposes of comparison, the committee decided to fit the affected litter data shown in Table 9-21 using the probit, logit, and Weibull dose-response models.

The computations relating to developmental risk, i.e., one or more pups found defective in a litter, were derived from a computer program prepared by John Kovar and Daniel Krewski of Health and Welfare Canada in 1981. The estimates are all based on the assumption that the response is additive in dose. The 95% lower confidence limit doses were developed by computing the confidence limits based on the variance of the reciprocal of the dose. The results of the curve-fitting are given in Table 9-22.

In previous volumes of *Drinking Water and Health*, the risk estimates were averaged to yield one composite number. If the data for all models

in Table 9-22 are averaged, the upper 95% confidence estimate of lifetime developmental risk is 7.1 x 10^{-6} and an average dose of 2.7 x 10^{-3} mg/kg/day which is the upper 95% confidence estimate for a lifetime development risk of 1 x 10^{-6}. Assuming that a 70-kg human consumes 2 liters of water daily and that 20% of the intake of nitrofen is derived from water, one may calculate the suggested ADI from this approach as

TABLE 9-22 Developmental Risk Estimates for Nitrofen Dose

	Dose to Produce a 1 x 10^{-6} Risk of Defective Offspring[a] (mg/kg/day)		Upper 95% Confidence Estimate of Lifetime
Model	MLE[b]	95% UCL[c]	Developmental Risk[d]
Probit	5.0 x 10^{-3}	2.9 x 10^{-3}	6.4 x 10^{-6}
Logit	5.4 x 10^{-3}	3.0 x 10^{-3}	6.1 x 10^{-6}
Weibull	4.0 x 10^{-3}	2.1 x 10^{-3}	8.8 x 10^{-6}

[a] Based on laboratory animals that produce litters with several pups as opposed to humans who usually produce a single offspring per birth.
[b] Maximum likelihood estimate.
[c] Upper confidence limit.
[d] Assuming daily consumption of 1 liter of water containing the compound in a concentration of 1 µg/liter.

$$\frac{2.7 \times 10^{-3}\ \text{mg/kg bw/day} \times 70\ \text{kg} \times 0.2}{2\ \text{liters}} = 0.19\ \text{mg/liter, or 19 µg/liter.}$$

This value then may be compared with the more conservative suggested ADI of 1.1 µg/liter obtained from the first approach. These two estimates of "safe" levels (assuming 1 x 10-6 lifetime risk provides a "safe" level) are provided to show the contrast in levels computed using two different reasonable estimation procedures. They are not proposed as a basis for setting appropriate levels for nitrofen in drinking water. Because nitrofen has been shown to be a carcinogen, this effect needs to be taken into account. Some data to support computations which include consideration of carcinogenicity are given earlier in Table 9-20.

It is difficult to estimate human risks from laboratory animal data since the animals examined produce several offspring per litter in contrast to humans, who usually produce only a single offspring per birth. In addition, when litter data are used for extrapolation, the potential for intralitter interactions cannot be reflected in the results, even though it is not clear what biological significance such interactions would have in risk extrapolation to humans.

Reproductive Effects

In a three-generation reproduction study, male and female weanling rats were given diets containing 0-, 10-, 100-, or 1,000-ppm dietary concentrations of technical-grade nitrofen. After 11 weeks on this regimen, 20 rats of each sex were mated to produce the successive generation. There were no adverse effects on fertility or gestation indices and no apparent effects on viability or lactation indices up to the 100-ppm (10-mg/kg/day) exposures. The viability index for rats on the 1,000-ppm (100-mg/kg/day) diet was significantly depressed, and no pups survived beyond the F_0 generation in this group. Likewise, viability was markedly depressed in the 100-ppm group in the progeny of the F_0 generation. The NOEL was 10 ppm (1 mg/kg/day) (Ambrose et al., 1971).

In a two-generation reproduction study, Sherman rats were fed dietary levels of technical-grade nitrofen at 0, 20, 100, and 500 ppm, which furnished doses of 1.1 to 1.8 mg/kg/day, 5.2 to 9.2 mg/kg/day, and 26 to 46 mg/kg/day, respectively. Pair-mating was started when the rats had been on the diets for 68 and 200 days to produce the F_{1a} and F_{1b} litters. Offspring were observed through weaning. The survival of offspring was not affected at the 0- and 20-ppm dietary levels. At 100 ppm the survival of offspring to weaning was reduced, and at the 500-ppm level no offspring survived the neonatal period in two breedings of the first generation (Kimbrough et al., 1974).

Technical-grade nitrofen was fed to four groups of 25 male Sprague-Dawley rats for 13 weeks at dietary concentrations of 0, 100, 500, or 2,500 ppm. Untreated female rats were mated with treated males, and fertility, gestation, and lactation indices were monitored. Testes, kidneys, and liver weights in the offspring were increased at 500 and 2,500 ppm (50 and 250 mg/kg/day), but histological changes—hypertrophy and cytoplasmic basophilia of centrilobular hepatocytes—were restricted to the liver at levels of 500 and 2,500 ppm. There were no effects on fertility, gestation, litter size, weight, or sex ratio in any group. Offspring health and survival to day 35 were unaffected (O'Hara et al., 1983).

Results from these reproductive toxicity studies indicate that exposure of female, but not male, rats to dietary levels of 100 ppm and higher for more than 12 weeks results in decreased neonatal survival. This would be equivalent to approximately 10 mg/kg/day, based on average food consumption values for the rat.

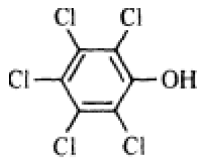
Conclusions and Recommendations

Nitrofen is teratogenic and carcinogenic in laboratory animals. Risk estimates were calculated for both the developmental and carcinogenic effects. Two different approaches were used by the committee in estimating developmental risks for humans exposed to nitrofen. In the first,

a NOEL approach with a conservative safety factor was used to estimate an acceptable daily intake. In the second, the probit, logit, and Weibull low-dose extrapolation models were used to calculate the developmental risk estimates. All three models gave very similar results, varying from 6.1×10^{-6} to 8.8×10^{-6}. The estimated lifetime risk and upper 95% confidence estimate of lifetime risk of cancer in humans are based on the generalized multistage model. The higher risk is due to cancer; however, the short duration of exposure in developmental studies, compared with chronic carcinogenicity studies, indicates that the most sensitive effect may be the developmental one.

PENTACHLOROPHENOL

CAS No. 87-86-85
RTECS No. SM6300000



Pentachlorophenol (PCP) was first reviewed in Volume 1 of *Drinking Water and Health* (NRC, 1977, pp. 750-753). The following material, which became available after the 1977 report was prepared, updates and in some instances reevaluates the information contained in the previous review. Also included are references that were not evaluated in the earlier report.

Pentachlorophenol is also known by the trade names Penta, Santophen 20, Dowicide 7 and G, Chlorophen, Penchloro, Sinituho, Weedone, Santobrite, EP 30, Liropren, Lauxtol, Fungifen, Durotox, Thompson's Wood Fix, Term-l-Trol, Permite, Penta-Kil, Pentasol, Penwar, Perotox, Permacide, Permagard, Permatox, and Chem-tol.

PCP's molecular weight is 266.3. It is a solid at room temperature, it has low volatility, and it is stable in aqueous solution with a solubility in water at 20 ppm at 30°C. In surface waters, PCP generally exists as the anion; some microbial and photodegradation may occur (EPA, 1984b). PCP can be expected to sorb to acidic sediment and leaf litter in surface waters (Hiatt et al., 1960). The odor threshold for PCP in humans is 0.857 mg/ml at 30°C (Hoak, 1957).

Pentachlorophenol and its salts (mainly the sodium salt) have been in commercial use since the 1930s and are the second most widely used pesticides in the country. Total PCP production in 1980 was 46,826,000

lbs (21,240 metric tons) (U.S. International Trade Commission, 1981, p. 231); imports in 1983 were 274,730 lbs (125 metric tons) (U.S. International Trade Commission, 1984, p. 98). PCP is also a substantial component of tetrachlorophenol (Schwetz et al., 1974).

Commercial PCP is most commonly produced by the direct chlorination of phenol. It is typically composed of 88.4% pentachlorophenol, 4.4% tetrachlorophenol, less than 0.1% trichlorophenol, and 6.2% higher chlorinated phenoxyphenols (Schwetz et al., 1974). The nonphenolic components fall into two chemical classes: polychlorinated dibenzo*p*-dioxins and polychlorinated dibenzofurans. One commercial product tested contained the following chlorinated dioxins: 2,3,7,8-tetrachlorodibenzo*p*-dioxin, less than 0.05 ppm; hexachlorodibenzo-*p*-dioxin, 4 ppm; heptachlorodibenzo-*p*-dioxin, 125 ppm; and octachlorodibenzo-*p*-dioxin, 2,500 ppm (Schwetz et al., 1974).

The typical dibenzofuran content of commercial pentachlorophenol is as follows: hexachlorodibenzofuran, 30 ppm; heptachlorodibenzofuran, 80 ppm; and octachlorodibenzofuran, 80 ppm (Schwetz et al., 1974). Hexachlorobenzene is also found at levels of 400 ppm in commercial pentachlorophenol (IARC, 1979; Schwetz et al., 1978).

More than 80% of the PCP produced in this country is used as a wood preservative. The compound may be used alone or in combination with other agents, such as the chlorophenols, 2,4-dinitrophenol, sodium fluoride, the dichromate salts, sodium arsenate, or arsenious oxide (EPA, 1984c). It is also used as a herbicide and defoliant. Approximately 12% of the PCP produced is used in making sodium pentachlorophenol—a wood preservative, fungicide, herbicide, and slimicide (IARC, 1979).

PCP has been detected in a variety of wildlife and in fish at levels ranging from 0.35 to 26 mg/kg (IARC, 1979; Renberg, 1974). In the State of Michigan, herds of dairy cattle became contaminated with PCP that was used to treat the walls and feed bins of their barns. The PCP levels in 18 of these cows ranged from 58 to 1,136 µg/kg (Anonymous, 1977; IARC, 1979).

Widespread human exposure to PCP occurs as a result of its extensive production, persistence in the environment, and varied applications. Most exposure arises from its use as a wood preservative; however, there is also extensive exposure from use in homes and gardens. There are many reports of occurrence in the food chain, general environment, and workplace as well as in the body fluids of the general population and exposed workers. And there are a number of reports of toxicity in people exposed chiefly in the home. The National Institute for Occupational Safety and Health noted that most workers exposed to PCP are employed in the gas and electric service industries and that hospital workers may also be exposed (NIOSH, 1977b).

The following paragraphs provide some examples of environmental concentrations.

