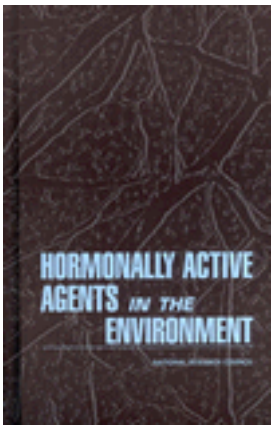

Hormonally Active Agents in the Environment

Committee on Hormonally Active Agents in the Environment, National Research Council

ISBN: 0-309-51777-X, 452 pages, 6 x 9, (1999)

HORMONALLY ACTIVE AGENTS *IN THE* ENVIRONMENT

Committee on Hormonally Active Agents in the Environment

Board on Environmental Studies and Toxicology

Commission on Life Sciences

National Research Council

NATIONAL ACADEMY PRESS
Washington, D.C.

NATIONAL ACADEMY PRESS • 2101 Constitution Avenue, NW • Washington, DC 20418

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This project was supported by Contract No. CX 824040-01-0 between the National Academy of Sciences and the U.S. Environmental Protection Agency and the U.S. Centers for Disease Control and Prevention, and Cooperative Agreement No. 1445-CA09-96-0027 between the National Academy of Sciences and the National Biological Service of the U.S. Department of the Interior. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the organizations or agencies that provided support for this project.

Library of Congress Cataloging-in-Publication Data

Hormonally active agents in the environment / Committee on Hormonally Active Agents in the Environment, Board on Environmental Studies and Toxicology, Commission on Life Sciences, National Research Council.
p. cm.
Includes bibliographical references (p.) and index.
ISBN 0-309-06419-8 (case binding)
1. Reproductive toxicology. 2. Endocrine toxicology. 3. Environmental toxicology. I. National Research Council (U.S.). Committee on Hormonally Active Agents in the Environment.
RA1224.2.H67 1999
616.4′071—dc21

99-50523

Additional copies of this report are available from:

Hormonally Active Agents in the Environment is available from the National Academy Press, 2101 Constitution Ave., NW, Box 285, Washington, DC 20418 (1-800-624-6242 or 202-334-3313 in the Washington metropoliton area; http://www.nap.edu).

Printed in the United States of America

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Preface

THERE HAS BEEN INCREASING public concern about potential adverse effects on human health of various environmental contaminants designated by some as "endocrine disruptors." In response, the National Research Council was asked by the Environmental Protection Agency, the Department of the Interior, the Centers for Disease Control and Prevention, and the United States Congress to help policy makers by independently evaluating the scientific evidence that bears on the issue.

This report is the culmination of a long and difficult process that began with the appointment of the Committee on Hormonally Active Agents in the Environment by the National Research Council more than 4 years ago on July 27, 1995. The formal charge to the committee was as follows:

> review critically the literature on hormone-related toxicants in the environment; identify the known and suspected toxicologic mechanisms and impacts on fish, wildlife, and humans; identify significant uncertainties, limitations of knowledge, and weaknesses in the available evidence; develop a science-based conceptual framework for assessing observed phenomena; and recommend research, monitoring, and testing priorities. To the extent practicable with available information and study resources, the committee [also was asked to] identify particular chemical substances, geographic areas, contaminant sources, human subpopulations, and fish and wildlife populations of special concern with respect to hormone-related toxicants.

The membership of the committee represents an attempt to obtain a balance of views regarding the subject as well as scientific expertise in the principal domains that comprise the study of hormonally active agents (HAAs) in the envi-

ronment. The efforts to unambiguously define "endocrine disruptors," by whatever name, and the reasons for renaming them "hormonally active agents" (HAAs), are detailed in the introduction of the report.

The committee met on five occasions and received briefings from Dr. Margaret Stasikowski (U.S. EPA), The Honorable Robert Perciasepe (U.S. EPA), Dr. Robert Kavlock (U.S. EPA), and Dr. Robert Hoover (National Cancer Institute). The committee proceeded with its study and deliberations, focusing on our charge, which was first and foremost the critical review of the literature on the subject. The work of the committee was organized to reflect the major biological systems affected by HAAs. These became the chapters of the report. Drafts of these chapters were extensively discussed and critiqued by the committee. The chair and the project directors, acting as editors, modified the original texts accordingly.

This process went through dozens of iterations in attempts to achieve a consensus document. This was readily achieved in some chapters but became extraordinarily difficult in others, most notably in the area of reproduction and development, including the issue of declining sperm production in human populations.

This report has been reviewed in draft form by individuals chosen for their diverse perspectives and technical expertise in accordance with procedures approved by the NRC's Report Review Committee for reviewing NRC and Institute of Medicine reports. The purpose of this independent review is to provide candid and critical comments that will assist the NRC in making the published report as sound as possible and to ensure that the report meets institutional standards for objectivity, evidence, and responsiveness to the study charge. The review comments and draft manuscript remain confidential to protect the integrity of the deliberative process. We wish to thank the following individuals, who are neither officials nor employees of the NRC, for their participation in the review of this report: Donald Brown, Carnegie Institution of Washington; Theo Colborn, World Wildlife Fund; Peter de Fur, Richmond, VA; Ronald Estabrook, University of Texas; Neal First, University of Wisconsin; Ronald Kendall, Texas Tech University; Ellen Ketterson, Indiana University; Dolores Lamb, Baylor College of Medicine; Paul Licht, University of California, Berkeley; Emil Pfitzer, Ramsey, NJ; Lorenz Rhomberg, Harvard School of Public Health; Herbert Rosenkranz, University of Pittsburgh; Antonio Sastre, Midwest Research Institute; George Seidel, Colorado State University; Ellen Silbergeld, University of Maryland; Paul Stolley, Columbia MD; Paolo Toniolo, IARC; and John Wingfield, University of Washington.

The individuals above provided many constructive comments and suggestions. It must be emphasized, however, that responsibility for the final content of this report rests entirely with the authorizing subcommittee and the NRC.

The work leading to this report, which has taken 4 years to complete, was a challenging and arduous exercise. It is hoped that the long delay in its publication will not unduly impair its utility for those who have the important responsi-

bility of making policy decisions regarding relevant research and public health agendas.

The chair is particularly grateful to Drs. Carol Maczka and David Policansky, the project co-directors, and their staffs for their truly Herculean labors.

Ernst Knobil, Ph.D.
Chair, Committee on Hormonally Active Agents in the Environment

Contents

Glossary

16a-OH-E2	estriol
ADI	acceptable daily intake
AF-2	activation function-2
AFP	α-fetoprotein
Ah	aryl hydrocarbon
AMS	USDA Agricultural Market Survey
APE	alkylphenol ethoxylate
ATSDR	Agency for Toxic Substances and Disease Registry
β-HCH	β-hexachlorocyclohexane
B[*a*]P	benzo[*a*]pyrene
BBP	butyl benzyl phthalate
BKD	bacterial kidney disease
BKME	bleached kraft mill effluent
BNBAS	Brazelton Neonatal Behavioral Assessment Scales
BPA	bisphenol A
BW	body weight
CB	chlorobiphenyl
CF "E"	Carworth Farm "E" strain
CI	confidence interval
CMI	cell-mediated immune
ConA	concanavalin A
CYP	cytochrome P450
CYP11	side-chain-cleavage enzyme
CYP17	17-hydroxylase
CYP19	aromatase

DBP	dibutyl phthalate
DDD	1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane
DDE	1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene
DDT	dichlorodiphenyltrichloroethane
DEHP	iethylhexyl phthalate
DES	diethylstilbestrol
DMBA	7,12-dimethylbenzathracene
DOI	Department of the Interior
DTH	delayed-type hypersensitivity
E1	estrone
E2	estradiol
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee (U.S. Environmental Protection Agency)
EGF	epidermal growth factor
EPA	Environmental Protection Agency
ER	estrogen receptor
ER–	estrogen receptor negative
ER+	estrogen receptor positive
ERE	estrogen responsive element
FDA	Food and Drug Administration
FSH	follicle-stimulating hormone
GLEMEDS	Great Lakes embryo mortality, edema, and deformity syndrome
GM-CFU	granulocyte-macrophage colony-forming unit
GnRH	gonadotropin-releasing hormone
GtH-II	gonadotropin hormone-II
HAA	hormonally active agent
HAH	halogenated aromatic hydrocarbon
HCB	hexachlorobenzene
hCG	human chorionic gonadotropin
HCH	hexachlorohexane
HpCDF	hepatochlorodibenzofuran
HQ	hazard quotient
HRT	hormone replacement therapy
IARC	International Agency for Research on Cancer
IOM	Institute of Medicine
IUGR	intrauterine growth retardation
IVF/ET	in vitro fertilization and embryo transfer
K_{ow}	octanol/water partition coefficient
LH	luteinizing hormone
LPS	lipopolysaccharide
MAFF	Ministry of Agriculture, Fisheries, and Food
MDI	[Bayley] Mental Development Index
$MeSO_2$	methylsulfonyl

MTD	maximum tolerated dose
NAE	National Academy of Engineering
NAS	National Academy of Sciences
NCI	National Cancer Institute
ND	none detected
NIEHS	National Institute of Environmental Health Sciences
NOAEL	no observed adverse effect level
NP	nonylphenol
NPE	nonylphenol ethoxylate
NRC	National Research Council
NTP	National Toxicology Program
NTR	no tumors reported
OR	odds ratio
PAH	polycyclic aromatic hydrocarbon
PBB	polybrominated biphenyl
PB-PK	physiologically based pharmacokinetic model
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PCDH	polychlorinated diaromatic hydrocarbon
PCR	polymerase chain reaction
PDI	[Bayley] Psychomotor Development Index
PeCB	3,3′,4,4′,5-pentachlorobiphenyl
PeCDF	1,2,3,7,9-pentachlorodibenzofuran
PFC	plaque-forming cell
PHA	phytohemagglutinin
PHED	Pesticide Handlers Exposure Database
PSA	prostate specific antigen
PWA	pokeweed mitogen
RfD	reference dose
RPF	relative potency factor
SAP	Scientific Advisory Panel
SBP	steroid-binding plasma protein
SHBG	steroid-hormone-binding globulin
SIR	standardized incidence ratio
SMR	standardized mortality ratio
sGnRH-A	synthetic gonadotropin-releasing hormone
SPI	Society for the Plastics Industry
SRBC	sheep red blood cell
STPE	sewage treatment plant effluent
T_4	3,3′,5,5′-tetraiodo-L-thyronine (thyroxin)
TBG	thyroxine-binding globulin
TCB	tetrachlorobiphenyl

TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDD-EQ	dioxin-like chemical
TCDF	2,3,7,8- or 1,3,6,8-tetrachlorodibenzofuran
TDS	FDA Total Diet Study
TEQ	toxic equivalent
TGF	transforming-growth factor
TIE	toxic identification and evaluation
TMRC	theoretical maximum residue concentration
tT_4	total T_4
TTP	time to pregnancy
USDA	U.S. Department of Agriculture
VLDL	very-low-density lipoprotein
WHO	World Health Organization
ZRP	zona radiata protein

HORMONALLY ACTIVE AGENTS *IN THE* ENVIRONMENT

Executive Summary

CONCERN HAS BEEN RAISED IN RECENT YEARS regarding potential adverse effects of various environmental contaminants often called "endocrine disruptors" and referred to in this report as "hormonally active agents" (HAAs). In part, this concern originated from the finding that some synthetic chemicals in the environment that are associated with adverse reproductive and developmental effects in wildlife mimic the actions of the female sex hormone estradiol. In addition, the effects of in utero exposure to the potent synthetic estrogen diethylstilbestrol (DES) in the offspring of treated women and the replication of these effects in mice have focused attention on embryonic development as a target for the potential disruptive effects of environmental agents with hormonal activity. Although it is clear that exposures to HAAs at high concentrations can affect wildlife and human health, the extent of harm caused by exposure to these compounds in concentrations that are common in the environment is debated.

Driven by considerable public interest, the U.S. Environmental Protection Agency (EPA), the U.S. Department of the Interior (DOI), the U.S. Centers for Disease Control and Prevention (CDC), and the U.S. Congress requested that the National Research Council (NRC) conduct an independent study of this topic. In response to that request, the NRC convened a multidisciplinary expert committee, the Committee on Hormonally Active Agents in the Environment, and charged it with the following tasks: (1) review critically the literature on HAAs in the environment; identify the known and suspected toxicologic mechanisms and impacts on fish, wildlife, and humans; identify significant uncertainties, limitations of knowledge, and weaknesses in the available evidence; develop a science-based conceptual framework for assessing observed phenomena; and recommend research, monitoring, and testing priorities; (2) to the extent practi-

cable with available information and study resources, identify particular chemical substances, geographic areas, contaminant sources, human subpopulations, and fish and wildlife populations of special concern with respect to HAAs; and (3) if possible and warranted, suggest general approaches for identifying and mitigating toxicologic problems.

The charge to the committee did not include, nor did the committee attempt to evaluate, risk-management policy options. Due to constraints of time and resources, the committee also did not consider approaches for mitigating toxicologic problems.

THE COMMITTEE'S APPROACH AND DIFFICULTIES ENCOUNTERED

To evaluate the endocrine-disruptor hypothesis, the committee's approach involved (1) identification of chemicals with hormonal or antihormonal activity; (2) evaluation of scientific literature on the effects in both the adult and the developing organism associated with those chemicals in vertebrates—humans, laboratory animals, and wildlife; and (3) consideration of whether the effects can be attributed to the hormonal properties of the chemicals and environmental exposure to them.

The committee focused its attention on compounds that have been reported to induce reproductive changes, developmental defects, neurobehavioral abnormalities, immunologic deficits, carcinogenesis, and ecologic effects.

It became clear as the work of the committee progressed that limitations and uncertainties in the data could lead to different judgments among committee members with regard to interpreting the general hypothesis, determining appropriate sources of information, evaluating the evidence, defining the agents of concern, and evaluating environmental and biologic variables.

Some of the differences reflect areas where additional research would help; others reflect differing judgments about the significance of the existing information. The differences are not confined to this committee but are reflected in the scientific community at large. Some differences appear to stem from different views of the value of different kinds of evidence obtained by experiments, observations, weight-of-evidence approaches, and extrapolation of results from one compound or organism to others, as well as allowable sources of information and criteria for arriving at meaningful conclusions and recommendations.

Other difficulties arise from questions about the observed effects: for example, is human sperm concentration really in decline? In other cases, the effect is clear (e.g., developmental abnormalities in some wildlife species), but the cause is in question. Often, organisms are exposed to many environmental chemicals as well as other environmental and ecologic perturbations. In addition, one must recognize that under multiple exposures, there is the potential for interaction among agents. These factors make assigning cause to specific chemicals

extremely difficult. Understanding the mechanism of action of various compounds is inseparable from defining HAAs and is one reason for the difficulty in defining HAAs. As an example, many compounds that have hormone-like activity might also affect organisms through pathways unrelated to any hormonal activity. Despite these differences, however, the many areas of consensus among the committee members are described below.

THE COMMITTEE'S EVALUATION

Mechanism of Action

For most associations reported between HAAs and various biologic outcomes, the specific mechanism of action is not well understood. However, lack of knowledge about a mechanism does not mean that a reported effect is unconfirmed or unimportant, nor does demonstration of a mechanism document that the resulting effects are unique to that mechanism or are pervasive in natural systems. It does suggest that additional studies are warranted. In general, the committee does not believe that HAAs can function only through receptor binding and recommends research to distinguish between direct and indirect effects and between primary and secondary effects of HAAs. Underlying mechanisms of action should be investigated with both in vitro and in vivo systems that can detect diverse responses.

Health Effects

Because of the difficulties associated with establishing cause and effect in humans and wildlife populations, for which exposure to HAAs is often the result of unintended releases of chemicals into the environment and involves exposures to multiple chemicals, the committee evaluated laboratory studies on individual HAAs in conjunction with available data from human studies and from field observations of wildlife. The following sections summarize the committee's consensus with respect to developmental, reproductive, neurologic, immunologic, carcinogenic, and ecologic effects of HAAs.

Developmental Effects and Reproduction

Adverse reproductive and developmental effects have been observed in human populations, wildlife, and laboratory animals as a consequence of exposures to HAAs. In humans, the effects of prenatal exposure to polychlorinated biphenyl (PCBs), 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE), and other contaminants from maternal consumption of contaminated fish or other food products have been studied in several populations in the United States and abroad. Collectively, these studies indicate that prenatal exposure to PCBs can cause

lower birth weight and shorter gestation and have been correlated with deficits in IQ and memory as well as delayed neuromuscular development. Prenatal and postnatal exposure to PCBs and polychlorinated dibenzofuran (PCDFs) from accidental contamination of rice oil in Yusho, Japan, and Yu-Cheng, Taiwan, have resulted in various developmental defects. Reported increases in the incidence of male reproductive disorders such as hypospadias (urethra opening found at the bottom rather than the tip of the penis), cryptorchidism (undescended testes), and testicular cancer cannot be linked to exposures to environmental HAAs at this time. With respect to the end point most closely studied, sperm concentration, retrospective analyses of trends over the past half-century remain controversial. When the data from large regions are combined and analyzed, some data sets indicate a statistically significant trend consistent with declining sperm concentrations. However, aggregation of data over larger geographic regions might not be an appropriate spatial scale for this analysis, given the significant geographic heterogeneity. The current data are inadequate to assess the possibility of trends within more appropriately defined small regions. Acquiring data at smaller regional scales is critical to assessing the significant geographic variation in sperm concentration.

Laboratory studies using male and female rats, mice, and guinea pigs and female rhesus monkeys have shown that exposure of these animals during development to a variety of concentrations of certain HAAs (e.g., DDT, methoxychlor, PCBs, dioxin, bisphenol A, octylphenol, butyl benzyl phthalate (BBP), dibutyl phthalate (DBP), chlordecone, and vinclozolin) can produce structural and functional abnormalities of the reproductive tract.

Many wildlife studies show associations between reproductive and developmental anomalies and exposure to environmental contaminants, some of which are HAAs. Reproductive and developmental abnormalities have also been observed in several populations of fish exposed to effluents from sewage treatment plants and paper mills and polluted waters of the Great Lakes. Effects observed include intersexes in trout exposed to sewage-treatment-plant effluent (STPE); increased egg and fry mortality in Great Lakes trout and salmon; thyroid enlargement in Great Lakes salmon; and changes in plasma sex-steroid concentrations, decreased egg and gonad size, and delayed sexual maturity in white suckers exposed to effluents from paper mills along Lake Superior.

Laboratory experiments with specific HAAs found in those effluents and polluted waters have produced effects consistent with those wildlife observations. For example, certain HAAs found in STPEs induce estrogenic responses in male trout. Specifically, ethinylestradiol and alkylphenol ethoxylates have been shown to induce vitellogenin synthesis, a hallmark of estrogen exposure, and to decrease the rate of testicular growth in male fish in tests that duplicate concentrations found in some effluents. Dioxin and structurally related compounds have been shown to induce blue-sac disease in developing trout and growth and sur-

vival reduction in salmon. Thyroid enlargement in salmon of the Great Lakes is hypothesized to be caused by exposure to PCBs, which also have been shown to induce goiter formation in laboratory rodents fed PCB-contaminated salmon from the Great Lakes. Finally, β-sitosterol found in paper-mill effluent has been shown to alter the reproductive physiology of goldfish under experimental conditions, in which goldfish were injected with 10-100 μg/g.

Laboratory studies are also consistent with some reproductive and developmental abnormalities (e.g., skewed sex ratios, behavioral modifications, and morphologic abnormalities of the gonads) observed among North American gull populations. Specifically, gull eggs injected with DDT at concentrations found in wild gull eggs induce gonadal abnormalities that are similar to those observed in contaminated gulls. Also, doves fed mixtures containing DDE and PCBs exhibit abnormal breeding behavior.

Similarly, defects seen in alligators from Lake Apopka (the site of a chemical spill containing dicofol and DDT), including small penis size and abnormal testes in males and abnormal ovaries in females, are consistent with structural and functional reproductive abnormalities that occur following perinatal exposure of laboratory rodents to estrogenic and antiandrogenic chemicals.

Recommendations

Wildlife and human populations should continue to be monitored for abnormal development and reproduction. Studies of wildlife species that exhibit population declines, abnormal sociosexual behavior, or deformities should be designed to investigate those phenomena with regard to chemical contamination. In human populations suspected of being affected by HAAs, prospective and cross-sectional studies using cohorts tracked from conception through adulthood are particularly needed on female and male reproductive end points, such as sperm concentration, cryptorchidism, and hypospadias. Regional differences in those end points should be studied prospectively to determine whether the differences can be associated with genetic and environmental factors.

Neurologic Effects

In humans, results of cognitive and neurobehavioral studies of mother-infant cohorts accidently exposed to high concentrations of PCBs and PCDFs and of mother-infant cohorts eating contaminated fish and other food products containing mixtures of PCBs, dioxin, and pesticides (such as DDE, dieldrin, and lindane) provide evidence that prenatal exposure to these HAAs can affect the developing nervous system. Similarly, monkeys exposed to PCBs in utero and during lactation have deficits in cognitive function when assessed at 14 months post-exposure and rats and mice exposed prenatally to PCBs suffer impaired locomotor ability and learning.

Recommendations

In human populations suspected of being affected by HAAs, longitudinal tests should be conducted on developmental milestones from conception through adulthood. A standardized set of criteria should be established to study neurobiologic and social development.

Immunologic Effects

Several HAAs affect diverse elements of the immune system in laboratory animals. There is evidence of suppression of the immune system by exposure to organochlorines (predominantly PCBs) in birds in the Great Lakes region. There is also evidence of suppression of innate and acquired immune responses in seals fed fish from the PCB-contaminated Baltic Sea. Such immunosuppression is believed to be the reason for the increased incidences of bacterial and viral infections in seals in similarly contaminated waters. In humans, data on the immunologic effects of HAAs are inadequate to support any definitive conclusions.

Recommendations

Studies are needed on the prevalence of autoimmune problems in cohorts suspected of being affected by HAAs, especially offspring whose mothers were exposed during pregnancy. Cohort studies that include various life phases should use clinically relevant immunologic assays to clarify the relationship between HAA exposure and human health. The immunologic activity of HAAs currently in use (e.g., endosulfan and lindane) should also be investigated in laboratory studies.

Carcinogenic Effects

With the exception of several bioassays in which mice were exposed to DDT during fetal life, during lactation, and after weaning without apparent adverse effects, perinatal exposure to environmental HAAs has not been assessed with respect to carcinogenesis in laboratory animals or humans, nor have transgenerational effects been investigated. Although some HAAs (e.g., toxaphene, DDE, DDT, TCDD, and endrin) have been associated with tumors of the thyroid, pituitary, or renal glands in particular species and strains of laboratory animals, HAAs in general have not been shown to induce tumors in reproductive or other endocrine organs after postnatal exposure, but the suitability of animal models might be in question. An evaluation of the available studies conducted to date does not support an association between adult exposure to DDT, DDE, TCDD, and PCBs and cancer of the breast. Although the current literature does not support associations between those HAAs and other hormonally sensitive can-

cers (testicular, prostate, or endometrial cancer), few studies have examined measured concentrations of those compounds in adults in relation to cancer risk. Moreover, no studies have been conducted to examine associations between risk of any cancer and exposure to any HAAs during development, particularly during fetal life. However, a recent study reported an association between dieldrin and breast cancer; additional epidemiologic and laboratory studies are needed to help confirm or refute this possible relationship.