Air PCP concentrations from 0.25 to 7.8 ng/m^3 have been detected in the air of two towns (Cautreels et al., 1977; IARC, 1979). In a contaminated home, the air contained PCP concentrations ranging from 0.14 µg/m^3 in a bedroom to 1.2 µg/m^3 in the attic (Sangster et al., 1982). In a wood preservative factory and wood treatment plant, air levels were 0.013 mg/m^3 (average) and 5.1 to 15,275 ng/m^3, respectively (IARC, 1979).

Water PCP has been measured in effluent streams (0.1 to 10 mg/liter) (IARC, 1979); in river water (1.23 µg/liter) (Wegman and Hofstee, 1979); and in surface ponds and drainage water near a wood treatment facility (1 to 800 µg/liter) (Wong and Crosby, 1981). Concentrations of 98 ppt have been detected in Dade County, Florida, municipal drinking water (Morgade et al., 1980). Levels up to 24 ppm have been detected in wells near a sawmill in Hayfork, California (Litwin et al., 1983).

Sediment In Europe, measured concentrations in sediment have been 20-fold higher than those found in surface water (EPA, 1984b; Wegman and van den Broek, 1983).

Soil Levels from 0.46 to 69.1 mg/kg have been observed in greenhouse soil treated with PCP (IARC, 1979).

Food In the United States, the estimated average daily intake of PCP from food in 1974 was 0.76 µg (IARC, 1979).

Animals Levels ranging from 0.35 to 23 mg/kg in fish and from 58 to 1,136 µg/kg in cattle have been found in animals contaminated with PCP used to treat wood (IARC, 1979).

Humans In nonoccupationally exposed dialysis patients, PCP levels in blood plasma were 15.7 to 15.8 µg/liter, compared with 15.0 µg/liter in controls (IARC, 1979). In the general, nonoccupationally exposed population, PCP has also been detected in seminal fluid (20 to 70 µg/kg), fingernails (IARC, 1979), blood, and urine (Dougherty, 1978). In a recent survey, PCP was found in 85% of the urine samples collected. The mean level was 6.3 µg/liter (Kutz et al., 1978). Adipose tissues from necropsy samples taken from residents of Dade County, Florida, contained PCP concentrations ranging from 10 to 80 ppb (Morgade et al., 1980).

PCP was detected in workplace air at 25 factories in which the compound was used to treat wood. The average level was 0.013 mg/m^3 (maximum

range, 0.004 to 1.0 mg/m^3). The level in urine of the workers exposed to these airborne concentrations ranged from 0.12 to 9.68 mg/liter (Arsenault, 1976; IARC, 1979).

Worker exposure to PCP at one wood treatment plant over a 5-month period resulted in serum and urine levels of 348.4 to 3,963 µg/liter and 41.3 to 760 µg/liter, respectively (IARC, 1979; Wyllie et al., 1975). Plasma levels of 0.02 to 2.4 µg/ml have been reported in PCP producers and applicators (Zober et al., 1981). Persons exposed to PCP in contaminated homes had plasma levels of 25 to 660 µg/liter. An average of 128.6 ± 134 µg/liter (mean ± SD) was seen in controls (Sangster et al., 1982).

PCP is registered in the United States as a wood preservative and for agricultural use in seed treatment. In 1978, however, the Environmental Protection Agency (EPA) issued a rebuttable presumption against registration for pesticide products containing PCP and in July 1984 limited their sale and use, also setting restrictions on dioxin levels in PCP-containing products (EPA, 1978). The American Conference of Governmental Industrial Hygienists (ACGIH) has established a threshold limit value of 0.5 mg/m^3 and a short-term exposure level (STEL) of 1.5 mg/ m^3 for dermal exposure (EPA, 1984c). The Occupational Safety and Health Administration's 8-hour time-weighted average is 0.5 mg/m^3 (IARC, 1979).

In 1980, EPA recommended alternative ambient water quality criteria of 1.01 mg/liter based on PCP's toxicity and 0.030 mg/liter based on the organoleptic properties of PCP. EPA has recently derived 1-day, 10-day, and lifetime health advisories for PCP in drinking water, all of which exceed 1 mg/ liter for adults and are less than 1 mg/liter for children. The agency recognized that the odor threshold for PCP is considerably lower (0.857 mg/ml at 30°C) (EPA, 1984b).

Metabolism

Rapid absorption of PCP has been reported in rodents, monkeys, and humans following oral, dermal, or inhalation exposure. In healthy volunteers ingesting a single dose of 0.1 mg/kg bw, the average half-life for absorption was 1.3 ± 0.4 hours. Peak plasma concentrations occurred after 4 hours (Braun et al., 1978).

The major tissue deposits vary somewhat between species. In humans whose deaths were not related to PCP exposure, the liver (containing PCP residues of 0.067 µg/g), kidney, brain, spleen, and fat (0.013 µg/g) appeared to be major deposition sites (Grimm et al., 1981). In the mouse, the gall bladder is a principal storage site. In the rat, it is the kidney.

The metabolism of PCP is generally similar in mammalian species. In rodents, more than 40% is excreted in urine unchanged. The remainder is excreted as tetrachlorohydroquinone and glucuronide conjugates of PCP.

In limited studies of humans, PCP, tetrachlorohydroquinone, and PCP-glucuronide have been found in urine (Ahlborg et al., 1974; Braun et al., 1977, 1978). *In vivo* retention of PCP by lipid-containing tissues may be attributable to conjugation with fatty acids (Leighty and Fentiman, 1982).

In all species tested, including humans, PCP is excreted principally in the urine; smaller amounts are found in feces and expired air. Excretion in rodents and humans is apparently biphasic (Bevenue et al., 1967). The excretion kinetics of PCP vary appreciably, depending on whether the exposure is acute or chronic. In humans, urinary excretion half-lives following chronic exposure are significantly longer than after single high-dose exposure (20 days versus 10 hours) (Casarett et al., 1969).

Blood and urine PCP concentrations were monitored in 18 workers (Begley et al., 1977) 1 day before taking vacation, on 4 different days during their vacations, and about 50 days after they had returned to work. During the vacation period, PCP levels in blood and urine fell to < 50% of the prevacation values. From these data, the estimated elimination half-life of PCP was calculated to be 19 to 20 days (EPA, 1984b).

In vitro studies have suggested the following possible mechanisms of toxicity (which are not necessarily mutually exclusive):

Uncoupling of oxidative phosphorylation: Weinbach (1954), Arrhenius et al. (1977), and Götz et al. (1980) showed that PCP uncouples mitochondrial phosphorylation in rat hepatocytes, indicating that hepatotoxic effects may be due to an interference with the energy metabolism of the cell. Uncoupling may result from the binding of PCP with mitochondrial protein (Weinbach and Garbus, 1965) or with fatty acids in the mitochondrial membrane (Leighty and Fentiman, 1982).

Disturbance of microsomal detoxification: PCP induces microsomal enzymes (Vizethum and Goerz, 1979). However, *in vitro* studies of rat liver microsomes have shown that PCP inhibits microsomal detoxification enzymes by disturbing electron transport from flavins to cytochromes. This suggests that PCP may synergistically increase the toxicity of many substances, such as polynuclear aromatic compounds (Arrhenius et al., 1977).

Perturbation of lipid membranes by PCP: Packham et al. (1981) have shown a correlation with phospholipid perturbation and toxicity (LD_{50}) for a series of compounds.

PCP's interaction with a number of other chemicals: The toxicity in *Pseudomonas fluorescens* was greater when PCP and 2,3,4,5-tetrachlorophenol were given sequentially than when PCP alone was given (Trevors et al., 1981). The antioxidant butylated hydroxyanisole (BHA) enhances the toxicity of PCP to *P. fluorescens* (Trevors et al., 1981). Hexachlorobenzene (HCB) at 1,000 ppm and 99% pure PCP at 500 ppm administered to female Wistar rats for up to 8 weeks resulted in an increased

accumulation of PCP in the liver. PCP also accelerated the onset of hepatic porphyria by HCB (Debets et al., 1980). Pretreatment with PCP inhibits the carcinogenic effect of hydroxamine acids and the hepatoxicity of *N*-hydroxy-2-acetylaminofluorene (Meerman and Mulder, 1981).

Health Aspects

Observations in Humans

There are numerous reports of toxic effects and deaths due to occupational and accidental exposures to PCP and sodium PCP (EPA, 1984b; IARC, 1979; Mercier, 1976). For example, factory workers exposed to PCP at a Winnipeg plant experienced sweating, weight loss, and gastrointestinal disorders (Bergner et al., 1965) as did herbicide sprayers in Australia (one person exposed for an extended period suffered hepatic enlargement) (Gordon, 1956). Plant workers in the Federal Republic of Germany reported skin eruptions and bronchitis following "acute" exposure (Baader and Bauer, 1951).

Other responses to extended occupational exposure to PCP that may be related to contaminants include persistent chloracne and disorders of the liver and nervous system (IARC, 1979). In addition, German workers (applicators and producers) had a significant rise in immunoglobulins, compared with controls, following chronic exposure (Zober et al., 1981).

Hematological effects, including *atteinte médullaire* (bone marrow damage), have been observed in workers exposed to products containing 5% PCP and 1% lindane in a petroleum solvent (Catilina, 1981; Catilina et al., 1981). Average serum and urine levels of PCP were higher (about 30% and 50%, respectively) in six exposed workers than in four controls, but no significant differences were seen in the incidence of chromosome aberrations (Wyllie et al., 1975).

Repeated dermal exposure to sodium pentachlorophenate may result in dermatitis, systemic intoxication, and, in a limited number of people, an allergic response (EPA, 1984b.)

Cases of acute nonfatal poisoning have followed the home or hospital use of products containing PCP. These include a 3-year-old child who, after exposure to PCP in a contaminated water supply, experienced delirium, fever, and convulsions (Chapman and Robson, 1965). Twenty neonates who were exposed to PCP used in laundering diapers and bed linen subsequently developed tachycardia, respiratory distress, and liver changes (Robson et al., 1969). In another study, people were found to have dermatitis after exposure to PCP-treated lumber in their houses (Sangster et al., 1982).

Fatal poisonings include a 58-year-old male employed for 1 week as a wood dipper. His death was most probably due to cutaneous absorption

and inhalation of PCP. Autopsy revealed degeneration of the liver and kidney (Bergner et al., 1965). Five fatal cases were reported among herbicide sprayers in Australia (Gordon, 1956); nine deaths occurred in sawmill workers in Asia (Menon, 1958). Two of the 20 neonates mentioned above died, and an autopsy indicated that they had degeneration of the kidney and effects on the liver (Robson et al., 1969).

Roberts (1963) has reported a fatal case of aplastic anemia in a 21-year-old male who handled treated lumber. More recently, he cited three additional cases of fatal aplastic anemia following exposure to PCP (Roberts, 1981). One of these cases also had Hodgkin's disease. Roberts (1981) also reported several cases of soft-tissue sarcoma, Hodgkin's disease, non-Hodgkin's lymphoma, and other malignant diseases after exposure to PCP, related compounds, and precursors.

A series of studies of chronically exposed workers has been conducted in Hawaii. The first involved workers in wood treatment plants and farmers or pest-control operators. Elevation of serum enzyme levels, i.e., serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and lactic dehydrogenase (LDH), and low-grade infections or inflammations of the skin, eye, and upper respiratory tract were found in the exposed groups (Klemmer et al., 1980). In a second study, plasma protein levels were found to be elevated in exposed, as compared with unexposed, workers (Takahashi et al., 1976). Begley et al. (1977) demonstrated an effect of PCP on the renal functions of 18 workers and an improvement following vacation. No long-term epidemiological studies of carcinogenicity were found by the committee.

Observations in Other Species

Acute Effects

At least 40 LD_{50} and LD_{Lo} studies have been performed, many of them with PCP of undefined purity. Therefore, it is difficult to determine the extent to which effects are attributable to PCP or to contaminants.

Acute exposure of laboratory animals to PCP results in vomiting, hyperpyrexia, elevated blood pressure and respiration rate, and tachycardia. Oral LD_{50}s ranging from 27 to 150 mg/kg bw have been reported, but no apparent differences in susceptibility have been observed between species at the lower level (Deichmann et al., 1942; Dow Chemical Company, 1969; Schwetz et al., 1978). No interspecies differences were reported for the higher level.

Subchronic/Subacute Effects

A significant number of subchronic/subacute studies have been conducted in rats. Both technical-and analytical-grade PCP were used, allowing attribution of some but not all observed

toxic effects to contaminants in technical PCP. In a study to determine the subchronic toxicity of the compound, PCP was fed in the diet to groups of Wistar rats at concentrations of 0, 25, 50, and 200 ppm for a 90-day period (Knudsen et al., 1974). Female rats receiving PCP at 200 ppm (10 mg/kg/day) had reduced growth rate. Liver weight in the female rats fed 50 and 200 ppm (2.5 and 10 mg/kg) daily was significantly higher. After 6 weeks, male rats fed 50- and 200-ppm concentrations of PCP had elevated hemoglobin and hematocrit values, whereas at 11 weeks hemoglobin and erythrocytes were significantly reduced in the same groups of animals. No PCP-related effects were observed in animals fed 25 ppm (1.25 mg/kg/day) (Knudsen et al., 1974; NRC, 1977).

In another experiment, male rats received technical or pure PCP in doses of 50 mg/kg/day for a 90-day period (Kimbrough and Linder, 1978). Both PCP formulations caused an increase in liver weight. More severe histopathological changes occurred in the livers of rats given the technical PCP than in those given the pure PCP (NRC, 1977).

In a 90-day study, increased liver and kidney weights, elevated serum alkaline phosphatase, and depressed serum albumin levels were observed in Sprague-Dawley rats that consumed technical-grade PCP at 3, 10, and 30 mg/kg/day (Johnson et al., 1973). When a sample of PCP containing substantially reduced amounts of dioxins was fed to rats, no adverse effects were found at 3 mg/kg/day. In animals receiving pure PCP, kidney and liver weights were elevated at 10 and at 30 mg/kg/day, but no adverse toxicological effect was found in animals receiving 3 mg/kg/day (NRC, 1977). Greichus et al. (1979) observed an increase in liver weights of pigs fed purified PCP in doses of 10 and 14 mg/kg bw daily for 30 days.

Subcutaneous administration of sodium pentachlorophenate (purity unknown) to three groups of six rabbits each for 60 days (at doses of 13.8, 27.5, or 68.8 mg/kg bw) caused a variety of effects, including secondary anemia, leukopenia, and numerous lesions in the brain and spinal cord. Doses were one-twentieth, one-tenth, or one-fourth the minimum lethal dose (275 mg/kg) (McGavack et al., 1941).

In vivo subchronic exposures of Wistar rats exposed to technical-grade PCP at 30 mg/kg bw resulted in alterations of mitochondria and nuclei of hepatocytes (Fleischer et al., 1980).

Chronic Effects

In a chronic study, alterations in liver morphology were observed at all doses in rats ingesting from 20- to 500-ppm concentrations of technical PCP over an 8-month period (Kimbrough and Linder, 1978). By comparison with pure PCP, more severe histopathological changes occurred in livers of rats given technical-grade PCP. With pure PCP, effects were not observed at the lowest dose.

Goldstein et al. (1977) administered both pure and technical-grade PCP to the diet of female Sherman rats for more than 8 months. Hepatic porphyria occurred at 100 and 500 ppm, whereas increased hepatic aryl hydrocarbon hydroxylase (AHH) and glucuronyl transferase (GT) activities were observed at 20-ppm concentrations of technical-grade PCP. By contrast, pure PCP at 500 ppm had an effect only on GT activity and body weight. Thus, the porphyria and other major liver changes induced by technical PCP are apparently due to contaminants, probably the chlorinated dibenzo-*p*-dioxins rather than the PCP.

In addition, Schwetz et al. (1978) observed decreased body weight (females only), increased urine-specific gravity (females only), and increased SGPT activity (both sexes) in 27 male and 27 female Sprague-Dawley rats fed 30-mg/kg bw doses of purified PCP for 2 years. Pigmentation of liver and kidneys was observed in females receiving 10 or 30 mg/kg bw daily and in males receiving 30 mg/kg bw each day. No-effect levels for females were 3 mg/kg/day; for males, 10 mg/kg/day.

In a 160-day study, cattle fed 20-mg/kg doses of technical PCP for 42 days, followed by 15 mg/kg/day for the remainder of the study, had decreased weight gain, progressive anemia, and immune effects. Only minimal adverse effects were observed after exposure to analytical-grade PCP (McConnell et al., 1980).

Immunotoxicity

Kerkvliet et al. (1982a,b) observed adverse effects on the immune system of B6 male mice given technical-grade and pure PCP at doses of 50 or 500 ppm of the diet for 10 to 12 weeks. The technical-grade PCP at 50 and 500 ppm increased both the incidence of transplanted tumors and susceptibility to virus-induced tumors. It also reduced T-cell cytolytic activity and caused a dose-dependent delay and suppression of peak splenic antibody production and serum antibody titers. The only effect of pure PCP at 500 ppm was to enhance development of splenic tumors (which were actually virus-induced tumor cells) following challenges with MSV/MB virus. By contrast to the enhancement of tumor susceptibility in PCP-exposed mice, PCP exposure did not significantly alter the susceptibility of animals to infectious virus-induced mortality.

Kerkvliet et al. (1982a) also observed immunotoxicity of technical-grade PCP in female Swiss-Webster mice. Animals were given diets containing technical-grade PCP for 8 weeks at 0, 50, 250, and 500 ppm (0, 7.5, 37.5, and 75 mg/kg). Immunosuppression was seen at the lowest dose. These effects were not found after administration of pure PCP.

Significant effects have also been found in the immune system of Sprague-Dawley rats (Exon and Koller, 1983b) and cattle (McConnell et al., 1980). Exon and Koller (1983b) fed pentachlorophenol (97%) in feed to female Sprague-Dawley rats from weaning until 3 weeks post partu

rition. Offspring from each treatment regimen were continued on PCP treatments until 13 weeks of age. Delayed hypersensitivity, suppression of humoral immunity, and increased phagocytosis by macrophages were observed at all dose levels (5, 50, and 500 ppm, i.e., 0.5, 5, and 50 mg/ kg) in both pre- and postnatally exposed animals.

Neurotoxicity

McGavack et al. (1941) observed effects on the central nervous system in rabbits after 60 days of exposure to subcutaneous doses of 5%, 10%, and 25% of the minimum lethal dose (275 mg/kg bw). Nervous system lesions were seen in all dose groups. Neurochemical effects were observed in 30 male Wistar rats given 20-mg/liter concentrations of technical-grade PCP in drinking water for 3 to 14 weeks. Thirty controls were also studied. Animals were sacrificed at 3 to 18 weeks after the experiment began. The main effects seen in the rat brain were transient biochemical effects (e.g., altered acid proteinase activity, superoxide dismutase, and glial glutathione content) (Savolainen and Pekari, 1979).

Mutagenicity

There are only limited data from *in vivo* genetic testing of PCP. In *Drosophila melanogaster*, there was no increase in sex-linked recessive lethal mutations (Fahrig et al., 1978; Vogel and Chandler, 1974). In the mouse spot test, there was evidence of definite but weak mutagenic activity (Fahrig et al., 1978).

In vitro testing produced negative results in numerous bacterial mutation assays with the exception of a positive *Bacillus subtilis rec* assay (Waters et al., 1982). In addition, positive results were observed in a yeast assay for forward mutation (*Saccharomyces cerevisiae*) (Fahrig et al., 1978). Chromosome abnormalities were seen in *Vicia fabia* seedlings (Amer and Ali, 1969). No increases in chromosome aberrations were observed in workers with elevated serum and urine levels of PCP (Wyllie et al., 1975). Interpretation of many of these studies is somewhat handicapped by inadequate reporting of dosages, ranges of background mutation, and other factors (Williams, 1982).

Carcinogenicity

PCP (technical-grade and commercial-grade) is currently under test by the National Toxicology Program (NTP, 1985b). No statistically significant differences between experimental animals and controls were observed in bioassays for cancer in which commercial-grade PCP was given to mice (18 per group, about 20 months exposure by gavage) (BRL, 1968a, p. 393); in Sprague-Dawley rats given dietary doses of commercial-grade PCP (27 per group, 22 to 24 months) (Schwetz et al., 1978); or in Wistar rats given both purified and technical-grade PCP by subcutaneous injection for 40 weeks (Catilina et al., 1981). In another study, however, the incidence of hepatomas in one of two strains of mice [(C57BL/6 x C3H/

Anf)F_1] tested was significantly increased following subcutaneous injection of commercial-grade PCP (IARC, 1979; BRL, 1968a). Commercial-grade PCP was not found to be a promoter of skin carcinogenesis in mice initiated with dimethylbenzanthracene (DMBA) (Boutwell and Bosch, 1959). As mentioned earlier, PCP enhances the transplacental carcinogenicity of ethylnitrosourea (Exon and Koller, 1982, 1983a).

The carcinogenicity studies conducted to date do not provide a basis for complete evaluation because of limitations, including inadequate duration, lack of concurrent testing in two species, and small numbers of animals (Williams, 1982).