Recommendations

Appropriately designed and conducted case-control and retrospective cohort studies are needed to document the presence or absence of associations between HAAs and various cancers in humans. If such associations are found, the possibility of causality must be investigated. Such studies should take into account the latency period between exposure and disease, the timing of likely exposure windows with respect to cancer, indicators of susceptibility, and the role of potential confounders. In addition, markers of total xenoestrogen exposure and chemical concentrations in blood or adipose tissue should be measured to provide an accurate assessment of internal dose and, therefore, to identify groups experiencing different exposures during the recent decade.

Research in appropriate animal models is needed on the role of prenatal exposure to suspected chemicals in inducing cancers later in life or in subsequent generations. Initial studies should focus on HAAs that have been shown to induce cancers of the thyroid, pituitary, and adrenal glands in some laboratory animals.

Ecologic Effects

Environmental HAAs probably have contributed to declines in some wildlife populations, including fish and birds of the Great Lakes and juvenile alligators of Lake Apopka, and possibly to diseases and deformities in mink in the United States, river otters in Europe, and marine mammals in European waters. Such contaminants, along with inbreeding, might have contributed to the poor reproductive success of the endangered Florida panther and the increased embryonic mortality of the snapping turtle in the Great Lakes.

Biologic communities in all the Great Lakes, including species from plankton to top predators, have undergone very large changes in the past 100 years. The changes have resulted from introduction of exotic species, pollution, fishing, development of the shorelines, changes in the lakes' hydrology and that of their tributaries, and other factors. To the degree that HAAs have affected population sizes of individual species, they might also have contributed to those changes in community structure, but it is difficult to account quantitatively for the various causal factors. There is epidemiologic and experimental evidence that some

persistent, bioaccumulative HAAs (also referred to as persistent organic pollutants) produce adverse effects on wildlife populations, but whether the primary effect is mediated by hormonal activity remains to be determined.

Recommendations

Long-term studies of populations subjected to HAA exposures are needed to assess the effects of these chemicals in altering population size, age structure, and dynamics. Observational and experimental studies of the linkages between chemical exposures and alterations of key aspects of life histories should be undertaken to understand how chemical exposures affect long-term ecologic attributes of natural systems. Ultimately, the physiologic and biochemical basis of these linkages, once established, should be determined.

Exposure, Dosimetry, and Screening and Monitoring

Determining the risk of environmental HAAs to humans and wildlife is difficult because exposure to these agents has not been routinely monitored, and effects that might be attributed to background concentrations could be complicated by endogenous hormones, pharmacologic estrogens (e.g., hormonal contraceptives), and naturally occurring HAAs (e.g., phytoestrogens) that are ubiquitous in the environment. Synthetic HAAs have been detected in all environmental media, although concentrations of some compounds, such as PCBs and DDT, have declined in some regions, because their use has been discontinued in those countries. However, those HAAs and others can persist in some media, such as sediments, for years and can contaminate areas far removed from the original site of contamination (e.g., via atmospheric transport). Some human populations have been found to have relatively high exposures to HAAs, primarily from diets that include frequent consumption of contaminated foods, especially fish, or foods containing phytoestrogens.

Understanding the relationships between exposure, absorption, disposition, metabolism, excretion, and response is important for predicting whether exposure to an agent will be harmful. Once inside an organism, the transport of an HAA to potential target organs is influenced by its binding to plasma proteins. Lipophilic HAAs will cross the placenta and can have effects on the developing organism. Adipose and other tissues can accumulate HAAs and serve as reservoirs or depots.

There are important differences among species and between adult and developing organisms in their responses to HAAs. These differences could have important implications when assessing toxicity studies or extrapolating data from one species or subpopulation to another. In addition, biologic responses to some HAAs might be greater at low doses than at high doses.

There are no generally accepted, adequately validated methods for routine

identification or monitoring of exposures to HAAs. In addition, most in vitro and in vivo screening assays do not address the full range of putative actions of HAAs.

Recommendations

Better monitoring of contaminated media is required to determine environmental concentrations of HAAs and to assess the persistence and recycling of HAAs in and between the various environmental media. Background concentrations of HAAs in humans, particularly in adipose tissue and blood, and other biota need to be established. In particular, routes of exposure and the effects of diet need to be assessed to provide a framework for examining the effects of these compounds in the general population and in highly exposed subpopulations.

Differences in response to HAAs among species and between adult and developing organisms need to be investigated further, especially at concentrations encountered in the environment. Dose-response relationships of recognized actions of various HAAs also need to be investigated at those concentrations in in vitro and in vivo studies.

The committee's recommendations for screening and monitoring are consistent, in principle, with those of EPA's Endocrine Disruptor Screening and Testing Advisory Committee. Those recommendations include (1) a battery of short-term assays for rapid and inexpensive screening of putative HAAs that need to be validated, replicated, and deployed appropriately; (2) the development and validation of additional biomarkers that screen for embryonic and fetal events that predict long-term, delayed effects; (3) the monitoring of wildlife as environmental sentinels; (4) further investigation of species- and tissue-specific effects resulting from exposure to HAAs; and (5) better characterization of dose-response relationships of various HAAs through in vitro and in vivo assays at concentrations encountered in the environment.

1

Introduction

In recent years, there has been increasing concern regarding potential adverse human health effects of various environmental contaminants designated by some scientists as "endocrine disruptors" (Thomas and Colborn 1992; Colborn et al. 1993) and referred to in this report as hormonally active agents (HAAs). These concerns have originated, in part, from observations of developmental and reproductive derangements in wildlife populations exposed to a wide range of synthetic chemicals and their by-products. Most notable are the adverse reproductive and developmental effects that have been observed in birds such as cormorants, herring gulls, Caspian terns, and bald eagles that feed on contaminated fish, which have led to drastically lowered reproductive success and population declines (Fox 1992; Giesy et al. 1994a).

Some of the environmental chemicals associated with adverse reproductive and developmental effects in animals mimic the actions of the female sex hormone estradiol and it has been hypothesized that human exposure to these compounds, generically referred to as xenoestrogens, may produce similar adverse effects on reproduction and development and be involved in the increasing incidence of breast cancer in human populations (Davis et al. 1993). It has also been hypothesized that environmental agents with estrogenic, anti-estrogenic, or anti-androgenic effects, or those affecting aryl hydrocarbon receptors may play a role in reported declines in sperm counts, increases in rates of testicular and prostatic cancer, and other urogenital tract anomalies in men (Sharpe and Skakkebaek 1993; Sharpe et al. 1995).

Historically, the focus on xenoestrogens as disruptors of reproductive function is based on an iatrogenic epidemic precipitated by administration of large doses of a potent synthetic estrogen, diethylstilbestrol (DES), to vast numbers of

pregnant women throughout the world. The intent of this treatment was to rectify hormonal imbalances perceived to be causal in the premature termination of pregnancy in women at risk because of histories of habitual spontaneous abortion. The physiologic rationale for this therapy, initiated some 50 yr ago when reproductive endocrinology was still in its infancy, is difficult to reconstruct. It was based, in part, on the perception that estrogen and progesterone production was decreased near the time of normal or premature labor and that estrogens, under some circumstances, can stimulate ovarian function in experimental animals (Smith et al. 1946; Smith 1948). Using the dosage regimen prescribed by the originators of the therapy, and followed by hundreds of physicians, increasingly large doses of DES were administered from early pregnancy until week 35; women who followed this regimen may have received a total dose as high as 1.625 kg of DES orally. Although the dosage protocols varied somewhat from clinic to clinic, millions of women were subjected to this hormonal assault beginning in the 1940s. It has been estimated that in the United States alone some 4 million pregnant women and their fetuses were exposed in this manner between 1947 and 1971 (Mittendorf 1995) and perhaps millions more elsewhere in the world.

In the United States, the use of DES in pregnancy was abruptly halted in 1971 by action of the Food and Drug Administration (FDA) following the finding by Herbst and his colleagues of the appearance of an otherwise rare tumor, clear cell adenocarcinoma (CCA), of the vagina in eight young women whose mothers were treated with DES during pregnancy (Herbst et al. 1971). This study, which identified more cases of CCA than had been previously reported in the medical literature in this age group, marked the initiation of intensive studies of the offspring of women treated with DES during pregnancy. As part of this effort, a Registry for Clear Cell Adenocarcinoma of the Genital Tract in Young Females was established to identify cases, regardless of exposure. As of April 1995, out of a total of 622 identified cases in the United States, ranging in age from 15 to 35 yr, prenatal DES exposure could be documented in 367 cases (Mittendorf 1995).

Adverse consequences of prenatal DES exposure on the human female genital tract, including structural abnormalities and epithelial changes, have been reviewed in detail by Herbst and Bern (1981) and by Mittendorf (1995), and are the subject of continued, intensive investigation. In addition to alterations of the female genital tract caused by DES, the fertility and reproductive performance of DES daughters have been impaired, and the risks of prematurity, spontaneous abortion, and ectopic pregnancy have increased, particularly in DES daughters, with genital tract changes, that were exposed in early pregnancy (Herbst and Bern 1981; Stillman 1982; Mittendorf 1995). Nevertheless, more than 80% of DES daughters who wished to become pregnant delivered at least one live baby (Herbst 1992). Appendix A of this report summarizes these additional findings.

While an increased incidence of malignancies or decreased fertility has not been consistently reported in the male offspring of DES exposed mothers ("DES

sons"), increased rates of genital tract abnormalities including cryptorchidism and hypospadias have been documented (Gill et al. 1976, 1979; Rothman and Louik 1978; Depue 1984; Mittendorf 1995; Wilcox et al. 1995). The available data on sperm concentrations in "DES sons" are inconsistent. The numbers involved, however, are small and, therefore, these studies have limited statistical power. Adverse reproductive outcomes in males exposed to DES during development are reviewed in more detail in Appendix A.

The effects of DES administration on the offspring of treated women and their replication in mice and rats (see Newbold 1995) focused attention on the estrogenic properties of HAAs as potential carcinogens and teratogens. Studies on mice in the early 1960s demonstrated the induction of long-term irreversible changes from exposure to estrogen during a critical developmental period (see Herbst and Bern 1981). These early findings demonstrate, in dramatic fashion, the vulnerability of the fetus during critical stages of development to unphysiologic perturbations in its environment with unforeseen outcomes later in postnatal life, and have focused attention on the fetus as a unique target for the potential disruptive effects of environmental agents with hormonal activity (Bern 1992a). It must be recognized, however, that DES, which is a potent synthetic estrogen, was given to pregnant women and experimental animals in very high doses relative to physiologic estrogenic activity and that great care must be taken in extrapolating the DES experience to other estrogenic substances and dosing regimens.[1] Additionally, laboratory studies indicate that nonestrogenic agents, such as antiestrogens, androgens, and progestins (Bern 1992a,b), may induce some of the same developmental changes that are seen after perinatal estrogen exposure.

The finding that in utero exposure to an estrogen could cause cancer and reproductive anomalies in adult offspring has served as the basis for hypotheses linking the effects of environmental contaminants with estrogenic and other endocrine-like activities to reproductive and developmental anomalies in wildlife and human populations (Colborn et al. 1993).

THIS STUDY

Although HAAs in high concentrations can affect humans and wildlife, whether environmental exposures to them are responsible for a variety of widespread adverse effects on the health of humans and wildlife remains a topic of debate. In response to considerable public and congressional interest in this matter, the U.S. Environmental Protection Agency (EPA), the U.S. Department of the Interior (DOI), the U.S. Centers for Disease Control and Prevention (CDC), and the U.S. Congress requested a National Research Council (NRC) study of

[1] Because DES, while not an "environmental estrogen," has been considered by some workers in the field as a model for the action of estrogens generally, its effects on reproductive organs are summarized in Appendix A.

this topic. In response to this request, the NRC, the working arm of NAS, assembled a multidisciplinary expert committee, the Committee on Hormonally Active Agents in the Environment, under NRC procedures to "review critically the literature on hormone-related toxicants in the environment; identify the known and suspected toxicologic mechanisms and impacts on fish, wildlife, and humans; identify significant uncertainties, limitations of knowledge, and weaknesses in the available evidence; develop a science-based conceptual framework for assessing observed phenomena; and recommend research, monitoring, and testing priorities. To the extent practicable with available information and study resources, the committee [was asked to] identify particular chemical substances, geographic areas, contaminant sources, human subpopulations, and fish and wildlife populations of special concern with respect to hormone-related toxicants in the environment. In addition, if possible and warranted, the committee [should] suggest general approaches for identifying and mitigating these toxicological problems" (Committee Statement of Task).

This committee (like many groups before it) had difficulty deciding on the proper descriptors and definitions of its subject. Historically, the conceptual construct underlying the charge to the committee centered on the finding that the compounds under consideration have estrogenic activity and the hypothesis that this activity disrupts normal developmental and reproductive processes by interacting with estrogen receptors, the so-called "estrogen hypothesis." When the committee began its work, however, it was clearly recognized that the compounds to be evaluated also had antiestrogenic and antiandrogenic properties as well as some other hormonal activities, such as effects on thyroid function, and that not all of their actions are mediated by known hormone receptors. Indeed, the mechanisms underlying many of the effects of environmental contaminants on a variety of systems in animals and humans have not been elucidated. It is for this reason that the widely used descriptor of these compounds, "endocrine disruptors," was considered by some members of the committee to be too restrictive and to imply modes of action that are in fact unknown.

Because the compounds in the "endocrine disruptor" inventory gleaned from the literature—the starting point of the committee's work—by definition have hormone-like activity in at least some test systems, we have chosen the term "hormonally active agents" to describe them without regard to their mode or mechanism of action. This new and unfamiliar terminology was arrived at following extensive discussion, and some discomfort with its use remains (see discussion at the end of this Introduction).

For the historical reasons already mentioned, compounds with estrogenic activity predominate in the list of HAAs but substances in the environment with other demonstrable endocrine activities have not been excluded. Chemicals in the environment that do not conform to the broad definition of HAA—that is, that do not have any hormone-like activity in the usual test systems—were excluded from our study.

It became clear as the work progressed that the same data could be approached with different viewpoints leading to different judgments. These differences are discussed in the next section.

The NRC's charge to the present committee did not include a request to assess risk management policy options, so the committee has not done so in this report.[2] However, the committee hopes that the critical evaluation of the data presented in this report will be helpful in informing those who must make the decisions about policy options.

The report consists of 11 chapters, one appendix, and one addendum. Chapter 2 presents the HAAs that are discussed in this report and describes the current state of knowledge regarding the different modes of action of HAAs. In this chapter, a discussion of the biology of estrogen action is presented as a model for understanding the mechanisms by which HAAs with estrogenic activity could interfere with normal endocrine processes. It has been assumed that lessons learned from the study of estrogenic HAAs might be relevant to understanding the mechanisms of action of other types of HAAs that act by binding to intracellular hormone receptors. Chapter 3 describes what is currently known with regard to exposures and monitoring of HAAs. Chapter 4 reviews the information on dosimetry—how the uptake, distribution, metabolism, and elimination of hormones and HAAs might be involved in the mechanisms leading to altered hormonal processes and their consequences. In Chapters 5-7, the committee identifies a number of vertebrate biologic systems for which there were data relating alterations in these systems to HAA exposure, and separate chapters were devoted to each system: reproductive (Chapter 5), neurobehavioral (Chapter 6), and immune (Chapter 7). Chapters 8 and 9 review what is known about HAAs and cancer in animals and humans, respectively. This is followed by a chapter on

[2] The National Research Council's policy on reports related to human-health risks (unpublished material) emphasizes the need for its study committees to separate matters of scientific fact and analysis from views and judgments about what public policies or measures, if any, should be taken to avoid or limit risk in a given situation. The NRC takes no position on any risk-management philosophy (e.g., the precautionary principle), but asks its study committees to avoid explicitly or implicitly advocating a position on the degree of caution that should be exercised in risk management. Weighing the relative importance of protecting public health and economic interests, for example, in the face of uncertainty is a public policy judgment, not a scientific one. The NRC does not discourage its study committees from addressing risk management questions when the charge calls for it, but recognizes that its proper role is to inform public-policy choices to the extent practicable by describing and interpreting relevant scientific facts and uncertainties for the benefit of citizens and their representatives who must make the policy judgments. NRC committees must carefully separate their proper role of informing policy choices from the different role of recommending policy. When a committee is asked to discuss policy options, the NRC recommends that various options be presented and their implications be explained in a neutral manner. This is not an easy task, but examples of such treatment are illustrated in *Science and Judgment in Risk Assessment* (NRC 1994) and *Science and the Endangered Species Act* (NRC 1995).

ecologic effects (Chapter 10). Chapter 11 describes techniques for screening and monitoring for HAAs. Appendix A provides a summary of reproductive disturbances in humans and experimental animals exposed to DES. In the addendum that follows the References, at the request of EPA, the committee comments on the recommendations made by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC). Because the NRC committee was unable to reach consensus on some important matters, the remainder of this chapter describes these difficult areas.

ISSUES THAT DIVIDED THE COMMITTEE

In its report, the committee's approach comprised three steps: (1) identification of chemicals in the environment with hormonal activity; (2) evaluation of the scientific literature on the effects caused by those chemicals in laboratory animals, humans, and wildlife; and (3) consideration of whether observed effects can be attributed to the hormonal properties of the chemicals and to observed environmental exposures.

There are many areas of consensus among the committee members, reflected throughout the report and in the Executive Summary. However, it became clear as the work of the committee progressed that the same data could be approached from different viewpoints. Those different views led to different judgments among the committee members about the significance of the threat posed by HAAs and are described in this section. Many of the differences reflect areas where additional research would be helpful, and the differences sometime illustrate the kinds of research that are needed. In other cases, the differences do not reflect the need for research but reflect differing judgments about the significance of information. The differences are not confined to the members of this committee but are also reflected in the scientific community at large and in the comments received during review.

Much of the division among committee members appears to stem from different views of how we come to know what we know. How we understand the natural world and how we decide among conflicting hypotheses about the natural world is the province of epistemology. Committee members seemed to differ on some basic epistemologic issues, which led to different interpretations and conclusions on the issues of HAAs in the environment.

In part, difficulty in agreeing on various aspects of the problem arises because many of the terms used are imprecise, with possible differences in meaning that lead to differences in interpretation. For example, the charge to the committee, most broadly and simply stated, was to evaluate the endocrine-disruptor hypothesis. Each term is open for interpretation. What does it mean to undertake an evaluation? What do we mean by endocrine disruptor? And, what exactly is the hypothesis that is to be evaluated? The charge to the committee required more than simply compiling facts and presenting a review of the current literature.

To fulfill its charge, the committee had to (1) interpret the hypothesis, (2) decide on which facts were germane to evaluating that hypothesis, (3) decide on allowable sources of information, (4) assign different weights to different kinds of evidence when data or interpretations were in conflict, (5) establish and evaluate alternative explanations that might account for observed patterns in the data, (6) decide on what type of errors were acceptable and which were not, (7) establish criteria for arriving at meaningful conclusions and recommendations based on understanding of the hypothesis and the status of the data pertinent to the hypothesis, and (8) reach consensus on all of these points. Unfortunately, the committee did not achieve consensus on all these tasks and related issues. In particular, the following issues were the focus of disagreements among the committee members or issues that need some explanation here: (1) in their identification of and approach to the endocrine-disruptor hypothesis; (2) how much to emphasize other hormonal systems (i.e., those not involving sex steroids), (3) identifying allowable sources of information; (4) how to evaluate the evidence, including how to deal with Type I and Type II errors; (5) how to define HAAs, including the importance of mechanism of action of toxicants and their relevance to the endocrine-disruptor hypothesis; (6) the importance of low-dose effects and the shape of the dose-response curves; (7) how to treat periods of susceptibility; (8) how to extrapolate information on one species to others, including humans; and (9) the definition and quantification of environmentally relevant doses, concentrations, and exposures.

The Endocrine Disruptor Hypothesis

The charge to evaluate the endocrine-disruptor hypothesis begins with ambiguity about what the exact hypothesis—and hence the charge to the committee—really is. In its simplest form, the hypothesis is that some chemicals in the environment mimic estrogens (and other sex-hormones) and hence interfere with (disrupt) endogenous endocrine systems, with adverse effects. Interpreted narrowly, it would pertain to one or more species in at least one place. Interpreted broadly, it would mean that the impacts of HAAs are uniform and global; this is probably not meaningful, and can certainly be falsified by a single counterexample. The more interesting alternative lies somewhere between these two extremes. The committee was divided as to the degree to which available data support an interpretation of the hypothesis between the two extremes. An analysis of the hypothesis helps to understand some of the differing judgments among committee members.

The hypothesis must be viewed in the context in which this committee was formed. Public and scientific concern has mounted over a perceived correlation between the presence of certain classes of chemicals in the environment and certain biologic effects. Often, the effects in question—for example, defects in development, declines in fertility, incidence of various cancers, possible popula-

tion declines in wildlife species—are alarming. In some cases, the *effect* is in question; for example, is human sperm concentration really in decline? In other cases, an effect is quite clear, for example, developmental deformities of organ systems in wildlife species, including reproductive organs, but the *cause* is in question. Because there is simultaneous ambiguity at the level of both causes and effects, it is hard to identify the real nature of the question to be reviewed, and the committee decided first to clarify the nature of the hypothesis under scrutiny. Clarifying the hypothesis is related to clarifying the definition of "endocrine disruptor" and agreeing on an appropriate term; that problem is discussed later in this chapter.

For the endocrine-disruptor hypothesis to be understandable, some effect of some chemical on or through the endocrine system must be documented. The endocrine system can be disrupted in many ways that would lead almost every chemical with a disruptive effect on the organism to be classified as an endocrine disruptor; however most proponents of the hypothesis have a narrower definition in mind. Their formulation of the hypothesis suggests that some chemical inputs can act analogously to specific hormones, and the chemicals either overmodulate or undermodulate specific activities of hormones, producing specific effects that are hormonally mediated. Even the term "disrupt" is subject to differing interpretations: exposure to chemicals can result in physiologic adaptations within the normal range of variation to the death of the organism, and all gradations between those extremes. There is not agreement on how to categorize those responses. The EDSTAC report (EPA 1998) described a similar disagreement among its membership, also involving the difficult problem of an objective judgment of what constitutes an "adverse" effect.

The above is why the committee refers to such chemicals as hormonally active agents rather than as endocrine disruptors. However, there is still ambiguity, because modulation of hormonal activity can occur through a variety of mechanisms.

The committee recognized that it could not undertake a general evaluation of the correlation between specific chemicals and general effects. By restricting its review to information about chemicals that produce effects through hormonal pathways, the committee did not intend to suggest that there are no correlations between a variety of chemicals and effects not mediated through hormonal pathways or that such effects are not important. The committee also made no judgment as to whether such chemicals produce effects mediated through other mechanisms. An alternative interpretation of the hypothesis would probably lead to a different review and interpretation of evidence.

Other Hormonal Systems

The early focus of the endocrine disruptor literature on compounds with gonadal steroid activity and the need to limit the scope of the study prompted the

committee to emphasize these activities and their consequences. It was recognized that interference by HAAs with other hormonal and non-hormonal physiologic systems may have major impacts on development and other functions but the literature on these was not abundant and there was some disagreement about the committee's availability of time and resources to further extend its purview. In addition, various HAAs have been reported to have profound effects on the biosynthesis and metabolism of thyroid hormones with consequent reductions in circulating thyroid hormone levels (Brouwer et al. 1998). Whether this disruption of thyroid hormone homeostasis mediates some of the developmental effects associated with exposure to these HAAs remains to be determined.

As an example, the retinoic acid system is another pathway the committee did not consider in detail that could result in serious developmental defects if disrupted. Retinoids and retinoid receptors are key factors in vertebrate development. TCDD, planar PCBs, and a variety of other compounds affect development through processes governing cell fate and organ development and function. Phenotypically, these effects in some species resemble effects resulting from excess or from deficient levels of retinoic acid (Birnbaum et al. 1989). That suggests that at least some developmental abnormalities could result from chemical effects on retinoid synthesis, retinoid inactivation, or on retinoid receptors or function.

Interactions between different hormonal systems can link effects on one system to outcomes through another system. For example, thyroid receptors form functional heterodimers with 9-cis retinoic acid receptors (RXRs) (Puzianowska-Kuznicka et al. 1997). Linkage of the thyroid system to estrogen-active compounds also is suggested, as the estrogen receptor-related orphan receptor alpha 1 stimulates the expression of the thyroid hormone receptor alpha (Trα) (Vanacker et al. 1998).

Allowable Sources of Information

The literature related to endocrine disruption is large and often controversial. Articles, reports, abstracts, and circulating manuscripts appear at an accelerating rate. The committee had to choose what to base its conclusions and recommendations on. The foremost problem of the committee then was to provide meaningful information with respect to this broad and highly controversial topic and yet keep the scope of its review manageable.

Many members of the committee are involved actively in research in this area, and so they often had access to unpublished results or manuscripts. Although such information could alert the committee to possible trends, unpublished reports could not be included in this evaluation because they had not undergone independent peer review. Similarly, other unreviewed information, including much literature posted on the Internet, many federal agency reports, and some reports from industry and nongovernmental organizations, were not

used in evaluating the data. However, such publications, including abstracts, have been cited in the report for informational purposes.

Restricting information sources to published, peer-reviewed material meant that some late-breaking results were not fully evaluated, including results that may be inconsistent with some of the report's conclusions, but that restriction was the clearest and most objective way to deal with the problem of unpublished data. Peer-review, of course, provides no guarantee of accuracy or reliability. In some cases, phenomena reported in peer-reviewed papers could not be observed by others, and some unreviewed reports have been influential and accurate.

The committee evaluated the conclusions of peer-reviewed papers based on the methods used by the investigator. Often, the assessment of a topic was revisited many times because investigations are ongoing on many of the more controversial topics, and so some conclusions are unavoidably tentative, although they are based on the best available information from peer-reviewed sources at the time this report was completed.

Evaluating the Evidence

Under ideal conditions, a critical experiment can be performed with an outcome that can definitively decide between two hypotheses. The Meselson-Stahl demonstration of the semiconservative replication of DNA (Meselson and Stahl 1957) and the Michelson-Morley experiment refuting the existence of a "luminiferous ether" (Michelson and Morley 1887) are classic examples of this sort. Similarly, Koch's postulates (Koch 1876; Yerushalmy and Palmer 1959) have been used as guides for designing critical experiments to establish causes of infectious disease.

Although such critical experiments can give elegant and definitive answers to important theoretic and practical questions, they are limited to hypotheses for which one can construct controlled environmental conditions that essentially isolate the process under investigation from all other processes. Koch's postulates, for example, depend entirely on the ability of the investigator to isolate putative causal agents from alternatives.

Many important hypotheses about causal mechanisms cannot be tested through critical experiments simply because the factors generating responses cannot be isolated from each other. This is often true when attempting to resolve environmental effects on biologic processes outside the laboratory setting. For example, potentially harmful chemicals seldom exist in isolation from other chemicals in the environment, and they usually interact with other physical environmental factors. The ability to deal with potential effects due to mixtures of chemicals continues to plague toxicologists, and these difficulties apply to HAAs. Many reports have recommended further research on mixtures of chemicals, and this committee agrees with that recommendation. However, the committee has

no illusion that the problem of how to apportion cause among the members of a mixture of environmental chemicals will soon be easily solved.

In systems where definitive critical experiments are difficult or impossible or are dominated by complex interactions under natural conditions, one can either withhold judgment or take a weight-of-evidence approach, in which experimental evidence is only one of several lines of evidence used in determining likely causal mechanisms.

The committee agreed that lack of evidence could not be taken as an indication that a proposed process does not operate. The greatest disagreement within the committee concerned how much we should rely on different positive lines of evidence in evaluating the endocrine-disruptor hypothesis or on the weight that negative evidence should receive. The committee often failed to agree on the relative importance of each class of evidence in reaching a common judgment on causation, or even whether a specific criterion could be applied to the hypothesis.

To a large degree, differences among committee members could be divided along two perspectives on the weight-of-evidence approach. Some committee members placed almost exclusive weight on experimental evidence and the establishment of a plausible mechanism of action. Other committee members placed less weight on the mechanism of action and more weight on consistency and coherence of results among studies and an analogy with other compounds in test systems, especially endogenous sex-steroid hormones. Because the weight-of-evidence approach does not by itself dictate how weights should be assigned to the factors, both of these positions are equally valid.

Type 1 and Type 2 Errors and the Precautionary Principle

Committee members had differing views on how analyses of risks to humans and wildlife should be influenced by the probability of making Type 1 and Type 2 errors. A Type 1 error is the conclusion that an association (between exposure and adverse health effects in this case) exists when in fact it does not. A Type 2 error is the conclusion that there is no association (between exposure and adverse health effects) when in fact there is. Research cannot remove all uncertainties in describing the real world, and so some assumptions must be made in statistical analyses. Specifically, some committee members felt that the precautionary principle requires statistical analysts to assume as the default hypothesis that an environmental agent has adverse effects. This approach, discussed in detail in an NRC report (NRC 1995), would mean that the alternative hypothesis of no effect would be rejected unless the probability of its occurrence by chance was less than 5%. Other committee members felt that the above approach would amount to trying to prove a null hypothesis of no effect for every chemical, a large and difficult task. Also, they considered that choosing the appropriate default hypothesis of an effect would present statistical difficulties, such as deciding how much of an effect to hypothesize. They considered that testing the null hypoth-

esis of no effect is statistically sound and widely accepted scientifically, and that choosing a default hypothesis of some effect amounted to a confounding of risk analysis with management.

Simberloff (1990) provided a thoughtful analysis of this matter, concluding that the automatic adoption of a null hypothesis, whether it is that there is no effect or that there is an effect, may not be the best way to analyze every scientific uncertainty. He pointed out that the decision to minimize either Type 1 or Type 2 errors should be based on a careful evaluation of each case, and not be accepted as a default of the analysis. We refer interested readers to that paper and to the NRC's 1995 report for a more detailed discussion of the problem.

Defining a Hormonally Active Agent

Early in its deliberations, the committee decided that in this report the term endocrine disruptor should be replaced, recognizing that the term is fraught with emotional overtones and was tantamount to a prejudgment of potential outcomes. Furthermore, because all the compounds initially considered by the committee possessed hormonelike activity in at least some test system, the committee adopted *hormonally active agents* (HAAs) as a more neutral mechanistic descriptor. Thus, the use of HAA was meant to describe these agents without regard to the outcome of any critical evaluation of their mode or mechanism of action. Any definition provides a semantic filter of sorts that limits the number of substances reviewed, and the committee members felt varying levels of discomfort with respect to the definition and change in terminology, because any attempt to clarify can either eliminate a major portion of the problem from consideration or expand consideration to compounds not originally contemplated.

Indeed, the problem of defining HAAs is inseparable from the mechanism problem: A single chemical can have multiple effects on an organism that act through several mechanisms, not all of which involve hormone receptors. Thus, some observers object to the characterization of such a chemical as an HAA, and others wonder if calling it an HAA predisposes a conclusion to the question of whether its effects are related to its hormonal activity. The example of DDT's role in the production of thin-shelled eggs by carnivorous birds is illustrative. There is no controversy about the observation that DDT (or its metabolites) causes shell thinning in carnivorous birds' eggs, such as cormorants and eagles. There is also no controversy about the observation that DDT and its derivatives can mimic some actions of estrogens. The difficulty arises because DDT can also have effects that are not directly related to these hormones. The controversy continues because many mechanisms have been proposed to explain how DDT causes eggshells to be thin, but none has been firmly established to be related to the action of estrogen (Chapter 5).

In approaching the issue of HAA mechanisms, therefore, the committee does not believe that HAAs can function only through receptor binding, although that

is the particular mechanism that has been most studied. Where mechanisms of HAA action are known they are presented in this report. Where the mechanisms are unknown, they often are not mentioned in this report. Lack of knowledge about a mechanism does not mean that a reported effect is unconfirmed or unimportant; it does suggest that additional studies are called for. This leads again to the question of how HAA should be defined. Is a compound an HAA if it can affect hormonal pathways, or is it an HAA only if in a particular case it does affect a hormonal pathway? If it is the former, then should all the biologic effects of such a compound be considered in this report, even if they are unrelated to hormonal pathways? Is it even theoretically possible to describe a biologic effect of an HAA, indeed of any chemical, as completely unrelated to hormonal pathways? The answers to these questions are not agreed on by all the members of the committee.

Dose-Response Relationships

In considering the consequences of HAA exposure, it is important to know the shape of the dose-response curve for a particular HAA. In evaluating the data, it became apparent that committee members opinions differed on the extent to which inverted U-shaped curves should be emphasized in this report, in light of the paucity of data regarding the actions of HAAs. All members agreed in principle that this phenomenon is potentially important for evaluating the results of bioassays and for designing toxicologic studies. If an underlying monotonic dose-response function (i.e., a function where response increases as dose increases or at least does not decrease) and a dose below which there is no effect (a threshold dose) are assumed when designing a toxicologic study, there is a risk of failing to detect a contaminant that is inactive at intermediate doses but does have an effect at low doses, in other words, one that does not display a monotonic dose-response function.

There is a published report that DES, a powerful estrogen, when administered prenatally to mice, elicits an inverted U-shaped curve in the prostate gland of the offspring (vom Saal et al. 1997). The higher doses administered reduced prostatic size whereas lower doses increased it; still lower doses had no effect. Other workers using the same strain of mice at the one dose of DES that caused a maximal increase in prostate weight in the vom Saal et al. (1997) study were unable to replicate that finding (Cagen et al. 1999).

Inverted U-shaped dose response curves have been reported in other in vivo studies. vom Saal et al. (1995) reported an inverted U-shaped-dose relationship between maternal doses of DES and territorial marking in male offspring. These investigators also reported an inverted U-shaped dose-response relationship with respect to prostate size when mouse fetuses are exposed to estradiol (vom Saal et al. 1997). Halling and Forsberg (1993) reported that uterine weight as a fraction of body weight following neonatal dosing displayed an inverted-U dose response

with DES but not estradiol. Fan et al. (1996) showed that TCDD induced an inverted U-shaped dose-response curve for its effects on cell-mediated immunity in rat; at low doses, TCDD enhanced and at high doses TCDD suppressed the delayed-type hypersensitivity reaction.

Periods of Susceptibility

The committee members agreed that there are important, critical periods during pre- and postnatal life during which an organism is particularly sensitive to exposure to various chemicals, but disagreed on how much emphasis should be placed on such critical periods in the absence of relevant data regarding HAAs. Despite growing information on the timing of events in differentiation in vertebrates, there is not much information that specifically links that timing to the actions of specific HAAs.

The issue is important because molecular events early in development determine phenotype later in development and in the adult. Disruption of these early events can thus produce effects that become apparent only later in development or in juveniles or adults. There are well-documented cases where exposure to chemicals such as PCBs and TCDD early in development causes severe defects later, whereas exposure later in development has no adverse effects (e.g., Vandersea et al. 1998).

Extrapolation Between Species

There were disagreements among committee members about the extrapolation of findings made with one species to others. In some cases, some members argued that if an adverse effect was observed in one species, then one should assume or suspect its occurrence in others. Other committee members considered that it was necessary to establish the adverse effect for each species of concern. In other cases, some committee members argued that the absence of an adverse effect in one or a few species meant that an adverse effect was unlikely in any species, while other members argued that it was necessary to establish the lack of an adverse effect for each species of concern. These disagreements led to disagreements about how to describe cross-species extrapolations and the comparative physiology of various species, and what to conclude based on incomplete information.

This issue is important because concern over particular effects ascribed to HAAs in the environment often is based on observations with one or a few species. The degree to which such observations apply to species other than those they were made on is crucial to any judgment about the seriousness of the risk of HAAs to wildlife, ecosystems, or humans. Although it is usually impossible to extrapolate qualitative dose-response relationships from one species to another, it is often prudent and useful to infer broad similarities in processes involved in toxicity

between species in the same taxonomic order and sometimes even in the same taxonomic class. The difficulty arises in deciding whether and when to generalize.

The difficulty is compounded because different processes behave differently with respect to inter-species differences. At one extreme, animals as distantly related to humans as nematodes have 40% of their genes in common with human genes. Genes and gene products governing pattern formation are structurally similar and appear to operate similarly even in evolutionarily distant species (e.g., Tomarev et al. 1997). At the other extreme, differences between genetic strains of a single species, due sometimes to a difference at only one genetic locus, are the basis of much of our understanding of development and physiology. An example of susceptibility differences among closely related organisms is the more than 5,000-fold difference in toxicity to TCDD among different mammal species (Pohjanvirta and Tuomisto 1994). A single allelic substitution (e.g., alcohol dehydrogenase, cytochrome P450) can affect responses to a toxicant within a single species (e.g., Meyer et al. 1990). The committee members did agree on the general principle that it is necessary to characterize the nature of interspecies differences.

Environmentally Relevant Doses, Concentrations, and Exposures

Committee members did not agree on the definition of "environmentally relevant" as applied to HAAs, or on how much information was required to establish environmental relevance. A related problem is the imprecise use of the terms "dose," "concentration," and "exposure" in toxicology literature in general. The two issues are discussed here together.

In this report, as in environmental toxicology in general, there is concern regarding the concentrations of suspect chemicals to which organisms are environmentally exposed. High doses often are used in the laboratory, as, for example, the use of maximum tolerated dose in early phases of evaluating carcinogenicity of chemicals (Goldstein 1994). However, our greatest concern in this report is whether the much lower doses of HAAs that result from environmental exposure cause adverse effects. "Environmentally relevant" is often used to refer to concentration and the resulting doses that organisms might encounter from exposures in nature or in the workplace. *Environmentally relevant* can apply to the presence of and exposure to chemicals in several contexts. The committee members did not agree on how much and what kinds of information are needed to establish how well laboratory experiments are environmentally relevant or what concentrations of chemicals in the environment are physiologically relevant. The following sections describe the relationship between environmental concentration, environmental exposure, and dose. In much of the published literature the committee reviewed, distinctions between environmental concentration, exposure, and dose often are not made. This failure can reduce the relevance and value of research results.

Environmentally Relevant Chemical Residues. For a compound to be environmentally relevant, it must exist in the environment. However, in the environment, one almost always finds mixtures. Experiments that use such mixtures, whether extracted from environmental matrices or artificially produced, may be relevant to contemporary environmental exposures and would account for possible interactive effects of chemicals in the mixture. Such experiments would not identify the specific agent or agents involved in effects of those mixtures. Furthermore, there is a lag between exposure and effect. For example, if exposure during development produces an effect only in the adult or if there is a long latent period, then it is usually too late to measure the exposure when the effect is observed, because the causative agent has long since disappeared. This may be of greater concern in long-lived species, including humans, and for conditions known to have a long lag time before diagnosis, such as some cancers.

Environmentally Relevant Chemistry. Chemicals of concern as potential endocrine disruptors or HAAs in the environment most often are organic chemicals that are hydrophobic to varying degrees. Such chemicals seldom are free in the environment but are complexed with organic or particulate matter. The chemistry governing that complexation may determine the bioavailability of the compounds (Farrington 1991). Moreover, different structures may be broken down by bacterial action at rates that depend on the structures.

Environmental Concentrations. This term refers to concentrations as they exist in the environment. The environmental chemistry processes will affect the concentration available. The effect of environmental chemicals depends on their concentration and the degree and method of exposure to them that organisms undergo. Experiments that use such concentrations may produce exposures typical of those experienced by organisms in the real world, but to faithfully reproduce such exposures, all routes of exposure must be considered and integrated.

Environmentally Relevant Routes of Exposure. Exposure refers to contact with an environmental chemical by a particular organism. The amount or degree of exposure is a function of the concentration of the chemical in the environment and the duration (time) of the contact (NRC 1991a; Ott 1995). Although there are many kinds of exposure, such as peak exposure, average exposure, minimum exposure, instantaneous exposure, integrated exposure, and so on (Ott 1995), our main concern here is with the fact of exposure and not with the detailed nature of the exposure, although the details are required to assess quantitative risks. We include all environments an organism experiences in our understanding of exposure, including workplace environments; we exclude the deliberate or accidental administration of chemicals by means of drug-delivery systems. The matrix in which chemicals occur and the lifestyle of an organism will determine the dominant route of exposure. If an objective is to examine the consequences of exposure in the environment, routes of exposure should be considered in experimental design. Exposure by intraperitoneal injection of adult females may be adequate to determine if a chemical or mixture has the capacity to elicit specific changes.

But if the major route of exposure is dietary, as it is for many chemicals for most vertebrates, then relating experimental exposure to that in the environment should include a dietary route.

Environmentally Relevant Doses. A dose is the amount of a chemical that enters an organism's body (often expressed as an amount per unit weight). This is also often called the "internal dose." An "environmentally relevant dose" as used in this report means a dose administered in an experimental setting similar to that which would result from exposure to the same chemicals at concentrations that occur in the environment. To determine whether an experimental dose of any given chemical is environmentally relevant requires knowledge of environmental concentrations of the chemical and the resultant doses experienced by organisms that encounter it in the environment.

2

Hormonally Active Agents

THE TERM HORMONALLY ACTIVE AGENTS (HAAs) is used in this report to describe substances that possess hormonelike activity, regardless of mechanism. Compounds with estrogenic activity received most of the initial concern regarding endocrine disruptors and are the most prominently studied HAAs. Therefore, they comprise the majority of the agents that could be critically evaluated by the committee. Antiestrogens, antiandrogens, aryl hydrocarbon (Ah) receptor agonists, and other toxicants acting through effects on hormonal systems are considered to a lesser extent but are no less important. Indeed, many published papers show that various hormonal systems (e.g., thyroid systems) are targets of HAAs. Thus, the emphasis of this chapter on xenoestrogens reflects the published literature, but does not imply any judgment about the relative importance of other HAAs.

This chapter identifies the synthetic HAAs that were evaluated by the committee and discusses naturally occurring HAAs, such as phytoestrogens. Although naturally occurring HAAs are not discussed extensively in the chapters on biologic effects, those compounds are ubiquitous in the environment and may confound background levels of exposure to HAAs (see Chapter 3).

One of the charges to the committee was to identify, if possible, the underlying mechanisms of action of HAA-related effects, and so this chapter also considers the mechanism of action of estrogenic compounds as a model of others wherein a ligand binds to a receptor and the resulting ligand receptor complex alters the transcription of mRNA, and ultimately cytoplasmic translation and protein synthesis. Thus, convincing evidence that an HAA can affect the endocrine system would be its ability to bind to classic hormone receptors and promote measurable responses, such as the induction of hormone-responsive genes or gene products. However, chemicals can disrupt hormonal processes, such as

hormone synthesis, metabolism, organ-system interactions, and hypothalamic-pituitary-gonadal-axis responses, by a variety of other mechanisms.

HORMONE-RECEPTOR-MEDIATED ACTIONS

If an environmental factor influences a biologic organism, there must be physical interaction between the factor and the organism. Most physiologic systems involve detector or sensory molecules called receptors (Jensen and Jacobson 1960; Gorski et al. 1968). Receptors are present in the target cells of various organisms and interact with specific regulatory molecules present naturally, or in some cases, unnaturally, in the target cells' immediate environment. Many receptors—neural, hormonal, and developmental—are involved in different aspects of an organism's development and physiology.

The estrogen receptor (ER) is located in the cell nucleus and is a member of the superfamily of steroid and thyroid hormone receptors that act as ligand-induced nuclear transcription factors (Evans 1988). Steroid-thyroid hormone receptors contain several common structural domains that are conserved between the various members of this superfamily (Gronemeyer 1991; Truss and Beato 1993; Beato et al. 1995; Ing and O'Malley 1995; Mangelsdorf et al. 1995). Figure 2-1 is a model of ER ligands moving into a target cell and interacting with the ER in the nucleus. Steroid-hormone-binding domains are located in the C-terminus of the ER and are required for hormone-induced activation of nuclear transcription factors. Steroid-hormone-receptor-mediated induction of gene expression is a complex process that involves formation of the nuclear receptor complex, binding to hormone-responsive elements, and interaction with other transcription factors and coactivators associated with the RNA polymerase II transcription-initiation complex (Murdoch and Gorski 1991). The transactivation process is complex and requires interactions of proteins that bind to proximal and distal sequences in the 5′-promoter region of target genes. Other influences, including various coactivators, also modulate steroid-hormone-induced gene expression (Beato et al. 1995).

The nuclear localization of steroid-hormone receptors and the lipophilic nature of their ligands means that estrogen mimics or toxicants can readily access fundamental gene regulatory mechanisms in target cells. This group of regulators and their receptors constitute a system that is accessible to a variety of environmental factors that can readily disrupt normal physiologic mechanisms. The sections that follow discuss in more detail the nature of the ER and other steroid or steroidlike hormone receptors.