Developmental Effects

Purified and commercial PCP administered orally to rats at daily doses of 5 to 50 mg/kg bw at various intervals during days 6 through 15 of pregnancy resulted in dose-related toxicity in the embryo and developing fetus. Early organogenesis (days 8 to 11) was the most vulnerable stage. Unlike other effects of PCP, these effects were somewhat greater with purified PCP than with the commercial product. At 5 mg/kg/ day administered on days 6 through 15 of gestation, commercial PCP had no adverse effects, but pure PCP caused a statistically significant increase in the incidence of delayed ossification of the skull. No other effects on embryonal and fetal development were noted (Schwetz et al., 1974).

A single 60-mg/kg bw oral dose of purified PCP was given to pregnant Charles River CD strain rats on days 8, 9, 10, 11, 12, and 13 of gestation. Treatment on days 9 and 10 had the greatest effect on fetotoxicity (Larsen et al., 1975). Mice given 25-mg/kg bw doses of commercial PCP in dimethyl sulfoxide by subcutaneous injection on days 6 through 14 of gestation (BRL, 1968b, p. 150) developed no teratogenic effects.

Pregnant Syrian golden hamsters given daily oral doses of PCP (unspecified purity) ranging from 1.25 to 20 mg/kg from days 5 to 10 of gestation experienced an increase in fetal deaths and resorptions. The no-effect level was 2.5 mg/kg/day (Hinkle, 1973).

Daily dietary exposure of Sprague-Dawley rats to 0, 3, or 30 mg/kg bw for a 62-day period before mating and continuing through lactation resulted in significant decreases in neonatal body weight, percentage of live births, and survival of pups at a dose of 30 mg/kg/day (Schwetz et al., 1978). Exon and Koller (1982, 1983a,b) administered PCP (0, 5, 50, or 500 ppm) to Sprague-Dawley rats in the diet beginning with the rats' own weaning through the weaning of their pups. They observed significant effects on the immune system (as indicated by decreased antibody titers, decreased delayed hypersensitivity to oxazolone, and increased peritoneal macrophage numbers) and reduced ethylnitrosourea-induced transplacental carcinogenesis.

Conclusions and Recommendations

Inadequate characterization of PCP preparations used in toxicity experiments has led to uncertainties about the extent to which observed effects are attributable to PCP or to contaminants, including dioxins.

The metabolism of PCP is generally similar among mammalian species. PCP can be absorbed through skin, by inhalation, and by ingestion. Chronic exposures result in longer retention of PCP than do acute exposures. PCP interacts with other chemical substances that may increase or decrease its toxicity.

In humans, case reports indicate neurotoxicity, immune system effects, liver and kidney damage, and hematological disorders. There are also reports relating aplastic anemia and malignancy (Roberts, 1963) to PCP exposure. Epidemiological studies indicate alterations in enzyme systems and renal function and decreased resistance to disease associated with chronic exposure. No reports of epidemiological studies of possible carcinogenicity were found by the committee.

Studies in experimental animals have demonstrated effects from acute, subchronic, and chronic exposure. These include damage to liver, kidney, and central nervous system; hematological and immune system effects; and fetotoxicity. Pure PCP was more fetotoxic than technical-grade PCP.

The results of mutagenicity testing are mixed. PCP gave weak results in the mouse spot test, was positive in yeast and *Bacillus subtilis* , but was negative in several other short-term *in vitro* assays.

Two-year chronic bioassays in two species have not yet been completed. Therefore, carcinogenicity bioassays to date do not provide an adequate basis for evaluation. Thus, EPA based its recent risk assessment on the National Cancer Institute bioassay of certain PCP contaminants, the hexachlorodibenzo-*p*-dioxins (EPA, 1984b; NCI, 1980). A PCP chronic bioassay is currently being conducted by the National Toxicology Program (NTP, 1985b). PCP should be reevaluated when the results of that bioassay become available.

Developmental toxicity is an end point of considerable current concern. Animal and human studies suggest that the fetus and neonate are particularly susceptible. In the one-generation reproduction study by Schwetz et al. (1978), 3 mg/kg/day was administered in the diet 62 days prior to mating, during 15 days of mating, and subsequently throughout gestation and lactation. This may be regarded as a no-observed-effect level (NOEL), although there was a trend toward decreased neonatal weight at this dose. This is consistent with other studies shown in Table 9-23 showing NOELs ranging from 1 to 3 mg/kg/day.

A previous Safe Drinking Water Committee calculated an acceptable daily intake (ADI) of 0.003 mg/kg by using an uncertainty factor of 1,000

TABLE 9-23 Summary of No-Observed-Effect Level (NOEL) or Lowest-Observed-Effect Level (LOEL) for Pentachlorophenol

Test Animal	Duration of Exposure	Dosage Levels	NOEL or LOEL (mg/kg/day)	Effect Measured	Reference
Rat	2 months	0-30 mg/kg/day[a]	3	Trend toward reduced neonatal weight; no other toxic effect	Schwetz et al., 1978
Rat	12 weeks	0-200 ppm[a]	1.25	No toxic effect	Knudsen et al., 1974
Rat	90 days	0-30 mg/kg/day[b]	3	No toxic effect; limited increases in organ weight	Johnson et al., 1973
Rat	90 days	0-30 mg/kg/day[a]	3	Increased organ weights; serum enzyme changes	Johnson et al., 1973
Rat	Pre-and post-natal	0-500 ppm[b]	0.5	Immunosuppression	Exon and Koller, 1983b
Rat	3-14 weeks	20 mg/liter of drinking water[c]		Transient biochemical changes in brain	Savolainen and Pekari, 1979
Mice	8 weeks	0-500 ppm[c]	7.5	Immunosuppression	Kerkvliet et al., 1982b
Mice	8 weeks	0-1,000 ppm[b]	50	Increased liver:body wt. ratios; increased spleen wt.; no immunosuppression	Kerkvliet et al., 1982b
Mice	10-12 weeks	0-500 ppm[b]	7.5	Some enhanced development of virus-induced tumors	Kerkvliet et al., 1982a

Test Animal	Duration of Exposure	Dosage Levels	NOEL or LOEL (mg/kg/day)	Effect Measured	Reference
Mice	10-12 weeks	0-500 ppm[c]	7.5	Enhanced development of transplanted and virus-induced tumors; effects on the immune system	Kerkvliet et al., 1982a
Rat	Days 6-15 of gestation	0-50 mg/kg/day[b]	5	Delayed ossification of the skull	Schwetz et al., 1974
Rat	Days 6-15 of gestation	0-50 mg/kg/day[a]	5	No developmental effects	Schwetz et al., 1974
Hamster	Days 5-10 of gestation	1.25-20 mg/kg/day[d]	2.5	No developmental effects	Hinkle, 1973
Rat	8 months	0-500 ppm[b]	25	Alterations in liver morphology	Kimbrough and Linder, 1978
Rat	8 months	0-500 ppm[c]	1	Alterations in liver morphology	Kimbrough and Linder, 1978
Rat	8 months	0-500 ppm[b]	25	Increased glucuronyl transferase (GT) activity and decreased body weight	Goldstein et al., 1977
Rat	8 months	0-500 ppm[c]	1	GT induction and increased arylhydrocarbon hydroxylase (AHH)	Goldstein et al., 1977
Rat	2 years	0-30 mg/kg/day[a]	3	No toxic effect	Schwetz et al., 1978

[a] Commercial-grade PCP (usually higher purity than technical-grade PCP).
[b] Pure (analytical) PCP.
[c] Technical-grade PCP.
[d] PCP (grade unspecified).

(NRC, 1977). Using this as a basis, and assuming that a 70-kg human consumes 2 liters of water daily, which contributes 20% of total intake, the present committee estimated a chronic suggested no-adverse-effect level (SNARL) as:

$$\frac{3 \text{ mg/kg/day} \times 70 \text{ kg} \times 0.2}{1{,}000 \times 2 \text{ liters}} = \begin{array}{l}0.021 \text{ mg/liter,} \\ \text{or } 21 \text{ µg/liter.}\end{array}$$

This value is consistent with current NOELs for pure PCP.

A SNARL may also be estimated for a 10-kg child consuming I liter of water daily, which contributes 20% of total intake:

$$\frac{3 \text{ mg/kg/day} \times 10 \text{ kg} \times 0.2}{1{,}000 \times 1 \text{ liter}} = \begin{array}{l}0.006 \text{ mg/liter,} \\ \text{or } 6 \text{ µg/liter.}\end{array}$$

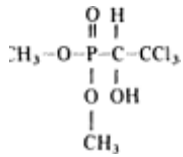
The committee noted that the toxicity of PCP is increased by impurities contained in the technical product. For example, the NOEL for pure PCP is 3 mg/kg/day (Johnson et al., 1973); however, the NOEL for technical PCP is 1 mg/kg/day (Goldstein et al., 1977; Kimbrough and Linder, 1978), indicating increased toxicity due to impurities. A SNARL for an adult may also be calculated for technical PCP as:

$$\frac{1 \text{ mg/kg/day} \times 70 \text{ kg} \times 0.2}{1{,}000 \times 2 \text{ liter}} = \begin{array}{l}0.007 \text{ mg/liter,} \\ \text{or } 7 \text{ µg/liter.}\end{array}$$

Likewise, a NOEL of 1.25 mg/kg/day for commercial PCP (Knudsen et al., 1974) could be used to calculate a SNARL of 9 µg/liter, which would fall in the range between the SNARLs for technical and pure PCP. The committee recommends the lower SNARLs for exposure to commercial-and technical-grade PCP. When the carcinogenicity data from the NTP bioassay become available, PCP should be reevaluated.

TRICHLORFON

Dimethyl (2,2,2-trichloro-1-hydroxyethyl)phosphonate
CAS No. 52-68-6
RTECS No. TA0700000



Trichlorfon is used both as a pesticide and as a chemotherapeutic agent for schistosomiasis. Trade names for the insecticides include Dylox,

Dipterex, and Clorfos. The pharmaceutical compound is usually called metrifonate, and commercial names include Bilarcil and Metriphonate.

In aqueous solutions, acid conditions promote the degradation of trichlorfon to dimethylphosphonic acid and trichloroethanol (IARC, 1983, pp. 207-231), whereas neutral-to-alkaline aqueous solutions result in a rearrangement of the compound to dimethyl(2,2-dichloroethenyl)phosphate (Dichlorvos) (Dedek et al., 1969; Metcalf et al., 1959). Ultraviolet light is also reported to degrade trichlorfon. The World Health Organization (WHO) reported that the half-life of trichlorfon sprayed on green plants is 1 to 2 days (WHO, 1972). The International Agency for Research on Cancer (IARC) reported that trichlorfon persists in soil for up to 2 weeks (IARC, 1983, pp. 207-231), while WHO cited a technical report stating that complete loss (below limits of detection) of a 10-ppm concentration of trichlorfon was achieved in 15 to 112 days, depending on soil type. Trichlorfon showed a "low" tendency to move to groundwater from soil (WHO, 1972).