Estrogen Receptor

The ER is unusual because it recognizes a wide spectrum of chemicals as ligands and can form different architectural structures with them (Brzozowski et

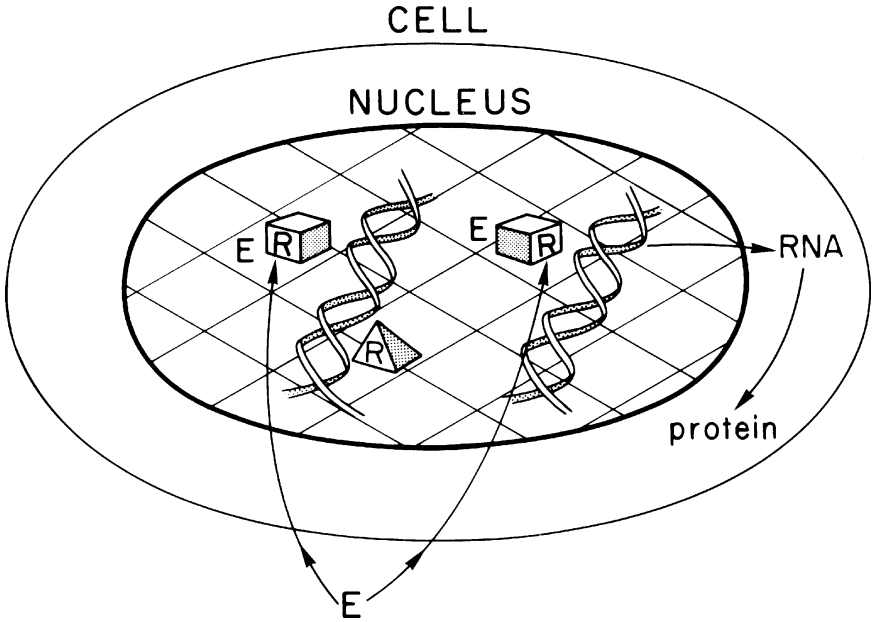

FIGURE 2-1 A simplified model of the cellular action of estrogens, androgens, thyroid hormone, or many HAAs. Most HAAs are assumed to be able to diffuse through cell membranes to reach the receptors located in the nucleus. R, receptor; pyramid, unoccupied receptor; box, occupied receptor; E, environmental compound such as estrogen, androgen, thyroid hormone, or HAA that can interact with the receptor. Source: Gorski et al. 1986. Reprinted with permission from *Recent Progress in Hormone Research;* copyright 1986, Academic Press, San Diego, Calif.

al. 1997). The shape of those structures affects the type of cellular and physiologic activities that can occur. For example, antiestrogenic versus estrogenic activities can occur because of the interactions of the ER with different ligands (Jordan and Murphy 1990). As is detailed in later chapters, many organic compounds have some estrogenic or antiestrogenic activity that can lead to a variety of biologic effects (see Chapters 5-10).

The diversity of ligands that interact with ER makes it difficult to predict structure-function relationships. Binding is extremely specific and capable of differentiating stereoisomers (Noteboom and Gorski 1965). Receptor affinity and occupancy are quantitative aspects of estrogen and antiestrogen binding to the ER that are important aspects of ER function and the effects of hormone mimics. Those quantitative aspects and the structures resulting from different ligand-receptor complexes are discussed below.

Receptor Affinity

The differences in dissociation rates of estrogens and antiestrogens from the ER have important biologic implications (Williams and Gorski 1972). The binding of estradiol to the ER has an equilibrium constant of ~ 0.1 nM because of a fast-forward rate of association and a relatively slow dissociation or off rate. At 0°C, the dissociation rate of estradiol is practically 0. At 37°C, the dissociation rate increases, resulting in a half-life of about 2 h (Kassis et al. 1986). Other ligands vary in their rates of dissociation; compounds such as estriol and estrone have rapid rates of dissociation and half-lives of just a few minutes (Pavlik and Katzenellenbogen 1980). There is little difference between the receptor affinity of human and other animal receptors, as would be expected because of the structural similarity of receptors in different species. It should be noted, however, that receptor half life is not the only determining factor relating the ligand to biologic activity. Systemic half life, which is effected by metabolism and clearance of the ligand, also must be considered when making assumptions with respect to biologic implications of the individual ligands.

Rapidly dissociating ligands, such as estriol, have been called "weak estrogens" (Stack and Gorski 1985). When they are injected in large quantities, these compounds often give a significant biologic response. However, only responses that occur shortly after the hormone is injected are observed. Cell replication and other, later, responses to estrogen are not observed. When the weak estrogens are injected at frequent intervals, changes in long-term responses also are detected (Stack and Gorski 1985). In cell culture, when weak estrogens are present at concentrations that cause a reasonable percentage of the receptors to be occupied, long- and short-term responses are observed (Stack and Gorski 1985). Thus, many compounds that can interact with the ER in a sustained manner can have a biologic effect even if they bind with a low-equilibrium affinity. However, in this case, the amount of such a compound must be great enough that it will occupy a significant number of estrogen-receptor-binding sites so as to induce an effect. A concentration 100 times that of estradiol would be necessary for a compound whose affinity is 1/100 that of estradiol. Many of the plant estrogens and environmental estrogens have affinities that are 1/100 to 1/1000 that of estradiol. Although ligands such as estradiol are classified as weak estrogens, that does not necessarily mean that they have fast clearance rates from the body. Dissociation rates and clearance are not directly related because multiple metabolic factors determine the rate of clearance. For example, clearance of a substance is related to plasma protein carrier kinetics, liver and kidney action, and metabolism of the agent within the target cell itself. In this regard, is it possible that estrogenic compounds with low receptor-binding affinities may still be biologically potent if they circulate for long periods within the body.

Accurately measuring rapidly dissociating estrogens is difficult when they

are bound to the receptor. Under true equilibrium conditions, an accurate estimate of the affinity and the number of occupied receptors can be made. However, most rapid assays of receptor binding are not true equilibrium measurements. For example, in assays in which either the receptor or the ligand is bound to some solid support, such as charcoal, the bound material is no longer in equilibrium when the unbound ligand is washed away from the receptor. Thus, a low-affinity ligand—as are most of the environmental estrogens—is apt to dissociate from the receptor during the assay.

Receptor Occupancy

A rat uterine cell has about 20,000 ERs, approximately 10 nM. Clark et al. (1972) showed that under physiologic conditions, up to one-half of the receptors were occupied. Ruh et al. (1973) demonstrated that intact uteri that are incubated in a medium containing estradiol, estriol, or estrone exhibit various quantities of occupied ERs, which correlates with the induction of a specific protein in this tissue. MCF7 cells are derived from a human breast-cancer line and are widely used as a model cell-culture system of estrogen-regulated growth. MCF7 cells have about 50,000 ERs each. Maximal growth response occurs at about 0.1 nM estradiol, and a substantial response occurs at concentrations as low as 1 pM (Welshons and Jordan 1987). Under these cell culture conditions, we would expect that only 1,000 to 5,000 ERs per cell would be occupied. Thus, small pools of receptors that could be difficult to distinguish in a cell could be important biologically.

Diversity of Ligand-Receptor Complexes

There is evidence that estrogenic and antiestrogenic ligands cause differences in the structure of the ER (Hansen and Gorski 1985). When estradiol binds to the ER, the receptor's surface properties change significantly from hydrophobic to hydrophilic (Fritsch et al. 1992). The extent of this change in surface properties is different when 4-hydroxytamoxifen binds to the ER. Other estrogenic and antiestrogenic compounds could be studied to determine whether the structural characteristics of the bound ER are different when bound to estrogen mimics.

ER Subtypes

ER-mediated transactivation depends not only on cell- and gene-promotor context, but on ER subtype. Before 1996, most studies characterized estrogen responses of one ER (ER_{α}); however, the recent discovery of an ER subtype (ER_{β}) extends the potential tissue- and ligand-specific induction of estrogenic and antiestrogenic responses (Kuiper et al. 1996; Mosselman et al. 1996; Tremblay et

al. 1997). ER_α and ER_β exhibit a common domain structure and high homology in the ligand-binding AF-2 and DNA-binding domains, but less than 25% homology in their N-terminal AF-1 domains. ER_α and ER_β exhibit overlapping and differential expression in various tissues and cells, and both proteins readily form homo- or heterodimers that bind estrogen-responsive elements (EREs) in gel mobility shift assays (Cowley et al. 1997; Pace et al. 1997; Pettersson et al. 1997). ER_α and ER_β bind estrogenic steroid hormones, naturally occurring estrogenic compounds, and synthetic estrogens, including many of those presented in Tables 2-1 and 2-2 (Kuiper et al. 1997, 1998; Watanabe et al. 1997). A recent report described the relative binding affinities of 60 different estrogenic compounds to both ER_α and ER_β and only a few compounds exhibited major differences in binding to the ER-subtypes (Kuiper et al. 1998). For example, the relative binding affinity of genistein for ER_β was greater than 20-fold higher than for ER_α; however, in transactivation assays dependent on ER_α or ER_β, the activity of genistein was similar for both ER subtypes. Additional studies will be required to characterize fully ligand-induced ER_α and ER_β estrogenic and antiestrogenic activities.

Other Steroid or Steroidlike Hormone Receptors

Among the many nuclear receptors for steroid and related hormones are receptors for the androgens, thyroid hormones, and adrenal glucocorticoids, all of which have been implicated as targets of HAAs. The receptors have many characteristics in common with the ER. There is some controversy about whether the glucocorticoid receptor is nuclear or cytoplasmic when not bound to a ligand (Yamamoto et al. 1988). In either case, the receptor is always nuclear when bound to a glucocorticoid. The androgen receptor has a higher affinity for a testosterone metabolite, 5α-dihydrotestosterone (DHT), than it does for testosterone itself. DHT is formed in some but not all androgen target tissues; thus, the effects of testosterone in a whole organism will vary from tissue to tissue, depending on the presence of the 5α-reductase enzyme, which converts testosterone to DHT (Wilson et al. 1993). The ability of other compounds to react with the androgen receptor therefore depends on the nature of the compound and on whether it would behave more like testosterone or DHT or is a substrate for the reductase.

The thyroid hormone receptor is the protooncogene for the oncogene Erb-a (Sap et al. 1986). The mutated oncogenic form does not bind thyroid hormone but is constitutively activated. There are not as many studies that consider the range of compounds that can bind to the thyroid hormone receptor as there are for the ER, but the possibility of environmental sources of such compounds must be considered.

TABLE 2-1 Identification of Synthetic Compounds with Estrogenic Activity[a]

Compound	In Vitro Bioassay			In Vivo Bioassay	Reference
	E-Screen	ER Binding	Induction		
Organochlorine Compounds					
DDT (technical)	√	√	√	√	Welch et al. 1969; Bitman and Cecil 1970; Soto et al. 1994, 1995, 1997
o,p′-DDT	√	√	√	√	Bitman et al. 1968; Bitman and Cecil 1970; Ecobichon and MacKenzie 1974; McBlain et al. 1977; Robison et al. 1984, 1985a,b; Galand et al. 1987; Johnson et al. 1988; Johnson et al. 1992; Soto et al. 1994, 1995, 1997; Brown and Lamartiniere 1995; Vom Saal et al. 1995
o,p′-DDE	√	√			Bitman and Cecil 1970; Johnson et al. 1992; Soto et al. 1995
p,p′-DDE[b]	√	√			Soto et al. 1995, 1997; Ren et al. 1996
p,p′-DDT	√	√		√	Soto et al. 1995, 1997
Chlordecone (Kepone)	√	√	√		Gellert 1978b; Hammond et al. 1979; Soto et al. 1994, 1995; Flouriot et al. 1995
Endosulfan	√	√			Soto et al. 1994, 1995; Sonnenschein et al. 1995; Arnold et al. 1996
α-Endosulfan	√	√	√		Soto et al. 1994, 1995
β-Endosulfan	√				Soto et al. 1994, 1995
Methoxychlor	√	√	√	√	Tullner 1961; Bitman and Cecil 1970; Bulger et al. 1978; Cummings and Metcalf 1994, 1995; Vom Saal et al. 1995; Soto et al. 1995
Toxaphene	√		√		Soto et al. 1994, 1995; Sonnenschein et al. 1995; Arnold et al. 1996

continued

TABLE 2-1 *Continued*

Compound	In Vitro Bioassay			In Vivo Bioassay	Reference
	E-Screen	ER Binding	Induction		
PCB mixtures	√			√	Bitman and Cecil 1970; Ecobichon and MacKenzie 1974; Gellert 1978a; Jansen et al. 1993
2-Chlorobiphenyl	√			√	Ecobichon and MacKenzie 1974; Soto et al. 1995
2,2′-Dichlorobiphenyl				√	Ecobichon and MacKenzie 1974
2,3,4-Trichlorobiphenyl	√			√	Soto et al. 1995
2,2′,5-Trichlorobiphenyl				√	Li and Hansen 1995
2,2′,4,5-Tetrachlorobiphenyl	√				Soto et al. 1995
2,3,4,5-Tetrachlorobiphenyl	√				Soto et al. 1995
2,4,4′,6-Tetrachlorobiphenyl	√				Soto et al. 1995
2,2′,5,5′-Tetrachlorobiphenyl				√	Jansen et al. 1993
2,2′,3,3′,6,6′-Hexachlorobiphenyl	√				Soto et al. 1995, 1997
2,2′,4,4′,6,6′-Hexachlorobiphenyl				√	Ecobichon and MacKenzie 1974
Lindane	√	√			Soto et al. 1995
Dieldrin	√				Soto et al. 1994, 1995; Sonnenschein et al. 1995; Arnold et al. 1996
Dichlorophenol			√		Jobling et al. 1995
Phenolics					
Alkylphenol ethoxylates	√	√	√		R. White et al. 1994; Sumpter and Jobling 1995; Jobling et al. 1996; Routledge and Sumpter 1996
4-Alkylphenols	√	√	√	√	Soto et al. 1991, 1995; Jobling and Sumpter 1993; R. White et al. 1994; Bicknell et al. 1995; Flouriot et al. 1995; Jobling et al. 1995, 1996; Sonnenschein et al. 1995; Sumpter and Jobling 1995; Ren et al. 1996; Routledge and Sumpter 1996

Bisphenol A	√	√	√	√	Brotons et al. 1995; Krishnan et al. 1995; Sonnenschein et al. 1995; Soto et al. 1995, 1997; Sumpter and Jobling 1995; Olea et al. 1996; Nagel et al. 1998
2′,5′-Dichloro-2-biphenylol	√				Soto et al. 1995
2′,5′-Dichloro-3-biphenylol	√				Soto et al. 1995
2′,5′-Dichloro-4-biphenylol	√				Korach et al. 1988; Soto et al. 1995
2′,6′-Dichloro-4-biphenylol		√			Korach et al. 1988; Soto et al. 1995
2,2′,5-Trichloro-4-biphenylol	√				Soto et al. 1995
3,4′,5-Trichloro-4-biphenylol		√			Korach et al. 1988; Soto et al. 1995
2′,4′,6-Trichloro-4-biphenylol	√	√	√	√	Korach et al. 1988; Jansen et al. 1993; Bergeron et al. 1994; Crews et al. 1995; Soto et al. 1995; Arnold et al. 1996
2′,3′,4′,5′-Tetrachloro-3-biphenylol	√				Soto et al. 1995
2′,3′,4′,5′-Tetrachloro-4-biphenylol	√	√	√	√	Korach et al. 1988; Jansen et al. 1993; Bergeron et al. 1994; Crews et al. 1995; Soto et al. 1995; Arnold et al. 1996
2-Chloro-4,4′-biphenyldiol		√			Korach et al. 1988
4-Biphenylol	√	√	√		Soto et al. 1995, 1997
3-Biphenylol	√				Soto et al. 1997
2-Biphenylol	√				Soto et al. 1997
Other					
t-Butylhydroxyanisole	√				Jobling et al. 1995; Sonnenschein et al. 1995; Soto et al. 1995
Diphenylphthalate	√				Jobling et al. 1995
Butylbenzylphthalate	√	√	√		Sonnenschein et al. 1995; Soto et al. 1995, 1997
Butylated hydroxyanisole	√	√	√		Jobling et al. 1995; Soto et al. 1995
Di-*n*-butylphthalate	√	√	√		Jobling et al. 1995; Soto et al. 1995; Soto et al. 1998

[a] The compounds listed in this table are only a representative sample of some of the best-studied synthetic compounds with estrogenic activity.

[b] This compound also is known to have antiandrogenic properties, as documented in cell culture studies using a recombinant chimeric androgen receptor and androgen-responsive promoter-reporter constructs (Kelce et al. 1995).

TABLE 2-2 Naturally Occurring Compounds with Estrogenic Activity[a]

Compound	Bioassay					Reference
	E-Screen	ER Binding	Induction	Recombinant	In Vivo	
Isoflavones						
4′,7-Dihydroxy daidzein		√		√		Shutt and Cox 1972; Verdeal et al. 1980; Sathyamoorthy et al. 1994
7-Hydroxy-4′-methoxy formonetin		√		√		Miksicek 1995
4′,5,7-Dihydroxy genistein		√		√		Shutt and Cox 1972; Verdeal et al. 1980; Miksicek 1995
5,7-Dihydroxy-4′-methoxy biochanin A		√		√		Miksicek 1995
Flavones and Flavonols						
4′,5,7-Trihydroxy apigenin		√		√		Miksicek 1995
3′,4′,5,7-Tetrahydroxy luteolin		√		√		Markaverich et al. 1988; Miksicek 1995
3,5,7,4′-Tetrahydroxy kaempferol		√		√		Markaverich et al. 1995
Flavanones						
4′,5,7-Trihydroxy naringenin		√		√		Miksicek 1995; Ruh et al. 1995
Chalcones						
2,4,4′-Trihydroxy isoliquiritigenin		√		√		Miksicek 1995
2′,4,4′,6′-Tetrahydroxy naringenin chalcone		√		√		Miksicek 1995
2′,4,4′,6′-Tetrahydroxydihydro phloretin		√		√		Miksicek 1995
4,4′-Dihydroxy		√		√		Miksicek 1995

Other						
Indolo[3,2-b]carbazole		√	√			Liu et al. 1994
Coumestrol	√	√		√	√	Martin et al. 1978; Verdeal et al. 1980; Soto et al. 1992a; Whitten et al. 1992; Nagel et al. 1998
Equol	√	√		√	√	Shutt and Cox 1972; Tang and Adams 1980; Sathyamoorthy et al. 1994; vom Saal et al. 1995; Nagel et al. 1998
Nordihydroguaiaretic acid	√	√		√		Sathyamoorthy et al., 1994
Zearalanol	√	√	√		√	Martin et al. 1978; Verdeal et al. 1980; Soto et al. 1992a
Zearalenone	√	√	√		√	Martin et al. 1978; Verdeal et al. 1980; Soto et al. 1992a
β-Sitosterol	√				√	Rosenblum et al. 1993; MacLatchy and Van Der Kraak 1995

[a]The compounds listed in this table are only a representative sample of some of the best-studied naturally occurring compounds with estrogenic activity.

HORMONALLY ACTIVE AGENTS

Environmental Estrogens

Synthetic Estrogenic Compounds

Studies in the 1960s and 1970s characterized the estrogenic activity of several synthetic compounds or pesticides, including *o,p′*-DDT (dichlorodiphenyltrichloroethane), Kepone, phenolic compounds, PCB (polychlorinated biphenyl) mixtures, and some PCB congeners (Tullner 1961; Bitman et al. 1968; Bitman and Cecil 1970; Ecobichon and MacKenzie 1974; Gellert 1978a,b; Hammond et al. 1979). PCB mixtures include planar/coplanar PCBs (i.e., PCBs with no ortho Cl substituents) and nonplanar PCBs (i.e., ortho-substituted PCBs). Hydroxy-PCB congeners were later shown to bind to the ER and induce estrogenic responses in the female rodent uterus (Korach et al. 1988). With the development of the E-screen assay (Soto et al. 1995) and other in vitro bioassays, the list of synthetic estrogens has greatly expanded to include a spectrum of organochlorine compounds, phenolics, phthalates, and antioxidants (Soto et al. 1991, 1994, 1995; Krishnan et al. 1993; R. White et al. 1994; Jobling et al. 1995). Table 2-1 lists a representative group of synthetic compounds that have been shown to have estrogenic properties in a variety of assays. The structures of these compounds differ markedly with respect to molecular size and volume; substituent structure, and placement, thus demonstrating the unexpected and unusual diversity of ligands that bind to the ER (Figure 2-2). The relative potencies of synthetic estrogens is highly variable and depends on the target organ or cell and the specific end point. Bioavailability can be modified by pharmacokinetic variables and preferential binding to other cellular factors, such as steroid-hormone-binding globulin, transthyretin, and other proteins that interact with lipophilic molecules. Relative estrogenic potencies for several xenoestrogens have been determined in the E-screen cell proliferation assay (Soto et al. 1995), and the results are highly structure dependent. The potencies of several estrogenic organochlorine pesticides were approximately 10^6 (molar basis) times lower than that observed for estradiol. In contrast, 2′,4′,6′-trichloro-4-biphenylol and octylphenol were 10^{-4} and 3×10^{-4} times less active than estradiol in the MCF7 cell-proliferation assay. Studies that use an in vitro assay that incorporates changes in bioavailability associated with interactions with components of blood have shown that, for some environmental estrogens, in vivo potency (effective doses), particularly in fetuses, can be significantly different from previous estimates (vom Saal et al. 1995; Nagel et al. 1997).

Some HAAs have been detected in human tissues (adipose, serum, and milk) and in food (Kutz et al. 1991; Winter 1992), and there is potential for human exposures to many of these chemicals. For example, Brotons and co-workers (1995) detected bisphenol-A in liquids from food cans that contained a plastic-

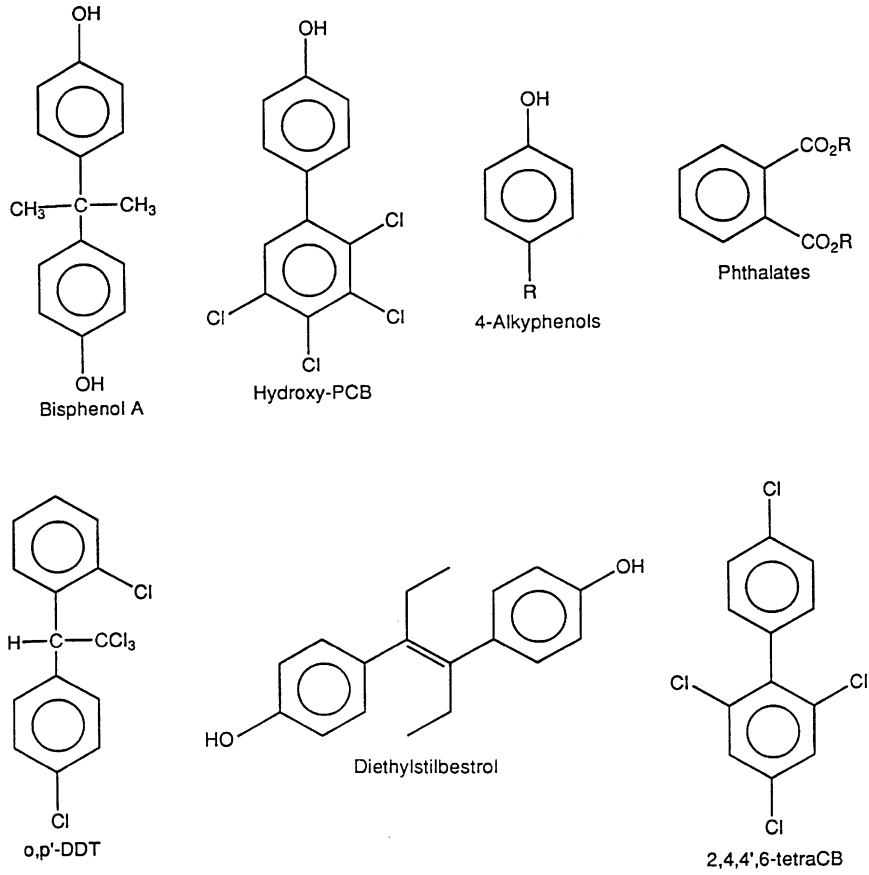

FIGURE 2-2 Some synthetic estrogens. The R values are limited relative to the potential estrogenic activity of the molecules.

coated liner; bisphenol-A also was detected in saliva due to leaching from composites and sealants used in dentistry (Olea et al. 1996).

Natural Estrogenic Compounds

Several structural classes of naturally occurring compounds in plants as well as fungal metabolites exhibit estrogenic activity in various bioassays (Figure 2-3, Table 2-2). Flavonoids are present in fruits and vegetables, and high concentrations have been identified in various soy products. Humans consume approximately 1 g of flavonoids each day (Kuhnau 1976), and high consumption of these compounds has been correlated with lower incidence of stomach, colon, breast, and prostate

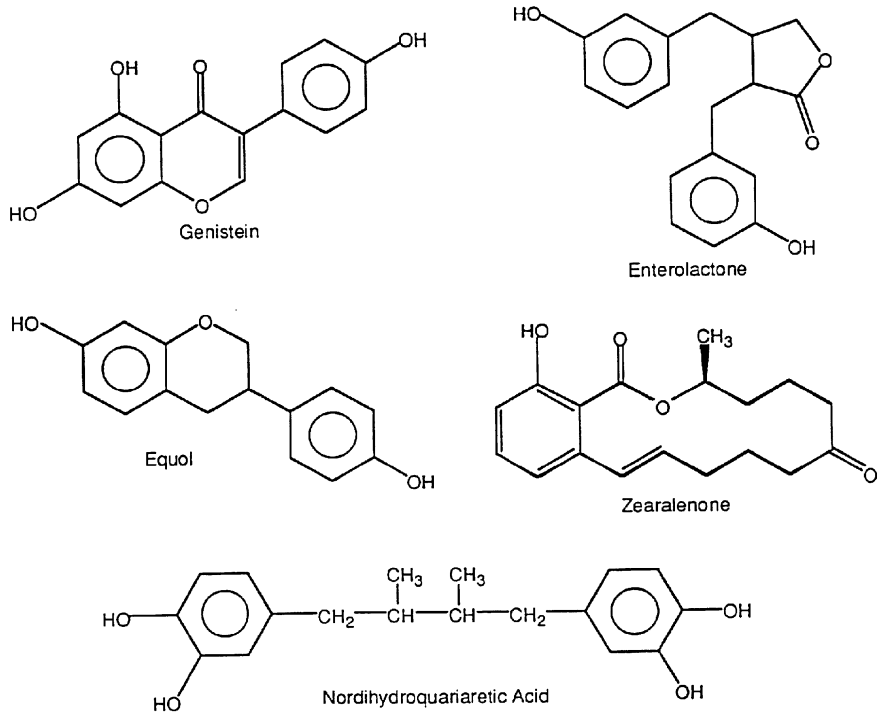

FIGURE 2-3 Naturally occurring estrogenic compounds.

cancers (Adlercreutz 1990). Two recent reviews summarize the health benefits of flavonoids (Bingham et al. 1998; Tham et al. 1998). Flavonoids elicit diverse ER-independent responses that could be important for their anticancer activity and that also could be responsible for estrogen-dependent adverse responses (Setchell and Adlercreutz 1988; Clarkson et al. 1995). However, it is apparent that, in addition to their estrogenic activity, flavonoids induce a broad spectrum of biologic responses that could contribute significantly to their anticarcinogenic activity (Adlercreutz et al. 1993a, 1995; Clarkson et al. 1995). Several studies characterized the estrogenic activity of flavonoids (Table 2-2); some of the compounds are weakly estrogenic in several assay systems. Flavonoid-derived compounds, such as coumestrol and equol; ligands, such as enterolactone and nordihydroguaiaretic acid (NDGA), which are present in food; and the fungal metabolites zearalenone, zearalenol, and zearalanol, also exhibit estrogenic activity.

As described above for synthetic estrogens, the potencies of naturally occurring estrogens are highly variable. For example, in the E-screen assay, zearalenol, zearalenone, and coumestrol were 100, 100, and 10^5 times less potent than was estradiol (Soto et al. 1995). In addition, the relative potency of phytoestrogens

can be modulated in vitro via binding to serum proteins. For example, in the presence of serum from adult men, the uptake and binding of equol to estrogen receptors in MCF7 cells was 11 times higher than it was in a serum-free medium, suggesting that assays conducted in the absence of serum would underestimate the estrogenic potency of equol (Nagel et al. 1998).

Dietary intake of naturally occurring estrogens and their relative contribution of estrogen equivalents to the diet has been discussed (Kuhnau 1976; Verdeal and Ryan 1979). The potential mass balance of human exposure to natural and xenoestrogens should be further investigated. Although estrogenic flavonoids are rapidly metabolized, these compounds can be detected in serum and in urine (Adlercreutz et al. 1986, 1993b, 1995; Setchell et al. 1997).

Bioassays for Estrogenic Compounds

Several assay systems have been developed to measure the potential estrogenic activity of xenoestrogens and naturally occurring estrogenic compounds: Cell proliferation, ER binding, induction of estradiol-responsive genes or gene products, and transient or stably transfected cell-bioassay systems are summarized below. In Chapter 11, these assays are described in more detail, and their advantages and disadvantages are discussed.

Receptor-Binding Assays

The estrogenic effects of various chemicals depend on initial binding to the ER. Direct binding studies are impractical because the assays require a radioligand with high specific activity; however, competitive binding assays are routinely used to measure ER binding (Jordan et al. 1985; Miksicek 1995). These types of assays are conducted by determining the binding affinity of a test compound for the ER relative to a radiolabeled competitor with a known binding affinity (Gray et al. 1997).

The binding of steroid hormones to their respective receptors in the intact animal is complicated by biotransformation of some hormones into sulfates, glucuronides, and other oxidation products. In addition, steroid hormones can interact with blood constituents, such as the serum proteins, which can alter transport and cellular uptake of HAAs. For example, Welshons et al. (1997) and Nagel et al. (1998) have shown, using cell-proliferation assays and competitive-binding assays, that the presence of serum significantly affects the uptake and activity of estrogenic compounds, such as coumestrol, genistein, bisphenol, and octylphenol. The competition for plasma-carrier proteins sites, as well as for the receptor, is an equilibrium-competition process. The equilibrium of a steroid between the plasma proteins and other blood constituents and the plasma water and the cellular receptors dictates the amount of ligand receptor, which is assumed to be the biologically active form of the receptor (Montano et al. 1995).

Cell-Proliferation Assays

The ability of estrogens to induce cellular proliferation in target organs is considered a hallmark of estrogen action (Hertz 1985). Therefore, a reliable bioassay for assessing estrogenicity would measure cell proliferation as an end point. This can be done in vitro by measuring mitotic indices in established cell lines derived from estrogen-responsive target organs. For example, Soto and Sonnenschein (1987, 1991) and Soto et al. (1994, 1995) have used the "E-screen assay" to investigate the effects of various estrogenic compounds in the proliferation of MCF7 cells, a human breast-cancer cell line.

Receptor-Dependent Gene Expression Assays

In vivo and in vitro studies have characterized several estrogen-induced genes or gene products that can be used as biologic markers of estrogen exposure: pS2, progesterone receptor, vitellogenin A2, cathepsin D, several protooncogenes, prolactin, transforming-growth factor α (TGFα), creatine kinase B, lactotransferrin, epidermal growth factor (EGF) receptor, calbindin D9k and D28k, heat-shock protein 27, uterine peroxidase activity, insulinlike growth factor, binding protein-4, lactate dehydrogenase, and complement C3. Although each of these genes or gene products is induced by estrogenic compounds, the induction response could be specific to the target organ or cell, or gene products could be induced by other classes of HAAs. For example, prolactin synthesis might be induced by epidermal growth factor, thyrotropin-releasing factor, and phorbol esters (Ramsdell and Tashjian 1985). The synthesis of another estrogen-inducible marker, ovalbumin, is stimulated by other steroids, such as progesterone, and by glucocorticoids (Palmiter 1975). EGF induces several prototypical estrogenic responses in the mouse uterus and vagina (Nelson et al. 1991; Ignar-Trowbridge et al. 1992). The induction of estradiol-induced genes or gene products can be useful as a screening bioassay for estrogenic compounds; however, there is a potential for false positives because of overlapping inducibility by other hormones or chemicals.

Recombinant Receptor-Reporter Gene Assays

Several in vitro bioassays that use recombinant receptor-reporter gene constructs have been developed to detect and quantitate estrogenic compounds and mixtures (Pons et al. 1990; Gagne et al. 1994; Jausons-Loffreda et al. 1994; Mäkelä et al. 1994; Jobling et al. 1995; Miksicek 1995; Ruh et al. 1995; Zacharewski et al. 1995). Transient transfection assays often use plasmids that contain 5′-flanking regions from estradiol-responsive genes, such as pS2 or vitellogenin A2, linked to reporter genes (chloramphenicol acetyl transferase, luciferase, β-galactosidase). In some cell lines, estrogen responsiveness also

requires cotransfection of a human ER expression plasmid, and ER levels are controlled by varying the amount of cotransfected ER. Results using recombinant receptor-reporter genes in human cell lines indicate that their sensitivity is comparable to that of the E-screen assay. However, it should be noted that the E-screen assay measures the proliferative activity of a test chemical, which involves transcription of all genes required for cell growth, whereas other in vitro assays either measure ligand binding or induction of a single gene or gene product.

Estrogen-Receptor Antagonists

Estrogen-receptor antagonists, or antiestrogens, have been extensively investigated as drugs for treatment of ER-positive, hormone-responsive breast cancer (Lerner and Jordan 1990). Antiestrogens characteristically bind to the ER; however, their subsequent activities as ER antagonists or agonists depend on the animal species, target organ or cell, and response. Tamoxifen, a widely used nonsteroidal antiestrogen for treatment of breast cancer, has been extensively characterized as an ER agonist and as an ER antagonist (Jordan 1988; Lerner and Jordan 1990). Tamoxifen and its active metabolite, 4-hydroxytamoxifen, stimulate uterine growth and progesterone receptor expression in rodents, induce proliferation of MCF7 cells, and increase expression of several estrogen-responsive genes (Jordan and Prestwich 1978; Westley et al. 1984; Sonnenschein et al. 1985; Katzenellenbogen et al. 1987; May and Westley 1987; Thompson et al. 1989; Lerner and Jordan 1990). In contrast, the steroidal antiestrogens ICI 164,384 and ICI 182,780 exhibit primarily antiestrogenic activity (Thompson et al. 1989; Wakeling et al. 1991).

The possible antiestrogenic activity of synthetic estrogens and bioflavonoids have not been investigated extensively. Table 2-3 lists a few of the compounds that have been studied.

Mäkelä and co-workers (1994) report that coumestrol, genistein, and zearalenone—which are estrogenic—did not exhibit antiestrogenic activity in several cell-culture assays. In contrast, Markaverich and co-workers (1988) report that luteolin and quercetin inhibited estradiol-induced uterine hypertrophy in rats and estradiol-stimulated growth of MCF7 cells. Quercetin does not exhibit estrogenic activity, whereas luteolin is weakly estrogenic (i.e., shows partial agonist activity) in ER binding and in vitro reporter gene assays (Markaverich et al. 1995; Miksicek 1995). Ruh and co-workers (1995) report that the weakly estrogenic bioflavonoid naringenin inhibited estradiol-induced uterine hypertrophy, peroxidase activity, PR concentrations, and [^{3}H]thymidine uptake in immature female rats and luciferase activity in MCF7 cells transiently transfected with the estrogen-responsive pS2-Luc plasmid. These data were obtained for individual bioflavonoids, and, coupled with a report suggesting that dietary soybeans are antiestrogenic (Mäkelä et al. 1995a), suggest that dietary compounds could be both estrogenic and antiestrogenic, depending on exposure.

TABLE 2-3 Compounds with Antiestrogenic Activity[a]

Compound	Inhibition of Estradiol-Induced End Points					Reference
	Uterine Hyperplasia	Growth of MCF7 Cells	Luciferase Activity in Transfected MCF7 Cells	Luciferase Activity in Transfected HeLa Cells	CAT[b] Activity in MCF7 Cells	
Synthetic Compounds						
2,2′,3,4′,5,5′-Hexachloro-4-biphenylol				√		Moore et al. (1997)
2,3,3′,4′,5-Pentachloro-4-biphenylol				√		Moore et al. (1997)
2′,3,3′,4′,5-Pentachloro-4-biphenylol		√		√		Moore et al. (1997)
2,2′,3,3′,4′,5-Hexachloro-4-bipheylol		√				Moore et al. (1997)
2,2′,3,3′,4′,5,5′-Heptachloro-4-biphenylol		√				Moore et al. (1997)
2,2′,3′,4,4′,5,5′-Heptachloro-3-biphenylol				√		Moore et al. (1997)
2,2′,3,4′,5,5′,6-Hepatchloro-4-biphenylol		√		√	√	Moore et al. (1997)
Natural Compounds						
Luteolin	√	√				Markaverich et al. 1988
Quercetin	√	√				Markaverich et al. 1988
Naringenin	√		√			Ruh et al. 1995

[a] The compounds listed in this table are only a representative sample of some of the best studied compounds with antiestrogenic activity.
[b] CAT, chloramphenicol acetyl transferase.

Hydroxy-PCB congeners have been identified in human sera (Bergman et al. 1994). Those compounds have been synthesized (Safe et al. 1995) and their ER agonist-antagonist activities have been investigated in MCF7 cells and in estradiol-responsive, stably transfected HeLa cells (Moore et al. 1997). The seven major hydroxy-PCBs in human serum exhibited minimal to nondetectable competitive binding to the rat uterine ER and did not induce proliferation of MCF7 cells (10^{-5} to 10^{-8} M). The estrogenic activity of the hydroxy-PCB congeners was further investigated by two estrogen-responsive in vitro bioassays: In one, HeLa cells were stably transfected with a Gal4:human ER chimera and a 17mer-regulated luciferase reporter gene, and in the other, MCF7 cells were transiently transfected with a plasmid that contained an estradiol-responsive vitellogenin A2 promoter and a chloramphenicol acetyl transferase (CAT) reporter gene. None of the hydroxy-PCBs (10^{-5} to 10^{-8} M) significantly induced luciferase activity in the stably transfected HeLa cells or CAT activity in MCF7 cells. The anti-estrogenic effects of the hydroxy-PCBs were investigated with the same bioassays in which the cells were treated with estradiol and with the hydroxy-PCBs. All of the hydroxy-PCB congeners inhibited one or more estrogenic response; one congener—2,2′,3,4′,5,5′,6-heptachloro-4-biphenylol—inhibited estradiol-induced cell proliferation, CAT activity (MCF7 cells), and luciferase activity (HeLa cells). Pentachlorophenol also inhibits estradiol-induced trout ER and vitellogenin mRNA levels in trout hepatocytes, and it binds competitively to the ER (Flouriot et al. 1995). The estrogenic and antiestrogenic activities of other xenoestrogens should be investigated for multiple end points and for target organs and cells because estrogen and antiestrogenic responses could be highly specific.

Environmental Antiandrogens

Kelce and co-workers (1995) reported that *p,p′*-DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) competitively binds to the androgen receptor; however, in utero exposure of pregnant rats to 100 mg/kg/d (gestation d 14-18) resulted in retention of thoracic nipples, an antiandrogenic effect. Treatment of 25-d-old male rats (100 mg/kg/d until d 57) delayed the onset of puberty and, in castrated adult rats treated with testosterone to control for effects on testes, treatment with *p,p′*-DDE (200 mg/kg/d for 4 d) decreased seminal vesicle and prostate weight. Each response is consistent with antiandrogenic activity, and the results were confirmed in cell-culture studies using a recombinant chimeric androgen receptor and androgen-responsive promoter-reporter constructs. Because antiandrogens and estrogens can induce some of the same adverse responses (such as demasculinization) in male rodents, the hypothesized effects of xenoestrogens in male reproductive problems (Sharpe and Skakkebaek 1993) also should include "xeno-antiandrogens." It is possible that other persistent organochlorine compounds are antiandrogens, and this should be further investigated. The identification of *p,p′*-DDE as an antiandrogen is important as well, because that compound is the major

persistent organochlorine pollutant in fish, wildlife, and human tissues; in places where DDT is still used as a pesticide, *p,p′*-DDE concentrations can be very high in human and wildlife tissues (Tanabe et al. 1994).

There also is evidence that the metabolites of vinclozolin, a dicarboximide fungicide, have antiandrogenic properties (Kelce et al. 1994). In vitro assays to determine the ability of vinclozolin to inhibit the conversion of testosterone to a more potent androgen (via 5 α-reductase) and to bind to the androgen receptor show that neither vincolzolin nor its degradation products (2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide) inhibit 5 α-reductase activity. Although the ability of vinclozolin to compete with androgen for binding to the androgen receptor is weak, its metabolites are effective androgen-receptor antagonists.

Aryl Hydrocarbon-Receptor Agonists

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related halogenated aromatic hydrocarbons (HAHs) are industrial or combustion byproducts that have been extensively characterized as HAAs (Colborn et al. 1993; Peterson et al. 1993; Safe 1995). HAHs work through the Ah receptor-signaling pathway. Their biochemical and toxic responses in laboratory animals are variable and depend on the age, strain, sex, and species of the animal. In utero exposure to TCDD results in adverse effects in male and female rat offspring, and many of the responses observed in males are similar to those reported for antiandrogens and estrogens (Peterson et al. 1993). For example, male rats exposed to TCDD in utero exhibited decreased reproductive capacity, reduced androgenic status, and feminization of sexual behavior (Peterson et al. 1993). There also is concern regarding human offspring exposed to relatively large quantities of HAHs in utero (Peterson et al. 1993; Rogan 1995). An overall risk assessment of TCDD and related HAHs should consider exposures from synthetic-derived compounds and from naturally occurring Ah-receptor agonists, such as indole-3-carbinol, present in vegetables and other plant products (Wilker et al. 1996; Safe 1998).

TCDD and related Ah-receptor agonists also cause antiestrogenic activity in rodent uterus and mammary and human breast-cancer cell lines (Safe 1995). In those target tissues, TCDD inhibits estrogen-induced hypertrophy and growth of the rodent uterus and breast-cancer cells and mammary tumor growth in rodents. In addition, Ah-receptor agonists inhibit a spectrum of estrogen-induced genes and related activities. The mechanisms associated with the antiestrogenic activity involves crosstalk between the Ah- and estrogen-receptor-signaling pathways (Safe 1985). Bertazzi and co-workers (1993) report that women exposed to TCDD as a result of an industrial accident in Seveso, Italy, exhibited a decreased incidence of mammary and endometrial cancer, suggesting that comparable antiestrogenic responses were observed in cellular and animal models and humans. There also is evidence that other Ah-receptor agonists, such as polynuclear aro-

matic hydrocarbons (PAHs) in tobacco smoke, induce antiestrogenic activity (Chaloupka et al. 1992). Two other studies (Lesko et al. 1985; Levi et al. 1987) report decreased incidence of endometrial cancer in tobacco smokers.

Other Hormonal Toxicants

Several other toxicants are hormone mimics or disrupt endocrine-response pathways. Hydroxy-PCBs and other hydroxylated organochlorine metabolites bind to transthyretin (Lans et al. 1993), and it has been suggested that these same compounds are thyroid-hormone-receptor agonists (Rickenbacher et al. 1986). A study of pregnant mice treated with 3,3′,4,4′-tetrachlorobiphenyl showed significant accumulation of 3,3′,4′,5-tetrachloro-4-biphenylol bound to fetal transthyretin, accompanied by decreased fetal plasma T_4 concentrations (Darnerud et al. 1996). The transplacental effects of hydroxy-PCBs and other compounds that bind to transthyretin should be studied.

MECHANISM OF ESTROGEN ACTION

The ER is a ligand-induced transcription factor and is a member of the steroid/thyroid/retinoid nuclear-receptor superfamily (Mangelsdorf et al. 1995). The ER mediates temporal- and tissue-selective expression of specific genes, and these responses are dependent on ligand structure, cellular and gene-promoter context (Katzenellenbogen et al. 1996). The classical mechanism of ER action involves binding of the ligand-bound ER homodimer with perfect or imperfect palindromic 5′-promoter ERE motifs and subsequent interactions with the basal transcription factor complex. More recent studies have demonstrated that estrogen responsiveness is more complex and involves ER interactions with other nuclear factors, including coactivators and proteins such as p300/CBP that influence histone acetylation and chromatin structure (Mangelsdorf et al. 1995; Katzenellenbogen et al. 1996). The ER can also modulate gene expression by interacting with other DNA-bound transcription factors, and estrogen-mediated transcriptional activation can be observed through ER-AP1 and ER- Sp1 interactions in which ER does not bind promoter DNA (Paech et al. 1997; Porter et al. 1997; Duan et al. 1998). The increasing complexity of ER action is consistent with the differential control of gene expression by ER and other members of the nuclear-receptor superfamily.

Even when it is not bound to an estrogen, the ER is found in the nucleus of its target cells. Although it is not clear what the ER is bound to in the nucleus, the complex chromatin structure of DNA and protein is a likely site. The chromatin of eukaryotic cells is arranged in an orderly manner, and different chromosomes have defined domains within the nucleus (Felsenfeld 1992). This is thought to be due to a network of proteins called the nuclear matrix, and there have been reports that ERs are associated with the nuclear matrix (Barrack 1987). ERs,

with or without estrogen, also have a high affinity for specific DNA sequences, the EREs (Murdoch et al. 1990; Beato 1991). These sequences of 15-20 base pairs can convey estrogen responsiveness to reporter genes when ligated upstream of an appropriate promoter sequence. They have all the characteristics of enhancer sequences that are common regulatory elements in genes. Quantitative studies indicate that the ER, with or without ligand, has an affinity for its ERE of about 0.1 nM, which is similar to the affinity of the ER for estradiol or for diethylstilbestrol (DES) (Murdoch and Gorski 1991; Furlow et al. 1993). Thus, there are at least two high-affinity ligand sites for the steroid receptors, one for the steroid and one for DNA. This is illustrated in Figure 2-4, in which the ER is shown interacting with an ERE upstream from the site where transcription is initiated. The ER is also shown interacting with another protein at the ERE site. This could be another ER unit to yield a homodimer, or it could be another protein resulting in a heterodimer. Finally, the ER is shown interacting with another protein, which in turn is part of the transcription machinery or is closely associated with it.