Most data on human exposure to trichlorfon come from reports on use of the compound as an antischistosomal drug (Lebrun and Cerf, 1960). For this purpose, the compound is normally ingested in doses ranging from 5 to 12.5 mg/ kg (Holmstedt et al., 1978). Originally, trichlorfon was given on consecutive days, but current regimens require three doses to be given over 2 to 4 weeks (Jewsbury et al., 1977; Reddy et al., 1975). Humans have also been reported to have been exposed occupationally (van Bao et al., 1974) or in suicide attempts (Hierons and Johnson, 1978; Senanayake and Johnson, 1982).

Metabolism

After humans were exposed to a 7.5- to 10-mg/kg dose of trichlorfon, presumably by the oral route, peak plasma levels of the compound were detected in plasma in 1 hour (Nordgren et al., 1981). Comparable LD_{50}s for experimental animals given trichlorfon orally and parenterally (500 and 250 mg/ kg, respectively) led Holmstedt et al. (1978) to speculate that the rate of absorption is similar via these routes. By this reasoning, the high LD_{50} of dermally administered trichlorfon (greater than 2,800 mg/ kg in rats, according to Edson and Noakes, 1960) would suggest that the compound is slowly or inefficiently absorbed from the skin.

In rats intravenously injected with $^{14}CH_3$-labeled trichlorfon, Dedek and Lohs (1970) found radioactivity in liver, lung, kidney, heart, spleen, and blood. The inhibition of esterases in the spinal cord and brain of hens suggested that trichlorfon or an active metabolite also enters the central nervous system (Hierons and Johnson, 1978).

Arthur and Casida (1957) suggested that the primary fate of trichlorfon is hydrolysis of the phosphonate bond to form 2,2,2-trichloroethanol, which undergoes glucuronidation, and dimethyl phosphate. Another major reaction is demethylation of the parent compound, which is probably mediated by glutathione. As reviewed by Holmstedt et al. (1978), nonenzymatic rearrangement of trichlorfon to dichlorvos is only a minor pathway but one of great importance, because it is the activating pathway in terms of antiesterase activity (Reiner, 1981). Once dichlorvos has been formed, the dichlorovinyl moiety may be removed by esterases, resulting in a vinyl alcohol that tautomerizes to dichloroacetaldehyde. Demethylation of dichlorvos also occurs, and the demethylated phosphate is apparently unstable, fragmenting into several products including dichloroacetaldehyde (Dedek, 1981). Nordgren et al. (1981), on the basis of pharmacokinetic models of data from human ingestion, suggested that a majority of the plasma and erythrocyte trichlorfon is eliminated through the formation of dichlorvos. The authors stated that in this manner trichlorfon becomes a "slow release formulation" of dichlorvos and results in prolonged inhibition of esterases relative to the duration of action of dichlorvos (see below). It may also have an impact on the safety of the compound in that dichlorvos may be a deoxyribonucleic acid (DNA) alkylating compound, and dichloroacetate and dichloroacetaldehyde are known mutagens.

Radiotracer studies indicate that lungs and urine are the major routes of excretion of trichlorfon in rats. Hassan et al. (1965) reported finding 28% of total radioactivity as $^{14}CO_2$ and 32% of total radioactivity in the urine within 24 hours after a dose of $^{14}CH_3O$-labeled trichlorfon. When ^{32}P-labeled compound was used, 75% to 85% of total radioactivity was recovered in the urine in 48 hours. They postulated that the urinary compounds were mainly di-and monomethyl phosphates and that the methoxy cleavage products were further metabolized to formate (a minor urinary metabolite) and from that compound to carbon dioxide (Hassan and Zayed, 1965).

Health Aspects

Observations in Humans

Trichlorfon has been used successfully as an antischistosomal agent in many humans. Oral therapeutic doses have ranged from 5 to 20 mg/kg and were formerly given for up to 23 consecutive days (see review by Holmstedt et al., 1978). The more recent dose regimen has been three doses separated by 2 to 4 weeks (Jewsbury et al., 1977; Reddy et al., 1975). These therapeutic doses inhibit serum and red blood cell (RBC)

cholinesterases to a great extent. Forsyth and Rashid (1967) reported approximately 90% inhibition of serum cholinesterase in three male patients within 1 hour after ingesting 10-mg/kg doses of trichlorfon. Inhibition reversed slowly and was not complete (10% to 30% inhibition remained) after 2 weeks. Hanna et al. (1966) reported comparable inhibition 30 minutes after dosing 10 patients with 5 mg/kg. The purity of trichlorfon used in these studies was unreported.

Nordgren et al. (1981) gave 98% pure trichlorfon to seven schistosomiasis patients. The researchers observed the rapid appearance of both the parent compound and its putative active metabolite dichlorvos (in quantities some 100-fold less than the parent compound) in blood after a single oral dose between 7.5 and 10 mg/kg. Plasma cholinesterase fell to near zero activity within 15 minutes. Recovery rate was reported to be slow. RBC cholinesterase was less inhibited than serum esterase, but recovery of activity was slower than that recorded for serum esterase. The authors stated that a second dose of 10 mg/kg after 14 days produced more RBC esterase inhibition than the original treatment.

Many of the reports on patients receiving trichlorfon therapy noted the presence of mild clinical signs of acetylcholinesterase inhibition. Gastrointestinal disturbances appear to have been the most prevalent effect. Hanna et al. (1966) noted decreased sperm counts and sperm motility in 10 men treated with metrifonate (5 mg/kg for 12 consecutive days) for urinary schistosomiasis. This effect was also noted by Wenger (WHO, 1972).

Certain organophosphorus esters produce axonal degeneration in peripheral nerves and long axon tracts of the spinal cord—a neuromyelopathy. Studies reviewed by Johnson (1981) indicate that humans ingesting high doses of trichlorfon have developed neurological dysfunction that appears to be organophosphorus neuropathy. The majority of these cases were reported in the Soviet literature, and Johnson questioned that impurities with greater toxicity might have contaminated the trichlorfon. It is well known that the higher chain length analogs of dichlorvos, particularly di-*n*-butyl-and di-*n*-pentyldichlorovinyl phosphate, are extremely neurotoxic and have little effect on acetylcholinesterase (Albert and Steams, 1974). Nonetheless, in at least one case (an attempted suicide), a syndrome identical to organophosphorus neuropathy was produced by ingestion of trichlorfon that was shown to have no toxic impurities (Hierons and Johnson, 1978). The dose that produced neuromyelopathy in this and other cases (e.g., Senanayake and Johnson, 1982) also caused severe clinical signs of acetylcholinesterase inhibition.

Van Bao et al. (1974) studied five people exposed to high levels of Ditriphon-50—a Hungarian insecticide preparation containing trichlorfon as the active ingredient and other unknown constituents. They found an

excess incidence of short-lived chromosome breaks and exchange figures relative to karyotypes of 15 healthy volunteers matched for age only. A significant increase in stable chromosome alterations was also found among the people exposed to Ditriphon 50.

Observations in Other Species

Acute Effects

In a study with trichlorfon, Edson and Noakes (1960) reported an acute oral LD_{50} at 649 mg/kg bw in male Wistar rats and a higher value in females. For dermal exposures, lethality (LD_{50}) was less than 2,800 mg/kg. The authors presumed that the cause of death was related to the inhibition of acetylcholinesterase. A review by Holmstedt et al. (1978) indicated that there were comparable or lower LD_{50}s in rats, mice, guinea pigs, and dogs given presumably pure trichlorfon by a variety of parenteral routes (lowest value, 150 mg/kg from intravenous doses in dogs; highest value, 650 mg/kg from intraperitoneal exposures of mice). DuBois and Cotter (1955) administered purified trichlorfon intraperitoneally to female rats and observed approximately 50% inhibition of brain acetylcholinesterase within 15 minutes after a 25-mg/kg dose. (Serum cholinesterase was 64% inhibited at this point.) Normal acetylcholinesterase activity was noted 1 hour after the dose. Greater inhibition and slower recovery occurred at higher doses. Edson and Noakes (1960) observed only 30% inhibition of RBC and serum esterases after an acute 500-mg/kg dose of a 50% trichlorfon solution and reported recoveries comparable to those noted by DuBois and Cotter (1955). The disparity between these findings and those reported for humans is notable: humans appear more sensitive to esterase inhibition, and the recovery of enzyme activity is slower.

Trichlorfon may potentiate the toxicity of other organophosphorus insecticides. The effect is presumably due to inhibition of enzymes that detoxify certain organophosphorus compounds (e.g., malathion) and is transient (DuBois, 1958).

Subchronic Effects

DuBois and Cotter (1955) maintained a steady-state 25% to 50% inhibition of rat brain acetylcholinesterase for 60 days with daily intraperitoneal 50-mg/kg doses of purified trichlorfon. At this dose level there were no deaths. However, 100 mg/kg caused progressive inhibition of acetylcholinesterase and death in two and five animals. All five rats died at doses of 150 mg/kg. In a 1955 study, Doull and DuBois (J. Doull, University of Kansas Medical Center, Kansas City, Kans., personal communication, 1985; WHO, 1972) reported cholinesterase depression in rats (13 females and 13 males per group) fed 300-ppm dietary concentrations of trichlorfon for 16 weeks but no effect at 100 ppm,

whereas Edson and Noakes (1960) observed no effects in male rats (10 per group) given up to 125-ppm concentrations of trichlorfon (11.3 mg/ kg/day) in the diet for 16 weeks. No changes in growth, food consumption, or gross appearance of tissues were observed at any dose in these two studies.

WHO (1972) cited two unpublished studies by Doull. In a 1962 study, cholinesterase was depressed in dogs (two males and two females per group) fed 500-ppm but not 250-ppm dietary concentrations of trichlorfon for 1 year (Doull, personal communication, 1985). In a 1958 follow-up, Doull and Vaughn fed 20-, 100-, 300-, and 500-ppm concentrations of trichlorfon to dogs (one female and one male per group) for 12 weeks followed by 4 weeks on unadulterated feed. At the end of this study, there was significant depression of serum and RBC cholinesterase in the 300-ppm (7.5 mg/kg/day) group, but not in the 100-ppm (2.5 mg/kg/day) group (Doull, personal communication, 1985). These data are comparable to those of Williams et al. (1959), who reported that plasma and RBC cholinesterase were inhibited in dogs (one male and one female per group) given trichlorfon at 500-ppm (12.5 mg/kg/day) in the feed for 12 weeks but that no effects were noted in animals given 200 ppm (5 mg/kg/ day) for the same period. In 1970 Löser (D. Lamb, Mobay Chemical Corporation, Stilwell, Kans., personal communication, 1985; WHO, 1972) performed a 4 year feeding study in dogs (four males and four females per group), in which plasma and RBC cholinesterase were inhibited in groups fed 200 ppm (5 mg/kg/day) but not in groups given 50 ppm (1.25 mg/ kg/day). Males in a group fed 800 ppm (20 mg/kg/day) had enlarged spleens and reduced adrenal size. (These data are summarized in Table 9-24.)