Response elements vary in their sequence. The ER can distinguish single base-pair changes with dramatic changes in affinity of orders of magnitude. It also can recognize some DNA sequences that are quite different from the canonical vitellogenin gene ERE. It is undoubtedly the surface of the ERE that is recognized and not the sequence directly. Thus, there could be other response element sequences yet to be recognized.

When the unoccupied receptors are extracted from cells, they are found as large complexes with heat-shock proteins, proteins that are produced in response to environmental stress (Toft and Gorski 1966; Toft et al. 1967; Smith and Toft

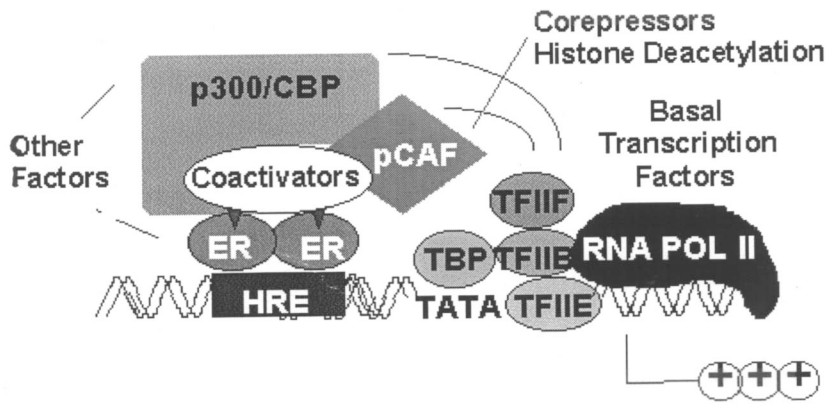

FIGURE 2-4 Transcriptional activation of estrogen-responsive genes: formation of ER-protein complexes at distal responsive elements.

1993). This is an area of considerable interest, and it is not clear how heat-shock proteins work in the functioning of the receptor. The heat-shock proteins could act as chaperons in the orderly folding of the receptor and translocation to the nucleus after its synthesis in the cytoplasm. It also has been suggested that the heat-shock proteins are present to prevent binding of the receptor to the DNA until the steroid binds to its receptor (Chambraud et al. 1990; Pratt et al. 1992).

After the estrogen or antiestrogen binds to the receptor in intact cells, the receptor ligand complex binds tightly to the nucleus and is difficult to extract. This complex is closely correlated quantitatively with the biologic response to estrogen. The binding of the ER to specific DNA sequences is part of the action of the ER but does not appear to be regulated by estrogens (Murdoch and Gorski 1991; Furlow et al. 1993). If the ER is bound to enhancerlike response elements without estrogen, the increase in affinity of the ER for the nucleus in intact cells exposed to estrogen must result from protein-protein interactions. The nature of these interactions has not been studied to any extent, but it is crucial for explaining how estrogenic compounds function.

There are published reports of the ER interacting with transcription factors associated with polymerase II, the RNA-transcribing enzyme known to synthesize mRNAs (Ing et al. 1992; Halachmi et al. 1994; Tsai and O'Malley 1994), as well as reports that corepressor proteins that interact with the transcription factors and with the ER are critical for the response to estrogens. This area is under active investigation, and more interactions have been reported than can be presented in a simple model. We can assume that, in the next few years, this area will be further elucidated so that a more detailed model of how estrogenic hormones activate transcription can be presented.

Another aspect of ER interactions with other proteins is that of self-interaction. Receptors are known to form homodimers, heterodimers, or both, at high concentrations in solution or when they bind to their respective response elements. In soluble systems, the ER forms homodimers that show cooperativity of estrogen binding (Notides et al. 1981; Sakai and Gorski 1984). However, there has been no evidence of cooperative estrogen binding or cooperative estrogen response in intact cells (Williams and Gorski 1974; Muller et al. 1985; Walent and Gorski 1990). This raises doubts about whether the ER is present as a homodimer in the intact cell. It could be that the ER pairs off with another protein to become a heterodimer that then binds to the ERE. Several other steroid receptors, such as the retinoic acid receptor, form heterodimers. This could be relevant to the problem of hormone mimics because the heterodimeric partner also might be influenced by environmental factors.

If ER interaction with nuclear proteins is the critical function of these receptors, then the possibility of variation in the nuclear composition of proteins that will interact with the ER becomes crucial for a cell's response to the ER and its ligands. Furthermore, if different ligands bound to the ER convey different

conformational states to the ER, then different ER-protein complexes could occur because of differences in the cell's nuclear protein composition and the estrogenic ligand.

Neural effects of estrogens could represent a more complex response to estrogen than that outlined above. Several reports indicate that estrogens regulate the neural system via changes in nuclear function similar to those described above. However, there are also reports in the literature of estrogen responses in neural cells that occur more rapidly than would be likely if they occurred as a result of changes in nuclear gene expression. Naftolin's group (Garcia-Segura et al. 1989, 1994) reported effects of 17β-estradiol on neural membrane that occurred within 1 min of estrogen administration. Tamoxifen blocked the response, and 17α-estradiol had no effect. Pappas et al. (1994) showed that small numbers of ERs are found in cell membranes. Small pools of ERs could be missed by immunocytochemistry and other methods of ER detection currently in use.

Studies in animal models have shown that estrogens and androgens control epithelial cell numbers in their target organs by inhibiting cell death (Martin 1980), by inducing cell proliferation (Step I), and later by inhibiting cell proliferation (Step II) (Stormshak et al. 1976). Those effects can be segregated in experimental models by manipulating sex-hormone concentrations; this suggests that they are controlled by discrete mechanisms (Soto et al. 1986; Sonnenschein et al. 1994).

Three hypotheses postulate the role of estrogens on induction of cell proliferation (Step I):

- The direct positive hypothesis proposes that the estrogens themselves trigger the proliferation of their target cells (Stack and Gorski 1984).
- The indirect positive hypothesis proposes that estrogens induce the synthesis of growth factors that, in turn, cause proliferation of estrogen-sensitive cells via stroma-epithelium paracrine (Dickson et al. 1986) or autocrine (Cooke et al. 1986) interactions. However, growth factors administered to ER knockout mice failed to induce a proliferative effect in the female genital tract (Curtis et al. 1996), which contradicts the indirect positive hypothesis.
- The indirect negative hypothesis posits that a plasma-borne inhibitory molecule (estrocolyone-I) inhibits the proliferation of estrogen-target cells (Soto and Sonnenschein 1985; Soto et al. 1992b; Sonnenschein et al. 1996). Estrogens could induce cell proliferation merely by neutralizing the effect of this serum-borne inhibitor.

Continued treatment with estrogens results in a proliferative shutoff (Step II) of their target cells (Stormshak et al. 1976). Step II is considered a direct effect of estrogens in that it is ultimately mediated by hormones rather than by growth factors or inhibitors as postulated for Step I (Amara and Dannies 1983; Soto et al. 1986; Sonnenschein et al. 1994).

MODULATION OF ESTROGEN-INDUCED RESPONSES

Estrogen-induced responses can be modulated by the internal cell environment. A complex of the antiestrogen 4-hydroxytamoxifen with the ER can have an agonistic effect in bone tissue; in breast tissue it is antiestrogenic (Jordan and Murphy 1990). The same cell type at different stages of maturity or development can react differently to the same steroid-ER complex because of the difference in internal cell environment. In molecular terms, this probably means interactions between the receptor and different cellular proteins present in the target cells. It is possible that some compounds might force a conformation of the ER that leads to interactions with different proteins in the nucleus. As the physiologic state of cells changes in response to a variety of influences, the complement of proteins present in the cell nucleus undoubtedly changes, and the ER complex then functions in an environment with a new array of proteins and transcription factors. This is an area of great interest to investigators that will influence the direction of research on hormone mimics.

An important relationship of ERs with environmental estrogens is seen in the presence of ERs in the very early stages of development of most vertebrates (Greco et al. 1993). This was brought to the attention of the medical community in the 1970s with the observation that the offspring of mothers who had been treated with large amounts of DES during high-risk pregnancies suffered abnormalities in various parts of male and female reproductive tracts. Most notable was the occurrence of vaginal adenocarcinomas, normally quite rare, that appeared after sexual maturity among some of the female offspring of DES-treated women. Although no similar cancers were observed in the treated mothers, daughters showed the response 15-20 yr after the mothers had received DES treatment. Thus, a combination of different cell environments and the presence of ERs in the early embryo fostered a totally unexpected problem that emphasized the importance of cellular environment influences on the response to chemical regulators.

The observations of embryonic effects were studied more carefully by McLachlan and co-workers (1980) in mice and rats and are detailed in the Appendix. The studies also point out that the presence of receptors in embryonic and fetal tissues presents a mechanism for estrogenic regulators to influence the developing organism. The normal function of the embryonic receptors is, as yet, not fully understood, but studies involving administration of estrogen agonists and antagonists during development suggest a role for estrogen receptors in the development of the brain, reproductive organs, and other tissues, such as bone (vom Saal et al. 1992; Greco et al. 1993; Newbold 1995).

Immunocytochemistry has shown that ERs are present in mouse blastocysts (Hou et al. 1996). Sensitive polymerase chain reaction (PCR) techniques showed that ER mRNA is present in the unfertilized mouse oocyte, and through the 32-64 cell blastocyst (Hou and Gorski 1993; Wu et al. 1992). PCR methods also were

used to show that ER mRNA is present in human oocytes (Wu et al. 1993). Somewhat later in development, ERs are present in male and female reproductive tracts at the indeterminate stage (12- to 15-d-old embryo) of reproductive-tract development (Greco et al. 1993). From 15 d of embryonic age to the neonate, ERs increase in females and decrease in males, although substantial numbers remain even in males (Greco et al. 1993).

Immunocytochemistry techniques revealed that embryonic ERs are present in all cells of the blastocyst (Hou et al. 1996). However, during mouse development, the presence of the ER in various cell types changes. ERs are present in the gonads of males and in the reproductive tracts of males and females throughout fetal development and after birth (Greco et al. 1993). Although quantitative changes do occur; qualitatively, both sexes have estrogen-response systems that can respond to estrogens from physiologic or environmental sources.

Only one case of an ER-deficient human has been authenticated (Smith et al. 1994). This human male showed definite signs of estrogen insufficiency, but the observations raise the question of why no other ER-deficient humans or other animals have been reported. One suggestion is that the ER normally has an early embryonic role and that a deficiency of ER is lethal to the embryo. However, a line of transgenic knockout mice that were engineered to have a defective ER gene survive to adulthood and have normal gross external phenotypes (Lubahn et al. 1993; Korach et al. 1996). Although both sexes are infertile and have defects of the gonads indicative of low-estrogen responsiveness, these findings support the conclusion that prenatal development of the reproductive tract of both sexes appears to be independent of an ER-mediated response.

It is not clear what the prepuberal appearance of ERs signifies or whether they have any physiologic function. It is apparent that embryonic ERs can damage the organism when estrogens or potential estrogenic mimics present themselves in the environment at sufficient concentrations.

SUMMARY AND CONCLUSIONS

The estrogen receptor and other steroid or steroidlike hormone receptors can bind with a range of compounds and as such are potential vehicles for HAAs to act upon and influence normal cellular pathways. Steroid hormone receptors control fundamental gene-regulatory mechanisms, and interaction of HAAs with these receptors may disrupt these processes. The potential for a biologic effect is dependent upon the cell type, concentration of an HAA in the target cell, and the binding affinity of that compound for a receptor. Most HAAs exhibit relatively low binding affinities for the ER, suggesting that relatively high concentrations of the compounds are required to induce a response. Some work suggests that HAAs may have a significant effect during cell growth and embryonic development, when organisms may be more sensitive to low concentrations of estrogens. HAAs, such as the partial agonist tamoxifen, may have different effects in differ-

ent tissues and species. However, most of the compounds that exhibit estrogenic activity appear to elicit similar cellular responses when experiments account for differences in measured response times and differences in rates of dissociation of the compounds from the ER.

The estrogenic properties and potencies of several HAAs discussed in this chapter are highly variable and structure dependent. It should be emphasized that although in vitro assays are important screening tools for determining estrogenic activity, the results cannot be used to state with certainty that a particular HAA will interfere with normal hormonal pathways in an organism (see Chapter 11).

The multiple mechanisms of ER-mediated responses include interaction of the ER with specific DNA sequences, such as estrogen-responsive elements, interaction with other transcription factors, crosstalk with other signaling pathways, and cell-membrane-mediated responses. Estrogen and HAAs appear to induce estrogen-responsive cell proliferation and related responses through common pathways. Although both males and females are responsive to estrogens, ER expression and estrogen responsiveness of various cell types can change between prenatal and postnatal life. The role of HAAs on hormonally regulated cellular pathways is further discussed in the other chapters of this report.

3

Exposures: Sources and Dynamics of Hormonally Active Agents in the Environment

THIS CHAPTER DISCUSSES HOW EXPOSURE to hormonally active agents (HAAs) can occur and how exposure to HAAs in the environment is measured or estimated. For the purposes of this chapter, exposure is defined as the "condition of a chemical contacting the outer boundary of [an organism]" (EPA 1992). Exposure of an organism to an HAA requires that the HAA be released into the environment and that it persist for a sufficient amount of time to bring about contact with an organism. Many of the HAAs are relatively persistent chemicals, and therefore, exposure to one or several of them is virtually guaranteed for most organisms.

Exposure assessment of some chemicals might be a difficult task, particularly when they remain in an organism for a long time. For females, that might be especially important because of exposure of offspring to chemicals absorbed or mobilized during pregnancy and particularly during lactation.

Understanding the relationship between exposure, absorption, disposition, metabolism, clearance, and repair and response is the key to predicting when exposure to an agent might result in a harmful dose.

General usage often confuses dose and exposure. To be precise about the terms, exposure generally is expressed in terms that describe concentration in a given medium (e.g., air or water) and, in some cases, duration (milligrams per liter, milligrams per cubic meter, parts per million, milligrams per day). Dose is based on exposure intake and body weight or surface area of the target organism (milligrams per kilogram of body weight per day or milligrams per square meter per hour).

This chapter has two major sections. The first section discusses the many sources from which HAAs can enter the environment, their persistence, and what concentrations have been found in environmental and biologic media. In the

second section, examples are given of some of the exposure concentrations for natural and anthropogenic HAAs for the general population and highly exposed subpopulations. The dosimetry and metabolism of HAAs are covered in Chapter 4.

SOURCES AND RELEASES

HAAs encountered in the environment can be produced synthetically or naturally, and exposure can occur from a variety of sources, both involuntary and voluntary. For example, virtually all humans and many animals are exposed to some phytoestrogens in their diet by eating plant products that contain these natural substances. Humans and animals can be exposed involuntarily to synthetic HAAs as a result of drinking contaminated water, breathing contaminated air, ingesting food, or contacting contaminated soil. Humans can also be exposed voluntarily to many synthetic HAAs by using HAA-containing commercial products, such as cleaners, pesticides, and food additives (domesticated animals can also be exposed to these products, but such exposure is assumed to be involuntary). Finally, many humans voluntarily ingest or apply chemicals to their skin for a specific beneficial or therapeutic purpose, and they might expose animals to these chemicals as well. These chemicals, which might act as HAAs, include such pharmaceuticals as birth control pills, herbal supplements, cosmetics, and pesticide products. Natural HAAs are generally considered to consist of plant-produced estrogens, the phytoestrogens. Synthetically produced HAAs have been used and are used in pesticides (e.g., dichlorodiphenyltrichloroethane (DDT) and endosulfan), plastics (e.g., bisphenol A), and other industrial applications (e.g., polychlorinated biphenyls (PCBs)). In either case, an exogenous HAA can supplement, inhibit, or be without measurable effect compared with typical concentrations of endogenous hormones. As discussed in Chapter 2, numerous chemicals have been identified that are known or suspected to mimic or inhibit hormones in humans and wildlife. Although this chapter discusses the potential for exposure to many of these chemicals, it is by no means exhaustive, and the extensive literature on the environmental concentrations of these chemicals should be consulted for a more comprehensive review.

Natural HAAs

Many plants contain HAAs, particularly the legumes. Some are produced by the plant itself (phytoestrogens), and others come from fungi that infect the plants (mycotoxins). Phytoestrogen compounds, such as lignins (e.g., matairesinol and secoisolariciresinol (SECO)) and isoflavonoids (e.g., isoflavones and coumestans), are common in human and animal food. Isoflavones are found in most plant tissues and include the estrogenic compounds genistein, diadzein, biochanin A, and formononetin, all of which have been detected in human urine (Adlercreutz and Mazur 1998). In addition to the legumes that contain relatively high concentra-

tions of phytoestrogens (up to 84,000 μg/100 g of dry weight in soybeans), oilseeds and nuts also contain highly variable concentrations of these substances (4-370,000 μg/100 g of dry weight in flaxseed). Significant concentrations of lignins, but not isoflavonoids, are also found in cereals, grains, berries, vegetables, and teas. Most vegetables do not contain isoflavones but do have high concentrations of lignins. Cruciferous vegetables, such as broccoli, also contain low but measurable quantities of isoflavones, in spite of having high concentrations of indole-3-carbinol, an anticarcinogen. Such fruits as apples, plums, and bananas have very low concentrations of isoflavonoids and lignins, the possible exception being exotic fruits, which might have high concentrations of isoflavones. Beer has detectable concentrations of isoflavones (Adlercreutz and Mazur 1998).

Concentrations of phytoestrogens can vary dramatically in plants. For example, peas and green beans contain coumestrol—0.40 μg/g and 1 μg/g of dry weight, respectively (Price and Fenwick 1985). Concentrations of diadzein and genistein in soybeans ranged from 22 to 1,915 μg/g and 69 to 1,897 μg/g of wet weight, respectively. Concentrations of both isoflavones generally exceeded 200 μg/g in most samples (Reinli and Block 1996).

Synthetic HAAs

Numerous synthetic chemicals have been implicated as HAAs. Many of these chemicals are no longer widely used in commerce; however, that is not true for all of them. A one-time common pesticide, DDT, was banned from use in the United States in 1992, and it is a banned or restricted pesticide in most developed countries. However, it continues to be produced and used in several developing countries, such as India, because of its effectiveness and relatively inexpensive production.

Historical use and disposal of PCBs have resulted in the presence of these chemicals worldwide. Current releases of PCBs should be confined to industrial accidents, improper disposal, and dissipation from environmental sites (e.g., sediments) containing high concentrations of PCBs. Minor releases of natural PCBs have been associated with volcanic activity.

It is estimated that over 4 billion pounds of PCBs have been produced worldwide (Hooper et al. 1990) since the early 1930s. Gunderson (1995) notes a significant decline in chlorinated pesticides and PCBs over the past two decades in most environmental media. Production of PCBs was banned in the United States in 1977, although products containing PCBs are still in use not only in the United States but also in other countries around the world.

In Table 3-1, the use and production of several representative HAAs are described. Many of the organochlorine pesticides that have been cited as HAAs are no longer used in the United States (e.g., DDT, endosulfan, dieldrin, and toxaphene), although they continue to be found in various environmental media.

TABLE 3-1 Use and Production of Several Representative Hormonally Active Agents

Chemical Name	Use	Production Volume Per Year	Fate	Reference
PCBs	Dielectric, flame retardant, hydraulic fluids, electrical equipment, pigments, numerous industrial applications	U.S. production banned since 1977; may be found in older equipment and applications	Slowly volatilizes from soil and water surfaces, removed from atmosphere via wet and dry deposition; very limited biodegradation; no other degradation; absorbs to sediments in water	Keith 1997
DDT	Insecticide	Since 1973, no longer used in U.S.; produced and used in several developing countries	Absorbs strongly to soil and sediment; photodegrades or evaporates on soil surfaces; photo-oxidizes on water surface; biodegrades in sediments but not water column	Keith 1997
Bisphenol A	Chemical intermediate for numerous industrial products including polymers, resins, dyes, and flame retardants; also used in dental sealants	Continued industrial use	Photodegrades; low-to-moderate mobility in soils, biodegrades under aerobic conditions following acclimation; in the particulate phase in atmosphere	Keith 1997
Chlordecone (Kepone)	Pesticide	U.S. production ended in 1975	Strongly binds to sediments and soils; subject to anaerobic biodegradation; atmospheric transportation bioaccumulates	ATSDR 1995
4-Alkylphenol (and polyethoxylates)	Surfactants, emulsifiers	360,000 tons produced worldwide in 1988	Polyethoxylates biodegrade under anaerobic conditions; greater than 99% removal in sewage treatment plant	Nimrod and Benson 1996; Keith 1997
Di-*n*-butylphthalate	Plasticizer for polyvinyl chloride; coatings; solvent; acaricide, leak detector, cosmetic component		Biodegrades in water ($t_{1/2}$, 2-3 wk), absorbs to sediment; transported in atmosphere with removal via rain; bioconcentrates	Keith 1997

However, the lack of U.S. production of these chemicals does not mean that they are not produced by other countries that have a need for inexpensive and effective pesticides, regardless of their long-term environmental effects. Other HAAs, such as various phenolics and phthalates, continue to be used in a variety of industrial applications in the United States and worldwide.

Manufacturing processes, such as textile wet processing, make wide use of alkylphenols (APEs) (Naylor 1995). Nonylphenol ethoxylates (NPEs) account for 80% of APE volume (Naylor 1995). U.S. production and use of APEs in 1990 exceeded 450 million pounds (Naylor et al. 1992).

Dioxins are unique among the HAAs considered in this report. They are not naturally produced by plants for any beneficial purpose, nor are they purposefully made by any industrial process. Rather they are formed naturally by the combustion of plant matter and are by-products of many industries, such as pulp and paper production, and other processes that use chlorine. In the United States, forest fires account for approximately 20 kg of dioxins per year, residential wood burning results in 20 kg/yr, agricultural burning releases 10 kg/yr, and incinerators contribute approximately 350 kg/yr for a total annual dioxin release of 400 kg (Spiro and Thomas 1994).

PERSISTENCE

Global deposition of various persistent organic chemicals has been observed for years, even in areas thought to be pristine. Concentrations of persistent organic chemicals, such as DDT, in rural areas are comparable to or higher than those in more-populated and industrialized regions of North America and Europe (Fellin et al. 1996). That is, to a large degree, due to atmospheric transport and condensation of these compounds at low temperatures. As a result, seasonal variations occur in deposition of these compounds from the atmosphere (D.J. Thomas et al. 1992), and deposition peaks in wintertime. Benzo[*a*]pyrene was detected at 20 pg/m^3 in the winter but only 1 pg/m^3 in the summer (Fellin et al. 1996). DDT concentrations measured in the water of the St. Lawrence River in 1991 were highest (mean concentration 3 ng/L) in April but had fallen by September (Pham et al. 1996). The principal DDT source was thought to be runoff from spring melt from the watersheds after atmospheric deposition during the winter. Concentrations were lower at other times of the year.

PCBs are very stable in the environment. They volatilize from water and thus can be transported in the atmosphere; however, most PCBs are associated with organic environmental components, such as soil and sediments. Environmental degradation of PCBs is dependent on the chlorine content of the various isomers. The greater the chlorine content of the PCB, the less degradation will occur in soil but the greater the potential for photodegradation from water surfaces and in the atmosphere (Delzell et al. 1994).

The half-life of relatively small, less chlorinated PCBs in the atmosphere has

been estimated to be 10-25 hr of direct noon-time sun (i.e., several actual days) (Delzell et al. 1994). For other more highly chlorinated PCBs, the atmospheric half-life (over the Great Lakes) is approximately 6 yr, in water the half-life is 3 to 9 yr, and in soil 3 to 17 yr (Hillery et al. 1997).

Kepone (chlordecone), a chlorinated pesticide, was released to the James River estuary in Virginia for 9 yr, beginning in 1966. It degrades very slowly and is resistant to destruction. Kepone concentrations in the estuary ranged from a high of 4.8 μg/g in zooplankton to 0.11 μg/g in bed sediments in 1977. The concentration in suspended material in the water column averaged 0.09 μg/g. Kepone accumulation was greatest in bed sediments of the middle estuary, located approximately 40 km downstream from the source, where concentrations ranged from 60 to 200 ppb. Approximately 42-90% of Kepone input was retained in the river system by entrapment in estuarine circulation and seasonal refluxing (Nichols 1990). Other persistent chemicals, such as PCBs, are also subject to recycling in the environment from sediments into the overlying water column.

MONITORING

HAAs have been detected in all environmental media as well as biologic tissue, but the concentrations can vary dramatically. Detecting the concentration and, in particular, determining changes in the concentration require monitoring of the various environmental media. Monitoring can be a one-time event (e.g., point sampling), periodic (e.g., annually), or continuous. It is used to show trends in deposition, production, or use. Monitoring may be determined for air, water (both ground and surface waters), soils, sediments, and biologic tissues, such as fish and human blood.

In the United States today, there are lower concentrations of PCBs and some chlorinated pesticides, such as DDT, than there were in the 1970s, before regulatory actions were taken to curb production and use of DDT and PCBs. However, during the 1990s, the rate of decline of these chemicals in some environments, such as the Great Lakes, has slowed because of recycling or continued input. These chemicals are found in rain water (Rapaport et al. 1985), providing a mechanism for their deposition into the Great Lakes and other regions of the United States because of their continued use elsewhere in the world (Iwata et al. 1993). This finding demonstrates the global nature of exposure and has raised concerns that it will be difficult to achieve further rapid decreases in these globally dispersed chemicals (Giesy et al. 1994b; Loganathan and Kannan 1994; Stow 1995; Williams et al. 1995).

Organochlorine compounds are virtually ubiquitous in the environment. Analysis of tree bark from over 90 sites worldwide indicated that DDT, endosulfan, chlordane, dieldrin, and hexachlorocyclohexanes (HCHs) were present at all sites at measurable concentrations, albeit at very low concentrations (0-10 ng/g of lipid) at some sites, regardless of how remote the site. Although DDT has been

banned from use in the United States since 1973, concentrations of DDE (a degradate of DDT) ranging from 1,000 to 10,000 ng/g of lipid continue to be found in tree bark taken from the midwestern United States. Simonich and Hites (1995) concluded that the more volatile the organochlorine (e.g., HCHs) the more readily it would move through the atmosphere from warmer climates and be distilled out onto vegetation, soil, and water in colder climates; however, this distillation process does not appear to operate as effectively for less volatile organochlorines, such as DDT and endosulfan, whose concentrations reflect current or past local usage.

Organochlorines have been measured and have been shown to be persistent in the environment. Iwata et al. (1993) measured concentrations of HCHs, DDT, chlordane, and PCBs in 1989-1990. HCHs showed higher ocean-air and surface-seawater concentrations in the northern hemisphere than in the southern hemisphere and higher concentrations closer to the poles than at more-central latitudes. Total DDT concentrations were highest near tropical Asia. The concentrations of chlordanes and PCBs were more uniform. HCH concentrations in water were in the range of tens of picograms to nanograms per liter; the other chemicals were detected in the range of picograms to tens of picograms per liter. It is generally accepted that these chemicals are transported in water vapor for long distances and then deposited as the water condenses in cold regions—explaining the higher concentrations found at the poles (Iwata et al. 1993; Lode et al. 1995; Muir et al. 1995).

Air

Toxaphene, endosulfan, *p,p′*-DDT, and *p,p′*-DDE were measured in the air above seawater and in seawater of Resolute Bay, Canada (Bidleman et al. 1995). Chlorinated bornanes, including the insecticide toxaphene, were detected in the air at 6.9 pg/m^3, endosulfan was measured at 4.0 pg/m^3, and *p,p′*-DDT was found at less than 0.3 pg/m^3.

Studies in the southern hemisphere (India and Australia) show interesting patterns in DDT contamination of air, water, agricultural soil, sediment, and fish (Kannan et al. 1995). In India, where DDT continues to be used, air and water concentrations were 3.5 ng/m^3 and 17.5 ng/L, respectively. In Australia, the concentrations were 0.017 ng/m^3 and 0.17 ng/L, respectively, demonstrating the tremendous difference that can be associated with continued DDT use.

PCBs are present in the atmosphere primarily in the vapor phase; however, a small portion also exists in the particulate phase. The proportion of PCBs in the particulate phase is dependent on the ambient temperature and the vapor pressure of the specific PCB. A greater concentration of PCBs in the particulate phase develops with lower temperatures and vapor pressures (Delzell et al. 1994). The total amount of atmospheric PCBs has been estimated to be between 10,000 and 100,000 kg. Atmospheric deposition might form the greatest source of PCB

contamination for surface waters, as these compounds can be transported unchanged for thousands of kilometers. Delzell et al. (1994) found that concentrations of PCBs in ambient air did not vary substantially between urban and rural areas (0.13 to approximately 10 ng/m^3), although urban areas with point sources for PCBs might have higher concentrations (up to 1.26 mg/m^3 at a contaminated site). However, Hillery et al. (1997) found that atmospheric gas-phase concentrations of PCBs around the Great Lakes ranged from 89 to 370 pg/m^3 in the early 1990s, with concentrations at a station near Buffalo, New York, an industrialized area, generally 2-3 times higher than those in more remote, less populated areas. PCB concentrations were also temperature dependent, with lower concentrations occurring during the colder months.

Indoor air generally has higher concentrations of PCBs than does outdoor air. Concentrations in office buildings, including research facilities and a shopping complex, ranged from 44 to 240 ng/m^3; buildings with electrical equipment containing PCBs had up to a threefold greater concentration. Houses were found to have PCB concentrations ranging from 39 to 400 ng/m^3. The authors concluded that PCB concentrations in indoor air in public buildings and homes were approximately 10 times greater than concentrations in outdoor air (McLeod 1981, as cited in Delzell et al. 1994).

Water

Studies of the Great Lakes region in 1980 reported environmental concentrations of PCBs that were approximately twice as high as those found in 1991 (Pearson et al. 1996). Total PCB concentrations in the open waters of Lake Michigan dropped markedly ($p < 0.05$), from 1.2 ng/L in 1980 to 0.47 ng/L in 1991. The amounts of PCBs measured over the past few decades are based on different methods of analysis, with isomer-specific analytic methods being used over the last decade. Much work since that time has been on the highly toxic coplanar PCBs (Patterson et al. 1994). The PCB concentration in the water of remote Siskiwit Lake in 1984 was 2.3 ng/L (Swackhamer et al. 1988). Surface-water concentrations are generally less than 10 ng/L, although some concentrations in urban areas might exceed that (Delzell et al. 1994). Huestis et al. (1996) and others have discussed the difficulty in comparing concentrations of PCBs reported in the 1970s or early 1980s (based on total PCB concentrations) with values reported over the last decade (based on measurement of selected congeners). PCB concentrations in the surface waters of Siskwit Lake were 2.3 ng/L (Delzell et al. 1994).

In seawater, *p,p′*-DDE was found at 1.0 pg/L and chlorinated bornanes, including toxaphene, were detected at 48 pg/L (Bidleman et al. 1995). Toxaphene was found at 23 pg/L in Lake Laberge, Canada (Kidd et al. 1995).

The alkylphenol ethoxylates (APEs) constitute another class of synthetic compounds that are being examined as possible HAAs. Nonylphenol (NP) is

estrogenic (Soto et al. 1991). The APEs are a group of widely used surfactants and detergents. A study of APE concentrations in 30 U.S. rivers was sponsored by the U.S. Environmental Protection Agency and the Chemical Manufacturers Association (Naylor et al. 1992). The studies measured NP and various NP ethoxylates (NPEs) in rivers receiving municipal or industrial wastewater discharges. The NPEs consist of an NP molecule with between 1 and 100 ethoxylate chains (NPE_1 to NPE_{100}) almost exclusively in the para position.

Most of the water samples (60-75%) were below the limit of detection (0.1 ppb for NP, NPE_1, and NPE_2; 1.6 ppb for NPE_{3-17}). The highest concentrations for NP, NPE_1, and NPE_2 were about 1 ppb; the highest concentration for NPE_{3-17} was 15 ppb. Sediment samples had higher concentrations (18 ppb for NPE and 162 ppb for NP) (Naylor et al. 1992).

Bennie et al. (1997) analyzed NP in the Laurentian Great Lakes and reported that 58% of the surface-water samples contained 4-NP, with values ranging from less than 0.02 to 7.8 μg/L.

Soil

Dioxin concentrations in the sediments of remote Siskiwit Lake on an island in Lake Superior peaked between 1940 and 1970, the period of greatest expansion in the industrial use of chlorine. Sedimentation rates for dioxins decreased by 30% between 1970 and 1983, as did industrial use of chlorine (Spiro and Thomas 1994). PCB concentrations in the lake were 48 ppb in 1983 (Swackhamer et al. 1988). PCB concentrations in this lake, which has no point sources, were 48 ng/g of sediment.

In general, PCB concentrations in sediment have reflected the increased use of chlorine chemicals between 1940 and 1970. Sediments from Dark Lake, Wisconsin contained PCBs at 2.2 ppb between 1935 and 1948, but concentrations increased to 19-20 ppb between 1962 and 1981. Sediments from larger bodies of water (e.g., Lake Michigan) showed similar trends; concentrations of PCBs between the 1960s and the 1980s were greater than 90 ppb (Swackhamer and Armstrong 1986). Highly contaminated sediments, such as those from Waukegan Harbor, Illinois, near Chicago, might contain PCBs in excess of 500,000 ppm (NRC 1997).

As part of the U.S. Geological Survey's nationwide assessment of contaminants in carp, Goodbred et al. (1997) analyzed stream-bed sediments for phthalates (including diethylphthalate and diethylhexylphthalate) and phenols (including alkylphenol). Phenol concentrations in 22 samples ranged from nondetectable in 3 samples to greater than 1,000 μg/kg of dry weight in 1 sample; 13 samples contained less than 100 μg/kg. Phthalate concentrations ranged from nondetectable in 1 sample to greater than 2,300 μg/kg of dry weight in another sample (South Platte River at Denver); 8 samples contained less than 100 μg/kg and 12 samples contained between 100 and 500 μg/kg.

Bennie et al. (1997) examined sediments from the Great Lakes and upper St. Lawrence River and found that 66% of the samples contained detectable concentrations of NP and NPEs at concentrations of up to 38 μg/g and 6.0 μg/g of dry weight, respectively.

Food

Coumestrol, an isoflavonoid, is found in many plants. Coumestrol concentrations range from 0.1 μg/g of dry weight for spinach to 71.1 μg/g for fresh soybean sprouts; most vegetables contain less than 1 μg/g (Verdeal and Ryan 1979). Analysis of human urine showed excretion rates of 4,700-34,000 nmol/d for lignins and 7,400 nmol/d for isoflavones. Zearalenone dietary intake was estimated to be 0.05-0.1 μg/kg/d (Bennett and Shotwell 1979; Kuiper-Goodman et al. 1987; Warner and Pestka 1987).

The Food and Drug Administration (FDA) monitors for pesticides and other potential food contaminants that could affect the human endocrine system. The FDA Total Diet Study (conducted on an annual basis) is conducted to determine typical intake of pesticides and other chemicals in the United States (Gunderson 1995). From 1986 to 1991, nearly 5,000 finished food samples were analyzed for hundreds of pesticides and other agents. Intake was estimated by age and body weight. Concentrations were typically below the level of detection. Based on the annual Total Diet Study conducted between 1965 and 1984 by FDA, average daily intake of DDT by teenage males decreased from a high of 31 μg in 1965 to 2.5 μg in 1984, and PCB intake decreased from 1.4 μg in 1971 to 0.03 μg in 1984 (Tao and Bolger 1998). The Total Diet Study conducted between 1985 and 1991 indicated that of 4,914 food items analyzed, DDE was found in 16%, dieldrin in 8%, and lindane (hexachlorocyclobenzene) in 4% of the samples (Tao and Bolger 1998).

Wildlife

The continued use of DDT in developing countries does not necessarily result in greater contamination of biota. For example, a study of DDT contamination of fish in India and Australia showed similar values for both countries: The concentrations in fish were 15 ng/g in India and 22 ng/g in Australia (Kannan et al. 1995).

The U.S. Geological Survey examined 578 male and female carp at 25 river sites in the United States (Goodbred et al. 1997). Organochlorine pesticide concentrations in fish tissue ranged from nondetectable (5-10 μg/kg of wet weight) at two sites to 1,310 μg/kg of wet weight for fish taken from the South Platte River in Colorado. Total PCB concentrations ranged from nondetectable at 10 sites to 72,000 μg/kg from fish taken from the Housatonic River in Massachusetts. PCB concentrations in fish were highest in the northeastern United States, where tissue concentrations generally exceeded 1,000 μg/kg. The only

other site with fish that exceeded that concentration was a section of the South Platte River near Denver that has been affected by urban development.

Examples of the content of persistent organochlorines—DDT or PCBs—in biota from various regions, including those far from known sources, are shown in Table 3-2. The data in the table show ranges in the concentrations of total DDT usually measured as DDE (a persistent DDT metabolite) and either total PCBs or the sum of multiple PCB congeners. The concentrations of individual estrogenic, proestrogenic, or antiestrogenic PCB congeners are not known or recorded, or they are below the limits of detection. In a Swedish study, Andersson et al. (1988) found that the highest concentrations of DDT and PCBs were in fish predators, such as raptorial birds and seals. The DDT concentrations were 0.14-57 μg/g of extractable lipid in fish, 5.5-400 μg/g in muscles from fish-eating birds (e.g., guillemot), 20-835 μg/g in eggs from high-trophic-level birds (e.g., peregrine falcon and sea eagle), and 1.7-66 μg/g in seals. PCB concentrations were 0.7-24 μg/g of extractable lipid in fish, 12.0-310 μg/g in muscles from fish-eating birds, 34-987 μg/g in eggs from high-trophic-level birds, and 1.9-75 μg/g in seals.

Geographic-distribution patterns, similar to those described above, are inferred from many studies by many investigators. Kannan et al. (1995) examined the concentrations of organochlorine residues in fish from many locations in tropical and subtropical Asia. As in other studies, the researchers reported that the concentrations of PCBs were higher near major urban areas (21-32 ng/g of wet weight for urban Australia compared with 2.4-7.6 ng/g for rural areas). Nevertheless, some species—even in remote areas—exhibit significant concentrations of persistent chemicals. The position of those animals in the food chain and the long biologic half-lives of the chemicals (see Table 4-1) explain the concentrations. The species include top carnivores (e.g., whales and polar bears) and organisms with abundant lipid content, such as some birds. The elevated concentrations of DDT found in more-remote regions, as well as urban areas, reflect its continued use in malaria-eradication programs for control of anopheles mosquitoes. The same geographic-use patterns probably contribute to higher concentrations of DDT in humans in such regions. DDT was the predominant organochlorine found in fish tissue from tropical countries (0.43-28 ng/g of wet tissue), whereas concentrations of the other organochlorines (PCBs and hexachlorocyclohexanes) were relatively low. Kannan et al. (1995) concluded that PCBs and DDT, being less volatile, persist longer closer to emission sources rather than being transported to more temperate climates.

As of 1994, data on organochlorine (and metal) contaminants in tissues of baleen whales (*Mysticetes*) had been published for approximately 1,000 whales in 10 species from the world's oceans. Toothed whales (*Odontocetes*) have also been examined worldwide. As summarized by O'Shea and Brownell (1994), the concentrations of contaminants in tissues of the filter-feeding baleen whales gen-

TABLE 3-2 HAAs in Biota from Various Regions

Species	Tissue Sampled	Location	Chemicals Detected and Concentration: Total DDT	PCBs	Reference
Cetaceans					
Beluga whale	Blubber	Arctic	1-3 μg/g (w)	2-3 μg/g (w)[a]	Muir et al. 1992
		St. Lawrence	19-100 μg/g (w)	50-75 μg/g (w)[a]	Muir et al. 1992
Killer whale	Blubber	N. Pacific		> 600 μg/g (w)[a]	Tanabe et al. 1994
Fish					
Pollock	Muscle	Bering Sea	0.002 μg/g (w)	0.007 μg/g (w)[a]	Kannan et al. 1995
Mixed species	Muscle	Bombay	0.044 μg/g (w)	0.003 μg/g (w)[a]	
		Sydney	0.055 μg/g (w)	0.12 μg/g (w)[a]	
Rattail	Liver	Carson Canyon		0.36 μg/g (w)[b]	Stegeman et al. 1986
		Hudson Canyon		2.73 μg/g (w)[b]	
Winter flounder	Liver	Fox Island		3.1 μg/g (d)[c]	Elskus et al. 1994
		New Bedford		333 μg/g (d)[c]	
Birds					
Adelie penguin	Liver	Antarctic	0.6 μg/g (d)	0.04 μg/g (d)[a]	Focardi et al. 1992
Polar skua	Liver	Antarctic	2.5 μg/g (d)	0.32 μg/g (d)[a]	Focardi et al. 1992
Peregrine falcon	Egg		10.4 μg/g (w)	9.2 μg/g (w)[a]	D.J. Thomas et al. 1992

NOTE: w, wet weight; d, dry weight. Concentrations based on dry weight are higher than those based on wet weight.

[a] Total PCBs or sum of multiple congeners.

[b] 24 congeners.

[c] 17 congeners.

erally are low (less than 0.10-587 ppm for total DDT; 1.9-27.8 ppm for PCBs) in comparison with those found in the carnivorous toothed whales (2.4-2695 ppm for total DDT; 1.4-800 ppm for PCBs). Concentrations of total DDTs and total PCBs in baleen whales are greater in the northern hemisphere than they are in the southern oceans, apparently because of greater contamination of northern ecosystems (O'Shea and Brownell 1994).

Given the concentrations of many contaminants that accumulate in cetaceans, these animals could be considered marker species for determining the geographic extent of HAA effects. Comparisons of different populations of beluga whales are particularly relevant. Male animals from a population in the St. Lawrence estuary had concentrations of PCBs and DDT that were 25- and 32-fold higher, respectively, than those in populations from other arctic locations (Muir et al. 1990a,b). The data in Table 3-2 show the range of concentrations of DDTs and PCBs in these animals. Several species of marine mammals from the east coast of Canada generally had substantially higher concentrations than did Arctic animals (Muir et al. 1992). Muir et al. (1996) found a 1.5- and a 1.9-fold decrease in DDT and PCB concentrations, respectively, in male beluga whales in the St. Lawrence estuary between 1982-1985 and 1993-1994.

However, studies of PCB and DDT residues and other organochlorines in marine mammals from around the globe also suggest that concentrations in animals are declining in some regions. In ringed seals from the Canadian arctic, for example, PCB concentrations decreased by more than 60% from 1972 to 1981 (Addison et al. 1986); in the same animals, total DDT decreased by 30%. Concentrations of PCBs in polar bears in some regions actually increased between 1969 and 1984 (Muir et al. 1988a). Interpretations of the significance of changes in contaminant concentrations are open to question if the number of animals is small. The residues of PCBs and of DDT and its metabolites detected in the blubber of harbor porpoise and bottlenose dolphin from the north and east coasts of Scotland between 1988 and 1991 ranged from quite low (0.28 μg/g for total PCBs and 0.14 μg/g for total DDT) to relatively high (23 μg/g for total PCBs and 10.2 μg/g for total DDT) (Wells et al. 1994). The concentrations of these compounds were found to be highly dependent on the age and sex of the animals; higher concentrations were found in males and older animals of both sexes. Females are thought to transfer up to two-thirds of the organochlorine concentrations normally found in their blubber to their offspring during gestation and lactation. The range of concentrations seen in this study emphasizes that data from single or small numbers of animals might be of limited value when comparing information on organochlorine residues in marine mammals within or between regions.

The concentrations of some regulated halogenated organic compounds have decreased since the 1970s. For many other chemicals, there are inadequate data upon which to evaluate trends. The most studied chemicals are PCBs and DDT, and the production of these compounds has been banned in the United States for

the past 20 yr, resulting in declines in environmental concentrations. Examples of declines in other areas include a progressive and substantial decline in PCBs and DDT found in eggs taken from bird colonies in the Canadian Atlantic region between 1972 and 1978 and a decrease in PCBs and DDT in Bering Sea fish from 1982 to 1992 (Kannan et al. 1995). Concentrations of these compounds in salmonries have ceased declining during the late 1980s and early 1990s, most likely due to atmospheric transport of these chemicals from their use in other countries (Miller et al. 1993). However, Stow (1995) reported that herring gull eggs collected from the Great Lakes are now showing steady-state concentrations of PCBs, associated with continued evidence of reproductive impairments in fish-eating birds (see also Giesy et al. 1995).

EXPOSURE

Sources of HAA exposure were discussed above. The resulting exposures to these environmental contaminants are reviewed below. The examples illustrate the significant potential for diverse routes and modes of exposure. They also demonstrate the range of possible concentrations. Some exposures may lead to large doses of HAAs. Each exposure to a given HAA or to a mixture of substances (e.g, all PCB congeners) must be considered separately to determine whether it poses a significant health risk, but the committee recognizes that wildlife and humans are exposed to complex mixtures of chemicals and that interactions between chemicals in mixtures cannot always be predicted by examining each chemical individually.

Daily human exposures have been estimated for some chemicals that have been reported to have HAA activity (Table 3-3). People who either work with HAAs or whose diets are very high in HAAs can receive substantially high doses. People who live in highly contaminated areas also can experience above average exposures.

The exposure estimates in Table 3-3 are based on several assumptions, many of which tend to overestimate exposure, such as water intake or dietary intake. Other assumptions can overestimate or underestimate potential biologic activity of the agents. Factors that influence response, such as receptor affinity, bioavailability, protein binding, and potency are described in detail in Chapter 4. One example of a likely overestimate is that DDT concentrations are typically reported for total-DDT-related isomers. Although many of those isomers have much lower estrogenic activity than does *o,p′*-DDT, the relative estrogenicity of DDT is fixed at the estrogenicity of *o,p′*-DDT. On balance, DDT is more persistent in body fat than are most natural estrogens. Tables 3-3 and 3-4 illustrate the orders-of-magnitude difference between the exposures to the estrogenic drugs and the exposures to the environmental chemicals reported to have estrogenic activity. Table 3-4 lists ranges for daily production of endogenous estrogen by humans.