In 1981 Coulston studied the effects of subacute trichlorfon exposures in rhesus monkeys. Erythrocyte cholinesterase was reduced in monkeys given 1-mg/kg oral doses of trichlorfon for 4 years and "possibly" reduced at a dose of 0.2 mg/kg. No alteration of body weight or "clinical chemistry" had been observed, although diarrhea was reported to occur at 5-mg/kg doses (F. Coulston, White Sands Research Center, Rensselaer, N.Y., personal communication, 1985).

Chronic Effects

Most chronic studies on trichlorfon are unpublished reports that were summarized by WHO (1972) or by Machemer (1981). A synopsis of those data is given in this section.

In 1962 Doull and colleagues fed Sprague-Dawley rats (25 of each sex) diets containing 0- to 1,000-ppm concentrations of technical-grade trichlorfon (Doull, personal communication, 1985; WHO, 1972). The dosing was intended to last for 24 months, but it was shortened to 17 months in males (due to mortality). Males in the 1,000-ppm (100 mg/kg/day) group

failed to gain weight equivalent to other groups, and life span was shortened in both sexes at the high dose. Serum cholinesterase, but not brain, submaxillary gland, or RBC cholinesterase, was inhibited by 25% in the group fed 500 ppm (50 mg/kg/day) but not in animals fed 250 ppm (25 mg/kg/day). Vascular lesions, including fibrous changes and necrotizing inflammation, were reported, but the dosing group in which they occurred was not identified. Histopathology was done on only five rats of each sex from each dose group.

TABLE 9-24 Effect of Subacute Administration of Trichlorfon on Cholinesterase Activities

Test Animal	Site of Inhibition	NOEL[a] (mg/kg/day)	Level Producing Inhibition (mg/kg/day)	Duration of Study	Reference
Rat	Brain	—	50	NR[b]	DuBois and Cotter, 1955
Rat	Unknown	10.0	30	16 weeks	Doull, personal communication, 1985[c]
Rat	RBC,[d] plasma, brain	11.3	—	NR	Edson and Noakes, 1960
Dog	Unknown	6.25	12.5	1 year	Doull, personal communication, 1985[c]
Dog	RBC, serum	2.5	7.5	12 weeks	Doull, personal communication, 1985[c]
Dog	RBC, plasma	5.0	12.5	12 weeks	Williams et al., 1959
Dog	RBC, plasma	1.25	5.0	4 years	Lamb, personal communication, 1985[c]

[a] No-observed-effect level.
[b] NR = not reported.
[c] See summary in WHO, 1972.
[d] Red blood cell.

A similar study was undertaken by Doull and coworkers again in 1965 (Doull, personal communication, 1985; WHO 1972). They gave Sprague-Dawley rats (25 males and 50 females per group) 100-, 200-, or 400-ppm concentrations of technical-grade trichlorfon in feed. The study was intended to last 18 months, but was apparently curtailed at about 70 weeks due to mortality in both the treated and control groups. There was a sex difference in cholinesterase inhibition: slight depression of RBC cholinesterase occurred in males at 200 ppm but not at 100 ppm (10 mg/kg/ day), whereas there was "very slightly" depressed RBC cholinesterase in females fed 100 ppm. Spleen and liver weights were reported to be reduced in the 400-ppm group (40 mg/kg/ day), and four females in the

high-dose group had pulmonary changes. (Other findings of this study are discussed in the pertinent sections below.)

Doull's findings contrast with those of Lorke and Löser in 1966 (Lamb, personal communication, 1985; WHO, 1972) and of Grundman and Hobik in 1966 (Lamb, personal communication, 1985; WHO, 1972). These researchers fed Long-Evans rats (50 males and 50 females per treated group and 100 of each sex for controls) 50-, 250-, 500-, and 1,000-ppm dietary concentrations of technical-grade trichlorfon for 2 years. Cholinesterase inhibition was seen only in the high-dose group. Coulston indicated that CD 1 mice (60 females and 60 males per group) given trichlorfon for 90 weeks were unaffected by 100 ppm in the diet, but that 300 ppm and 1,000 ppm (45 and 150 mg/kg/day) decreased weight gain and cholinesterase in both sexes (F. Coulston, White Sands Research Center, Rensselaer, N.Y., personal communication, 1985).

Neurotoxicity

Johnson (1970) and Olajos et al. (1979) produced clinical signs of neuropathy in adult laying hens by giving them 200 mg/kg trichlorfon followed 3 days later by a dose of 100 mg/kg (both doses subcutaneous). Both investigators had to protect the animals from cholinergic toxicity with atropine. (Johnson also used physostigmine and an oxime reactivator.)

Mutagenicity

The genetic toxicity of trichlorfon has been reviewed by IARC (1983, pp. 207-231) and by Moutschen-Dahmen et al. (1981). A disturbing aspect about the information relating to the genetic toxicity of trichlorfon is the number of contradictory reports regarding its activity in different assays. For most compounds, a profile of genetic activity for specific types of end points becomes clear as more tests are performed. The occasional contradictory result can usually be dismissed by examining differences in protocol or by demonstrating inconsistency with other reports. In the case of trichlorfon, opposite results are often reported with no clear indications as to which is correct. This may be related to its instability, to contaminants, or to different formulations of pesticides tested.

Trichlorfon apparently has the ability to react directly with DNA *in vitro* (Rosenkranz and Rosenkranz, 1972). This property has been confirmed in mice whose tissues were examined for 7-methylguanine following exposure to trichlorfon (Dedek, 1981; Dedek et al., 1975, 1976). The methylating capacity of trichlorfon was found to be 10-fold less than that of dichlorvos and 1,000-fold less than that of dimethyl sulfate. The binding in the testicles was approximately an order of magnitude lower than the binding in liver, kidneys, or lung. In similar studies (Segerbäck and Ehrenberg, 1981), the genetic risk to patients treated for schistosomiasis

with a therapeutic 15-mg/kg dose of trichlorfon was estimated to be on the same order of magnitude as the risk from 100 mrad of gamma radiation. This is approximately equal to the annual background dose and was characterized as a "low or very low" risk.

Both positive and negative results have been obtained in bacterial mutagenesis assays with trichlorfon. When activity is reported, it is usually in base-pair substitution strains without the need for exogenous metabolic activation, consistent with direct alkylating activity (IARC, 1983, pp. 207-231). There was some indication that mutagenic activity increased during storage, suggesting the production of a mutagenic breakdown product such as dichlorvos.

There is one report that trichlorfon induces mutations in yeast (Gilot-Delhalle et al., 1983), and one report that it does not (Morpurgo et al., 1977). IARC (1983, pp. 207-231) reported mild activity inducing mitotic crossing over in yeast, without the need for exogenous metabolic activation. Trichlorfon was negative in *Drosophila* mutagenesis assays. Because of high toxicity, however, only low doses could be examined (IARC, 1983, pp. 207-231).

Results from tests for mutagenesis and cytogenetic damage in cultured mammalian cells are generally positive for trichlorfon (IARC, 1983, pp. 207-231). Results from cytogenetic assays in mice with trichlorfon are, once again, mixed (IARC, 1983, pp. 207-231): what appear to be well-conducted studies give negative results in both bone-marrow and spermatogonial chromosomes (Moutschen-Dahmen et al., 1981). Chromosome analysis was done on blood samples from several human subjects following self-intoxication by large doses of trichlorfon. Chromosome damage was approximately eightfold greater than that of controls soon after exposure, but returned to near control levels by 6 months (van Bao et al., 1974). The small numbers of subjects and the lack of appropriate controls prevent determination of a causal effect in these cases (IARC, 1983, pp. 207-231). Other reports of chromatid aberrations, such as sister-chromatid exchange (SCE), have been reported in factory workers exposed to trichlorfon. However, elevations in this end point were also seen in workers from the same plant not exposed to trichlorfon, suggesting airborne exposure to other chemicals as well (IARC, 1983, pp. 207-231).

Reports of mutagenicity, along with the observation that trichlorfon can affect sperm morphology (Wyrobek and Bruce, 1975), indicate potential germ cell mutagenic activity for trichlorfon. Both positive and negative results have been reported with trichlorfon in the dominant lethal assay in mice (IARC, 1983, pp. 207-231). In what appear to be well-conducted dominant lethal studies, no statistically significant activity of trichlorfon (Moutschen-Dahmen et al., 1981) or dichlorvos (Epstein et al., 1972) was observed.

Information generated thus far suggests that trichlorfon is a weak, direct-acting mutagen. The assays may be influenced by cytotoxic activity and the presence of breakdown products or contaminants. The data are so mixed that it is difficult to conduct a risk assessment based on mutagenic activity at this time.

Future research should include examination of the dose-response relationship by studying the induction of cytogenetic damage in blood from patients before and after therapeutic exposure to trichlorfon. The potential germ cell mutagenicity of trichlorfon should be reexamined with an emphasis on altered sperm morphology in patients before and after trichlorfon therapy for parasitic infestations.

Carcinogenicity

Bioassays of trichlorfon for carcinogenicity have produced mixed results, and some studies have been criticized (Machemer, 1981). Furthermore, details of the older studies have not been reported, but have only been summarized in reviews (IARC, 1983, pp. 207-231; Machemer, 1981; WHO, 1972).

IARC (1983) cited a carcinogenesis bioassay in progress, and the National Toxicology Program (NTP, 1984b) reported a carcinogenesis bioassay being conducted by the Gulf South Research Institute under contract to the National Institute of Environmental Health Sciences. NTP (1984a) reported a slight increase in mammary tumor incidence and earlier onset in female rats given 250-, 500-, and 1,000-ppm concentrations of trichlorfon in the diet for 24 months. No statistical analysis was reported by these investigators. Five animals from each dose group were examined microscopically. Mammary tumors of three different types were seen in three of the five 1,000-ppm animals; androblastoma was found in two of the five. Three animals from the 500-ppm group and two from the 250-ppm group had developed mammary tumors. In 1965, Doull and coworkers reported mammary tumors in 15%, 11%, and 8% of female rats (50 per group) fed trichlorfon for 18 months at a level of 400, 200, and 0 ppm, respectively (Doull, personal communication, 1985; WHO, 1972). Microscopic examination of the tumors indicated that they were benign.

In 1966 Lorke and Löser (Lamb, personal communication, 1985; WHO, 1972) found no increased incidence in malignant tumors in Long-Evans rats given up to 1,000-ppm concentrations of trichlorfon in feed for 2 years. Gibel et al. (1971, 1973) gavaged 40 Wistar rats (unspecified sex) with 15-mg/kg doses of trichlorfon twice weekly for life. The average life span for treated animals was shortened, and 7 of 28 treated animals had malignant tumors (of unspecified type) as compared to no tumors in controls. Benign tumors were found in 19 of 28 rats and in 3 of 36 controls. Teichmann and Hauschild (1978) gave AB/Jena mice (30 males and 28 females) intraperitoneal 30-mg/kg doses of trichlorfon twice weekly for

75 weeks and found no significant difference in tumor incidence between treated and control animals. The same investigators (Teichmann et al., 1978) gave albino rats (30 males and 35 females) 22 mg/kg intraperitoneally twice weekly for 90 weeks and reported no increased tumor incidence over controls (25 males and 26 females). Teichmann and Schmidt (1978) found no increased tumor incidence in hamsters (23 male and 25 female treated animals; 22 male and 23 female controls) given 20-mg/kg doses of trichlorfon intraperitoneally twice weekly for 90 weeks.

The possible mechanisms of toxicity or interaction responsible for the majority of the effects described above are not known. In contrast, the mechanism by which acute toxicity proceeds, i.e., by inhibition of acetylcholinesterase, is understood at the molecular level. Inhibition of acetylcholinesterase by phosphorylation of its active site and the sequelae of acetylcholine accumulation are reviewed in Chapter 4. This action is probably a property of dichlorvos, a metabolite of trichlorfon (Reiner, 1981). A comparable reaction probably occurs between dichlorvos and other esterases such as serum cholinesterase and some carboxylesterases.

Developmental Effects

Staples and Goulding (1979) reported teratogenic effects (edema, herniation of the brain, hydrocephaly, micrognathia, cleft palate, shortened radius or ulna, hypophalangism, and syndactyly) in rats and hamsters given trichlorfon. Malformations were seen in CD rats given 480 mg/kg/day (in three divided doses) on days 6 through 15 of gestation but not if doses were given only on day 8 or 10. Teratogenicity in hamsters was produced by doses of 400 mg/kg/day (in divided doses) on days 7 through 11 of gestation. At 300 mg/kg/day, fetus weight was reduced in hamsters; no effect was seen at 200/mg/kg/day. Doses of 400 mg/kg/day given on day 8 of hamster gestation were embryotoxic but not teratogenic. Increased incidence of cleft palate only was induced in CD1 mice given 600 mg/kg/day on days 10 through 14 or 12 through 14 of gestation. The doses that were teratogenic in hamsters and rats were higher than the dose required to produce cholinergic signs in the dams. The incidence of teratogenic effect was still significant when compared with food-restricted controls in which weight gain was depressed as much as in treated dams. Treated mice did not develop cholinergic signs or terata relative to the more sensitive species (rat and hamster). CD1 mice had mixed response to 200, 300, and 400 mg/kg/day (Courtney and Andrews, 1980). Fetuses had extra ribs at the low dose, were normal at 300 mg/ kg/day, and had fewer ribs at the high dose.

Reproductive Effects

WHO (1972) reported that trichlorfon administered to rats at 1,000 and 3,000 ppm (100 and 300 mg/kg/day) had effects on reproduction, but details were sparse. The effect of trichlorfon on

reproductive organs has been studied by two investigators with mixed results. Female rats were given trichlorfon at 500 or 1,000 ppm (50 or 100 mg/kg/day) in feed for 24 months (Doull, personal communication, 1985; Lamb, personal communication, 1985; WHO, 1972), and five animals per group were examined. The investigators found that the rats were without primitive ova or primary follicles. The same finding was reported in one of five females from the group given 250 ppm (25 mg/kg/day). Two of five rats at 1,000 ppm (100 mg/kg/day) had tubular androblastomas. Three of five males examined from the 1,000-ppm group had focal aspermatogenesis. Cystic ovaries were observed in 40%, 33%, 14%, and 8% of rats given 400-, 200-, 100-, or 0-ppm concentrations of trichlorfon in the diet for 70 weeks, respectively (Doull, personal communication, 1985; Lamb, personal communication, 1985; WHO, 1972). Results of statistical analysis were not reported. Microscopic examination of tissue from four of five animals from the 400-ppm (40-mg/kg/day) group revealed the absence of follicles and ova. No change in gonads was found in Long-Evans rats given 50- to 1,000-ppm concentrations of trichlorfon in the diet for 24 months (Doull, personal communication, 1985; Lamb, personal communication, 1985; WHO, 1972). Dogs given trichlorfon at 1,000 ppm (25 mg/kg/day) in the diet for 1 year were reported to have a decrease in spermatogenesis (Doull, personal communication, 1985; Lamb, personal communication, 1985; WHO, 1972). The investigators also reported decreased gonad weight in male dogs fed trichlorfon at 800 ppm (20 mg/ kg/day) and females given 3,200 ppm (80 mg/kg/day) in the diet for 4 years. There were, however, no morphological changes in these organs.

Conclusions And Recommendations

The human genotoxic risk from trichlorfon exposure is not at all clear from the reported studies on the carcinogenic, teratogenic, or reproductive effects of the compound in humans or animals. Large variability exists in the observations, quality of the studies, detail of the reports, and even the purity of the test compound. Multiple reports that trichlorfon or some of its metabolites or breakdown products are mutagenic suggest that at least the potential for genotoxicity exists. The committee therefore strongly recommends that a well-conducted carcinogenesis bioassay of trichlorfon be performed as well as a meticulous assessment of its teratogenicity and reproductive toxicity.

Trichlorfon probably produces neurodegeneration (organophosphorus neuropathy), but the risk of this effect is small relative to other actions of the compound. The unequivocal effect that occurs at the lowest dose is inhibition of cholinesterases. That the inhibition of acetylcholinesterase by trichlorfon (or its metabolites) can produce a toxic syndrome is un

deniable. The significance of inhibition of other esterases as a toxic effect in and of itself is yet to be determined. In a study with monkeys, Machemer (1981) reported the lowest dose in which an effect on cholinesterase was observed (0.2 mg/kg/day for 4 years). Extrapolation to humans from data on nonhuman primates would be desirable; however, because the study has only been summarized, there is not enough detail to evaluate for this purpose.

Among studies reported in more detail, the lowest concentration at which an action of trichlorfon on cholinesterases has been reported is 200 ppm (5 mg/kg/day) in the feed of dogs (eight per group) for 4 years (Doull, personal communication, 1985; Lamb, personal communication, 1985; WHO, 1972). The highest no-effect concentration in this study was 50 ppm (1.25 mg/kg). Again, this report has only been summarized, although in a more complete manner. It would be better if an actual level of inhibition had been described in the report, but it is assumed that fairly significant inhibition of serum cholinesterase was encountered. This opinion is based on the observation that in most studies serum cholinesterase tended to be more sensitive to trichlorfon than was RBC cholinesterase and that the latter enzyme was inhibited as well (Doull, personal communication, 1985; Lamb, personal communication, 1985; WHO, 1972).

In determining a safety factor to be applied to the dose level, it is worth recalling that interspecies differences in trichlorfon effects may have existed both within and between studies. More importantly, comparison of rat and human response indicates that humans are more sensitive than rodents in terms of magnitude and duration of cholinesterase inhibition. The committee recommends a conservative safety factor of 100. Assuming that a 70-kg human consumes 2 liters of water daily and that 20% of the intake of trichlorfon is derived from water, one may calculate the suggested acceptable daily intake (ADI) as:

$$\frac{1.25 \text{ mg/kg bw/day} \times 70 \text{ kg} \times 0.2}{2 \text{ liters} \times 100} = \begin{array}{l}0.088 \text{ mg/liter,} \\ \text{or } 88 \text{ µg/liter.}\end{array}$$

An ADI may also be estimated for a 10-kg child on the assumption that I liter of water is consumed daily and that 20% of the trichlorfon intake is derived from water:

$$\frac{1.25 \text{ mg/kg/day} \times 10 \text{ kg} \times 0.2}{1 \text{ liter} \times 100} = \begin{array}{l}0.025 \text{ mg/liter,} \\ \text{or } 26 \text{ µg/liter.}\end{array}$$

The committee recommends that a new assay for carcinogenicity of trichlorfon be conducted following current protocols and methods. In addition, monitoring of workers exposed to trichlorfon for genetic effects such as SCE should continue.

CONCLUSIONS AND RECOMMENDATIONS

Table 9-25 lists the substances reviewed in this volume and presents either an ADI, a SNARL, or cancer risk estimate where data were sufficient for calculation. The statistical methodology for the risk estimation is described in Chapter 8. Further information on methodology can also be found in Volumes 1 and 3 of *Drinking Water and Health* (NRC, 1977, 1980). When reviewing this table, the reader should refer back to the discussion on individual compounds for specific details.

The committee recommends that the following studies be conducted:

Acrylamide:

- Chronic studies to evaluate the neuropathic effects of acrylamide.

Aldicarb:

- Research on the unconfirmed effects of low-level inhibition of acetylcholinesterase and other esterases.

Diallate:

- A carcinogenicity study using an improved protocol with more than one dose level.
- Studies of metabolism and developmental effects.

Dibromochloropropane:

- Oral feeding studies to determine clear dose-response relationships for reproductive effects.

Di(2-ethylhexyl) Phthalate (DEHP):

- Investigations of the role of DEHP-induced hepatocellular changes, such as peroxisomal and cellular proliferation, in carcinogenesis.
- Examination of the structure-activity relationships of DEHP and similar surfactants with cell membrane-altering properties in cell transformation bioassays and correlation of those relationships with aneuploidy induction as well as carcinogenicity.

1,2-Dichloropropane, 1,2,3-Trichloropropane, and 1,3-Dichloropropene:

- Additional studies on the toxicity of the chloropropanes and chloropropenes, focusing on pure substances rather than on mixtures.

Ethylene Dibromide:

- A carcinogenicity bioassay to demonstrate a reliable dose response from oral exposure.
- Additional studies on reproductive toxicity.

TABLE 9-25 Summation of Acceptable Daily Intakes (ADIs), Suggested No-Adverse-Response Levels (SNARLs), and Cancer Risk Estimates for Chemicals Reviewed in This Volume

		Estimated ADIs (mg/kg) or SNARLs (µg/liter)		Upper 95 Confidence Estimate of Lifetime
Compound	Response	Adult	Child	Risk
Acrylamide	Carcinogenicity			2.1×10^{-5}[a]
Aldicarb	20% Cholinesterase nhibition	0.7 µg/liter[b]	0.2 µg/liter[b]	
	30% Cholinesterase inhibition	3.5 /g/liter[b]	2.1 µg/liter[b]	
Diallate		[c]		[c]
Sulfallate	Carcinogenicity			1.6×10^{-6}
Dibromochloropropane	Carcinogenicity			9.9×10^{-6}
1,2-Dichloropropane		[c]		[c]
1,2,3-Trichloropropane		[c]		[c]
1,3-Dichloropropene	Carcinogenicity			1.1×10^{-6}
Di(2-ethylhexyl) phthalate	Carcinogenicity			2.1×10^{-7}
	Liver changes	0.003 mg/kg/day[d]		
	Liver function increases	0.003 mg/kg/day[d]		
	Liver enzyme alterations	0.01 mg/kg/day[d]		
	Teratogenicity	0.50 mg/kg/day[d]		

		Estimated ADIs (mg/kg) or SNARLs (µg/liter		Upper 95% Confidence Estimate of Lifetime
Compound	Response	Adult	Child	Risk
Mono(2-ethylhexyl) phthalate		[e]		[e]
Ethylene dibromide		[c]		[c]
Nitrofen	Carcinogenicity			5.6×10^{-5}
	Teratogenicity	1.1 µg/liter[d]	[f]	7.1×10^{-6}
Pentachlorophenol (pure)	Teratogenicity	21 µg/liter[d]	6 µg/liter[d]	
(technical)	Teratogenicity	7 µg/liter[d]	[g]	
Trichlorfon	Cholinesterase inhibition	88 µg/liter[d]	26 µg/liter[d]	

[a] Average risk assessment value.
[b] Upper 95% confidence limit from logit and probit models.
[c] Insufficient data for calculation.
[d] Based on NOELs or LOELs and the use of safety factors.
[e] Metabolite of di(2-ethylhexyl) phthalate (DEHP).
[f] Not calculated. The value for an adult is provided for comparison purposes.
[g] Not calculated. The value for an adult is provided for comparison purposes and is dependent on the impurities in the technical-grade material.

Nitrofen:

- Additional studies on mechanisms of teratogenicity and carcinogenicity.

Pentachlorophenol:

- Additional carcinogenicity bioassays.
- Studies to resolve uncertainties concerning the effects of contaminants, e.g., chlorinated dioxins and dibenzofurans.

Sulfallate:

- Additional carcinogenicity studies with improved protocols.
- Additional studies on metabolism and developmental effects.

Trichlorfon:

- A well-conducted carcinogenesis bioassay.
- Assessments of teratogenicity and reproductive toxicity.

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Committee Biographies

DANIEL B. MENZEL, *Chairman*, is professor of pharmacology and medicine at Duke University and director of chemical carcinogenesis and of environmental toxicology in the Duke Comprehensive Cancer Center. He has served as chairman of the Safe Drinking Water Committee of the NRC from 1981 to 1986, as chairman of the WHO working group on airborne carcinogens and other expert toxicology groups for the National Library of Medicine, National Institute of Occupational Health and Safety, National Institutes of Health, and the World Health Organization. He was also a member of the EPA Science Advisory Board. His active research is in chemical carcinogenesis, mathematical modeling, and estimation of human health risks.

BYRON E. BUTTERWORTH, *Vice Chairman*, is chief of genetic toxicology for the Chemical Industry Institute of Toxicology in Research Triangle Park, North Carolina. His numerous publications include papers on the development of new methods to determine the genotoxic effects of chemicals in the whole animal and in exposed individuals. He serves as an advisor on several national and international committees dealing with toxicology. He is a member of the American Association for Cancer Research and the Environmental Mutagen Society. Dr. Butterworth is also an adjunct associate professor in the Department of Pathology of the University of North Carolina in Chapel Hill.

MELVIN E. ANDERSEN is a research pharmacologist, Toxic Hazards Division, Armstrong Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio. Dr. Andersen specializes in developing physiologically based models for examining the pharmacokinetics and

pharmacodynamics of volatile chemicals. He is presently a member of the editorial board and in the past was associate editor of *Toxicology and Applied Pharmacology*. He is a member of the Society of Toxicology and a diplomate of the American Board of Toxicology and the American Board of Industrial Hygiene.

JAMES V. BRUCKNER is associate professor of pharmacology and toxicology and director of the Toxicology Program at the University of Georgia. Dr. Bruckner is an environmental toxicologist whose research areas include development of *in vitro* testing systems for hepatorenal toxins, comparison of the relative pharmacokinetics and toxicity of inhaled and ingested organics, and investigation of the toxicity of halogenated hydrocarbons. Dr. Bruckner has served as consultant to state and federal agencies and to industry and as a member of expert panels for these groups. He serves on editorial boards of the *Journal of Toxicology and Environmental Health, Toxicology*, and *Toxicology and Applied Pharmacology*.

SEYMOUR L. FRIESS is president of Drill, Freiss, Hays, Loomis & Shaffer, Inc., a firm of consultants in toxicology. His research has been in the fields of physical biochemistry, pharmacology, toxicology, and chemical hazard assessment/safety evaluation. He has served as president of the Society of Toxicology, the International Union of Toxicology, and the certifying body, American Board of Toxicology, Inc. He has also served on the editorial boards of major journals in toxicology.

MICHAEL A. GALLO is an associate professor and director of toxicology and environmental health in the Department of Environmental and Community Medicine at the University of Medicine and Dentistry of New Jersey—Rutgers Medical School, and associate director of the Rutgers University/Rutgers Medical School Joint Graduate Program in Toxicology. Dr. Gallo is associate editor of *Drug and Chemical Toxicology*, a member of the Board of Scientific Counselors of the National Toxicology Program, a member of the New Jersey Governor's Advisory Toxicology Panels on dioxin and radon, and chairs the Governor's Pesticide Control Council. He served as a member of the NRC Committee on Drinking Water and Health, Volume 5, and currently serves on the NRC Committee on Complex Mixtures. Dr. Gallo's research interests address the cellular and molecular mechanisms of toxicity of dioxins, chlorinated pesticides, and the molecular interactions of photoactive compounds and tumor promoters as they relate to risk assessment.

JOHN N. HATHCOCK is currently chief of the Experimental Nutrition Branch, Food and Drug Administration, and until recently was professor in the Department of Food and Nutrition, Iowa State University. Dr. Hathcock is a member of the American Institute of Nutrition and the Society of Toxicology and serves on the editorial boards of *Nutrition Reviews* and *Drug-Nutrient Interactions*. He has edited volumes on

nutritional toxicology and drug-nutrient interactions and has served as a consultant to the Pesticide Tolerance Study Group of the Environmental Protection Agency.

CLARK W. HEATH, JR., is director of the Bureau of Preventive Health Services at the South Carolina Department of Health and Environmental Control. He was formerly professor of Community Health at Emory University and director of the Chronic Diseases Division, Center for Environmental Health, Centers for Disease Control. Dr. Heath is a physician epidemiologist with particular experience in the epidemiology of cancer, reproductive disorders, and environmental health. He is a fellow of the American Epidemiological Society, the Society for Epidemiologic Research, and the International Epidemiological Association.

MICHAEL E. HOGAN is assistant professor in the Department of Molecular Biology at Princeton University. He received his undergraduate degree in biology at Dartmouth College, his Ph.D. from the Department of Molecular Biophysics and Biochemistry at Yale, and was a postdoctoral fellow of the Damon Runyon-Walter Winchell Cancer Fund at Stanford University in the Magnetic Resonance Laboratory (1979-1980). His principle field of interest is the physical biochemistry of carcinogen adducts with genetic material.

DAVID J. JOLLOW is a professor of pharmacology and director of the Laboratory of Molecular Toxicology at the Medical University of South Carolina. For the past 15 years, Dr. Jollow has conducted research on the molecular mechanisms underlying tissue necrosis caused by drugs and other xenobiotic compounds. He is a member of the American Society for Pharmacology and Experimental Therapeutics, a past member and chairman of the Toxicology Study Section of the National Institutes of Health, and a councillor of the International Society for the Study of Xenobiotics.

JEANNE M. MANSON is director of Reproductive and Developmental Toxicology at Smith Kline and French Laboratories, Philadelphia, Pennsylvania. Dr. Manson also serves as an adjunct associate professor in teratology at the University of Cincinnati and Thomas Jefferson University in Philadelphia. She is a member of the National Toxicology Program's Board of Scientific Counselors and has served on the Science Advisory Board of EPA and the Advisory Council of NIEHS. Her research has addressed the developmental toxicity of drugs and environmental agents.

FREDERICA P. PERERA is assistant professor of Environmental Sciences at the Columbia University School of Public Health. She is project director of several molecular epidemiology studies of chemical carcinogenesis in humans. Senior scientist at the Natural Resources Defense Council, Inc., from 1979 to 1983, Dr. Perera currently acts as a science advisor to that organization. She is senior author of a book on air pollution,

as well as articles on molecular epidemiology, risk assessment, and environmental policy, and serves on advisory committees including the National Toxicology Program's Board of Scientific Counselors and the U.S. Environmental Program.

MARVIN A. SCHNEIDERMAN is professor of biostatistics at the Uniformed Services University of the Health Sciences, Bethesda, Maryland, and a private consultant in matters relating to environment and health. Until 1980 he was at the National Cancer Institute, first as a theoretical (research) statistician, later as associate director for Field Studies (Epidemiology) and Statistics, and then as associate director for Science Policy. He has been on the editorial board of several journals. He was chairman of the Epidemiology Subcommittee of the first NRC Safe Drinking Water Committee. He is past chairman of COPSS (Committee of Presidents of Statistical Societies) and has served on the Board of Directors of the American Statistical Association and on the Council of the (International) Biometrics Society. He is the author of about 130 scientific papers and the editor of several books relating to the environment and health—one in Russian, which was developed as a consequence of his being chairman of the U.S. group in a series of scientific exchanges with Soviet scientists on matters relating to the epidemiology of cancer.

PETER S. SPENCER is professor of neuroscience and director of the Institute of Neurotoxicology at The Albert Einstein College of Medicine of Yeshiva University, The Bronx, New York. He is a member of the Board on Toxicology and Environmental Health Hazards of the NRC, a former member of its Committee on Toxicology, and past president of the specialty section on neurotoxicology of the Society of Toxicology. His research activities are focused on molecular and cellular mechanisms of chemical neurotoxicity.

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