TABLE 3-3 Potential Daily Human Exposure to Various Estrogens

Source	Route	Exposure (μg/d)	Reference
Oral contraceptive, mestranol or ethinyl estradiol	Oral	20-50	Hardman et al. 1996
Hormone replacement therapy, E_2	Oral	50-200	Scott et al. 1991
DES, to prevent spontaneous abortion	Oral	5,000-150,000	Dieckmann et al. 1953; Wilcox et al. 1995
Bisphenol A in food cans	Oral	6.3	Howe and Borodinsky 1998; Howe et al. 1998; Wingender et al. 1998
Bisphenol A in beverage containers	Oral	<0.75	Brotons et al. 1995; Howe and Borodinsky 1998; Howe et al. 1998; Wingender et al. 1998
Bisphenol A in dental sealant	Oral	90-931 in first hr	Olea et al. 1996
Nonylphenol in river water	Oral	0.7	Weeks et al. 1996
DDT in total diet	Oral	0.01	Gunderson 1995
PCBs in total diet	Oral	0.002	Gunderson 1995
Phytoestrogen, 100 g of wheat with 2 ppm zearalenone	Oral	200	Verdeal and Ryan 1979
Phytoestrogen, total bioflavonoids	Oral	1,000,000	Kuhnau 1976
DDT, India, environmental	Inhalation Water Diet, as fish	0.0798 0.035 1.5	Kannan et al. 1995 Kannan et al. 1995 Kannan et al. 1995
DDT, Inuit, arctic	Diet high in animal fat	24.2	Kuhnlein et al. 1995
DDT, Sahtu Dene/Metis, arctic	Diet low in animal fat	0.51	Kuhnlein et al. 1995

TABLE 3-4 Endogenous Estradiol Levels in Humans

Source	Level (pg/mL)	Reference
Girls before puberty	0.6 ± 0.6	Wilson et al. 1998
Boys before puberty	0.08 ± 0.2	Wilson et al. 1998
Adult male	15-40	Greenspan and Strewler 1997
Adult nonpregnant women	60-700	DeGroot et al. 1995
Pregnant women	500-15,000	DeGroot et al. 1995

Many exposure values are the result of simplistic assumptions, but they demonstrate several important points about exposure estimates for HAAs. First, one route of exposure often provides the dominant contribution to total dose. Oral exposure through food is generally greater than other exposures, in part because of a large daily intake of synthetic HAAs in fish and because of increased consumption of plant matter containing phytoestrogens by some populations, such as vegetarians. Second, inhalation is rarely a significant contributor to the dose of these chemicals from environmental exposures.

Human Background Concentrations

Steroid hormones, such as corticosteroids, androgens, and estrogens, are under feedback control from the pituitary gland. Therefore, as endogenous steroid hormone concentrations increase, the pituitary feedback control signals the endocrine organ (the adrenal gland or gonads), through pituitary hormones, to cease production or release of the endogenous steroid or to stimulate the release of opposing hormones. This homeostatic control in response to endogenous hormones is critical for maintaining proper hormone concentrations.

There is significant endogenous hormone production in males and females. The concentrations of endogenous estrogen hormones in humans change rapidly over short periods. Endogenous hormones can be active at concentrations as low as picograms per milliliter (pg/mL) in the blood, and the concentrations change with the reproductive cycle in females and episodically in males. As shown in Table 3-4, for example, adult men and prepubertal boys and girls have between 0.08 and 40 pg/mL of estradiol (Greenspan and Strewler 1997; Wilson et al. 1998). Nonpregnant women produce between 60 and 700 pg/mL of estradiol (DeGroot et al. 1995); the wide variation is due to the normal reproductive cycle. Estrogen concentrations increase dramatically during pregnancy. Pregnant women have between 500 and 15,000 pg/mL (DeGroot et al. 1995).

Circulating concentrations of estradiol range from 10 to 175 pg/mL during the female menstrual cycle, with the highest concentrations at the late follicular phase (Thorneycroft et al. 1971). Progesterone ranges from less than 1 to 10 ng/mL

(Thorneycroft et al. 1971). Studies of the concentrations of estrone and estradiol show that both hormones are present in the follicular phase of premenopausal women at about 50 pg/mL; whereas, postmenopausal women have concentrations of 10-30 pg/mL (Yen 1977; Slemenda et al. 1996). Blood concentrations of estradiol and estrone in normal adult men are similar to those in postmenopausal women (Zumoff et al. 1982).

Human exposure to PCBs from various environmental media in an urban setting has been determined (Table 3-5) using the assumption that an individual spends 56% of his or her time outdoors and 44% indoors (a substantial overestimation of outdoor time but illustrative nonetheless). This exposure assessment does not include intake from diet, which may be substantial as PCBs tend to bioaccumulate in higher trophic levels, such as in fish, dairy products, and meat. Exposure to PCBs from diet decreased from a high of 6.9 μg/d in 1971 to 0.05 μg/d in 1989. Combining dietary exposure with other routes of exposure yields a total daily exposure, on a body-weight basis, of 0.11 μg/kg of body weight per day for 1971 and 0.008 μg/kg of body weight per day in 1989, a decrease of 138-fold (Delzell et al. 1994).

An analysis of human adipose tissue taken from surgical patients and cadavers between 1972 and 1983 found that 95.3% of the U.S. population had detectable concentrations of PCBs, 28.9% had PCBs at greater than 1 ppm, and 5.1% had concentrations greater than 3 ppm. Concentrations increased with age, with children younger than 14 having less than half the concentrations of adults, possibly reflecting the banning of PCBs in 1976. More than 95% of all the U.S. population had detectable concentrations of PCBs, regardless of age, sex, race, or geographic location (Robinson et al. 1990).

TABLE 3-5 Estimated Human Exposure to Polychlorinated Biphenyls (PCBs)

Environmental Media	PCB Concentration	Exposure to Media	Bioavailability (%)	Exposure to PCBs (μg/d)
Soil	0.015 μg/g	0.05 g/d	50	0.000375
Air			43	
Outdoor	9.63 ng/m^3	13 m^3/d		0.054
Indoor	100 ng/m^3	10 m^3/d		0.41
Drinking Water	< 20 ng/L	2 L/d	50	< 0.02
Total				0.48

SOURCE: Data from Delzell et al. 1994.

Highly Exposed Populations

Although many of the synthetic HAAs discussed in this report are no longer in general commerce, some of them are still produced in developing countries. As a result, there is continuing exposure for production workers and waste handlers, as well as the general population. Even for HAAs that are not longer manufactured, exposure might occur as a result of earlier production and use. For example, although PCBs are no longer produced in the United States, they are still found in older electrical equipment, and maintenance workers can be routinely exposed to them.

The issue of detection limits of the assays used is critical with regard to presenting information about the incidence of exposure to specific chemicals. The issue is covered in more detail in Chapter 11. Hill et al. (1989) analyzed 12 chlorinated phenols in 197 children living in Arkansas using a detection limit of 1 ppb. They reported a much higher percentage of exposure to the organochlorine chemicals measured than some other studies that used higher detection limits. For example, 54% of the children were found to have the organochlorine 2,4,5-trichlorophenol in their urine with a detection limit of 1 ppb, but with a detection limit of 5 ppb, only 9% of the children tested positive, a result similar to prior studies conducted with the higher detection limit.

Maternal milk is discussed in Chapter 4 as a route of excretion for nursing women. Breast milk is an important mode of exposure for nursing infants. Organochlorines tend to concentrate in fat (Rogan et al. 1991; Jensen and Slorach 1996), and human milk is about 3.3% fat. Organochlorine concentrations in milk might vary substantially, depending on the exposure of the mother. However, the benefits of breast milk are typically seen as outweighing the potential for chemical exposure, even for those at the high end of the exposure range (Rogan et al. 1991; Lederman 1996).

Although the dose to the fetus is of critical importance in determining HAA effects, the dose is dependent on the exposure of the mother. An analysis of PCBs in umbilical cord blood of women who had and had not consumed Great Lakes fish, known to have high PCB concentrations, indicated that the average mean total PCB concentration in the cord blood was approximately 1.0 ppb for all women. However, those women who ate Great Lakes fish had higher absolute concentrations of highly chlorinated PCBs in the cord blood of their neonates, and the concentrations were correlated with consumption relative to the time of pregnancy (Stewart et al. in press). (For more information on fetal doses, see Chapter 4.)

Based on an inhalation exposure factor of 22.8 m^3/d (ICRP 1981) and DDT concentrations in air of 3.5 ng/m^3 and 0.017 ng/m in India and Australia, respectively, the DDT exposure by inhalation—making no assumptions about deposition in the lung, absorption, or disposition—would be 79.8 ng/d in India and

0.388 ng/d in Australia. Assuming water intake of 2 L/d, the DDT exposure from consumption of water containing 17.5 ng/L in India and 0.17 ng/L in Australia would be 35 ng/d and 0.34 ng/d, respectively. Assuming a fish intake of 100 g/d and assuming that the analyzed part of the fish is also the part consumed, DDT intake would be 1,500 ng/d in India and 2,200 ng/d in Australia. The sum of exposures would be 1,615 ng/d for India and 2,201 ng/d for Australia.

Investigators have examined the dietary exposures of people living in polar regions, especially those who consume foods high in animal fat. It is reasonable to believe that such populations would receive high dietary exposures to organochlorines because of the higher concentrations of such chemicals in polar regions and because of accumulation of such chemicals through the food chain. One study compared Inuit women from Baffin Island in the eastern arctic and Sahtu Dene/Metis women from the western arctic. The Inuit women ate a diet high in ringed seal meat and blubber and high in walrus, mattak, and narwhal blubber, and the Sahtu Dene/Metis women ate a diet of caribou, whitefish, trout, and duck (Kuhnlein et al. 1995). Inuit women exceeded acceptable daily intakes of chlordane-related compounds and toxaphene more than 50% of the time and frequently exceeded acceptable intakes of dieldrin and PCBs. Inuit women had a mean intake of 24.2 μg of DDT per day, compared with 0.51 μg of DDT per day by Sahtu Dene/Metis women.

Other studies showed greater concentrations of organochlorines in Inuit women than in Caucasian women from urban areas south of Quebec (Dewailly et al. 1993a). DDE in human milk fat averaged 1,212 ng/g and 336 ng/g in Inuit and Caucasian women, respectively. Total PCB concentrations in maternal milk were 1,052 ng/g and 157 ng/g in Inuit and Caucasian women, respectively. Assays of foodstuffs showed that polar-bear fat had total PCB concentrations of 7,002 ng/g; beluga blubber, seal blubber, and arctic char muscle had total PCB concentrations of 1,002 ng/g, 527 ng/g, and 152 ng/g, respectively. These data show various sources for dietary exposure that could explain the higher doses and concentrations of organochlorines found in the Inuit population.

A comprehensive review of PCB exposure for humans living in the Great Lakes region was conducted by the Agency for Toxic Substances and Disease Registry (ATSDR 1999). It found that people who consumed fish taken from the Great Lakes had PCB body burdens that were 2-4 times greater than those of the general U.S. population. Furthermore, PCB concentrations in breast milk of women who ate Great Lakes fish were almost twice those of a control group (Fitzgerald et al. 1998, as cited in ATSDR 1999). PCB concentrations in blood were correlated with the number of years an individual had consumed Great Lakes fish. Individuals who consumed less than 6 pounds of fish per year had a geometric mean for PCB blood concentrations of 6.8 ppb, whereas those who consumed more than 24 pounds of Great Lake fish had mean blood PCB concentrations of 19 ppb (Hovinga et al. 1993).

Phytoestrogens

Human and animal exposures to the phytoestrogens, particularly isoflavones, can be very high, because these compounds are found in many foods. Genistein, daidzein, formononetin, and equol are all present in clover. Infertility in sheep, "clover disease," has been traced to isoflavone concentrations as high as 5% of the dried weight of clover (Verdeal and Ryan 1979).

The recent practice of feeding infants soy-based formula has raised concerns with regard to the long-term health effects of exposure during development (Setchell et al. 1997; Irvine et al. 1998). For example, it has been recognized for some time that feeding infants soy-based formula was associated with goiter (thyroid enlargement associated with thyroid hormone deficiency) in animals and human infants (Shepard et al. 1960). One mechanism by which isoflavonoids, such as genistein, reduce thyroid hormone concentrations and result in goiter is by inhibiting thyroid peroxidase activity; this enzyme catalyzes thyroid hormone biosynthesis (Divi and Doerge 1996). The concentration of soy phytoestrogens that inhibited thyroid hormone biosynthesis is within the range of exposure of infants maintained on soy formula. Soy-based formulas contained isoflavones at 32-47 μg/mL, which corresponded to a daily exposure to total isolfavones of 4.5-8.0 mg/kg of body weight per day for a 4-mo-old infant. That concentration is 6- to 11-fold higher than concentrations known to cause hormonal effects in adults. (Divi et al. 1997; Setchell et al. 1997). In a study by Irvine et al. (1998), the phytoestrogen content of soy-based formulas and cereals were compared with dairy-based formulas and human breast milk. Again, infants received approximately 3 mg/kg of body weight per day from the soy-based formula, but a single daily serving of infant cereal could increase the isoflavone intake by more than 25%. Dairy-based formula and human breast milk contained isoflavones below the limit of detection. Human breast milk had undetectable concentrations of phytoestrogens regardless of the diet of the mother, including women who were vegetarians and consumed greater than 50 g of soy products in a 48-hr period before sampling.

Potential exposure to plant estrogens found in wood has been assessed by various in vitro and in vivo bioassays. Wood-derived estrogens, such as beta-sitosterol, could represent environmental hormone exposures, particularly from pulp and paper mill effluents, downstream of wood-processing facilities. Mellanen et al. (1996) used two breast-cancer cell lines in vitro (MCF7 and T-47D) and expression of the vitellogenin gene in rainbow-trout livers to estimate estrogenic activity of wood-derived compounds. Some compounds, such as beta-sitosterol, were estrogenic in human and fish bioassays, but some phytoestrogens, such as betulin and pinosylvin, were estrogenic only in humans.

Synthetic Agents

Not all HAAs are pesticides, nor are all pesticides considered to be HAAs. However, many organochlorine compounds do have pesticidal uses and, therefore, have a significant exposure potential for nontarget populations.

Pesticides

Pesticides are listed among the common examples of HAAs. The organochlorine pesticides DDT, endosulfan, dieldrin, and vinclozolin have all been reported as having hormone-mimicking or hormone-inhibiting activity (Kupfer and Bulger 1980; Soto et al. 1994; Kelce et al. 1995). Pesticide exposures occur by various routes. Some routes of exposure are considered voluntary, such as dermal or inhalation exposure for someone who mixes, loads, or applies a pesticide. Other exposures are involuntary or unwitting, such as ingestion of food stuffs that have pesticide residues. Involuntary exposure can also occur as a result of aerial pesticide spray that drifts to nearby areas.

Sources of the greatest consumer exposure to dietary pesticide residues have been listed by the U.S. Department of Agriculture (USDA) (Kuchler et al. 1996). Exposures are summarized for on-site farm use, postharvest use of pesticides, pesticides used on imported foods, and canceled pesticides that are environmentally persistent (Kuchler et al. 1996). In that report, the USDA Agricultural Market Survey (AMS) used food-consumption data and residue data for 50 pesticides found on 12 fruits and vegetables to estimate dietary exposure. The data were analyzed as "commodity-pesticide pairs"; the residue value was determined for each of the 50 pesticides paired with each of the 12 commodities. Particular attention was paid to special categories, such as exposure for children of various ages. Samples were collected from markets. Before the residues were analyzed, the food was handled (washed, peeled, or cooked) as a typical consumer would prepare it. Several of the 50 pesticides have been implicated as HAAs (DDT, atrazine, dicofol, endosulfan, lindane, methoxychlor, and vinclozolin).

The AMS makes several important points about dietary exposure to pesticides (Kuchler et al. 1996):

- Residues on many samples were below the limits of detection.
- Postharvest application accounted for a large proportion of the residues detected.
- Canceled pesticides (such as DDT) were present on the foodstuffs.
- Detection does not necessarily equate to the potential for adverse effects. The concentration on the commodity, the amount ingested, and the inherent toxicity of the pesticide all must be considered.
- Consumption patterns are different for different groups. For example, children generally eat substantially more fruit (such as bananas or grapes) and

weigh considerably less than adults do, and children probably eat fewer brussels sprouts than adults do. Therefore, children would ingest more of the pesticides, on a body-weight basis, than adults for some crops and less for others.

The FDA Total Diet Study determined intake of selected pesticides, synthetic chemicals, and radionuclides from 1982 to 1991. The study focused on table-ready food rather than unwashed raw agricultural commodities or partially prepared fruits and vegetables (Gunderson 1995). It allowed FDA to determine exposures after various food preparations had been done. A market basket of 234 foods was collected periodically between 1986 and 1991 and tested for some 300 organic chemicals. The mean daily intake for these compounds was calculated based on milligrams of substance per kilogram of body weight per day. Eight age and gender groups were assessed for daily exposure. Compounds that have been identified as potential HAAs were included in the group of chemicals for which there were detectable residues (chlordane, chlorbenzilate, DDT, dicofol, dieldrin, endosulfan, endrin, heptachlor, methoxychlor, PCBs, pentachlorophenol, toxaphene, and vinclozolin). The largest exposure when corrected for body weight was almost always seen in the 2-yr-old group. Even in that age group, dietary exposure to DDT was less than 0.05 to 0.0001 μg/kg/d. PCBs were about 0.002 μg/kg/d. The Total Diet Study showed that even though use of DDT is prohibited and its concentrations were low, it was still detected in 16% of the items analyzed. Dieldrin was found in 8%, and endosulfan was found in 7%. The Total Diet Study also demonstrated that dietary exposures to banned pesticides (DDT and dieldrin) dropped by 50% or more from 1982-1984 to 1986-1991 in all age and gender groups; no such drop was seen for malathion or parathion, which remain in use (Table 3-6). Most (61-80%) of the dietary contribution of DDT and dieldrin came from meat and dairy products. Foods with higher lipid content tended to have higher concentrations of contaminants. The Total Diet Study included imported foods that might have been from countries using some U.S. banned pesticides; however, these imported foods were analyzed for the presence of banned chemicals. In 1997, DDT was found in 24% (244 samples) of 1,036 food samples analyzed for pesticides and other chemical residues. Endosulfan was found in 11 (13%) of the baby-food samples at concentrations of 0.0004-0.0145 ppm, according to the FDA 1997 Pesticides Monitoring Database.

Synthetic Chemicals

Exposure to synthetic chemicals also can present risk of potential HAA activity. Several organochlorine chemicals, in addition to the pesticides mentioned above, have potential HAA activity. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (and related halogenated dibenzodioxins and dibenzofurans) and PCBs are persistent in the environment; some congeners are toxic at low concentrations, and some have hormonal (thyroid, antiestrogenic) activity (EPA 1994a,

TABLE 3-6 Mean Daily Intake[a] for Some Pesticides, 1982-1991

Pesticide	1982-1984	1984-1986	1986-1991
DDT, total	0.03	0.02	0.01
Dieldrin	0.007	0.004	0.003
Malathion	0.07	0.08	0.07
Parathion	0.001	0.001	0.001

[a] In milligrams per kilogram of body weight per day. From the FDA Total Diet Study.
SOURCE: Gunderson 1995.

1996). These compounds have been detected in various environmental and biologic media.

There is a potential for exposure to other synthetic chemicals that have measurable hormonal activity. One example is bisphenol A (BPA). BPA is a plasticizer used in epoxy resins that line various food and beverage cans and in polycarbonate resins (Table 3-1), which are used in microwave ovenware, returnable water and milk containers, refrigerator crisper drawers, and other food storage applications, including baby bottles. Bisphenol A is one of the top 50 chemicals produced in the United States, with over 1.6 billion pounds being produced in 1995 (Jennings 1994; Kirschner 1996). BPA is not usually used for disposable containers because of its cost.

The potential for exposure to BPA from these applications has been studied by Brotons et al. (1995) and by the Society for the Plastics Industry (SPI) (Howe and Borodinsky 1998; Howe et al. 1998; Wingender et al. 1998). These groups have investigated the potential for migration of BPA from epoxy-lined cans and beverage containers into the liquid in the cans (Brotons et al. 1995). Neither group measured the migration of BPA into the solid food in the cans. Brotons et al. (1995) found that a 300-g can of peas contained 50 mL of fluid with a BPA concentration of 23 μg, and the fluid was found to have weak but measurable estrogenic activity as determined by the human MCF7-cell-line bioassay. They also ran tests to determine whether high temperatures (as one might use for sterilization) would increase migration of BPA. Estrogenic activity was detected in such cases. The authors reported no estrogenic activity in fresh vegetables used as controls; however, some activity might have been expected considering that many of the vegetables tested contain phytoestrogens (MAFF 1996a). It has been reported that the MCF7 cell line is sensitive to dietary or plant estrogens and will detect coumestrol with a 50% response at 0.48 ng/mL (Welshons et al. 1990).

SPI also measured BPA concentrations (Howe and Borodinsky 1998; Howe et al. 1998; Wingender et al. 1998). SPI ran studies using FDA protocols to develop a worst-case potential exposure. The analytic data from the two groups

were quite similar; SPI originally found BPA concentrations at a range of undetectable (less than 5 ppb) to 121 ppb, with an average of 63 ppb for the food cans. All samples for the beverage cans were below the limit of detection (less than 5 ppb) (Howe and Borodinsky 1998; Howe et al. 1998; Wingender et al. 1998). SPI calculated a worst-case exposure based on the analytic-data assumptions about food-consumption patterns and food-type (as it relates to container type) distribution factors. Subsequent studies by SPI, however, demonstrated that its analytic method did not eliminate analytic interference, and its method over estimated the BPA concentrations in food cans in some analyses (Howe and Borodinsky 1998; Howe et al. 1998; Wingender et al. 1998). The SPI method was tested in nine laboratories. After elimination of interference, average BPA residues from food cans dropped to 36 ppb, indicating that the analytic method used to determine the presence and concentration of HAAs in various environmental samples can be an important factor when assessing the exposure concentrations.

SPI made a worst-case dietary exposure assessment, assuming that BPA residues were present at 5 ppb for beverages, even though none was detected, and present at 37 ppb for other foods. That assessment resulted in a total dietary concentration of 2.1 ppb. Using FDA assumptions for dietary intake (3,000 g/d) and body weight (60 kg), a worst-case oral exposure to BPA at 6.3 μg/d or 0.105 μg/kg of body weight per day was calculated.

Polycarbonate resins were not found to release detectable (less than 5 ppb) concentrations of BPA under high-temperature, worst-case conditions (Howe and Borodinsky 1998; Howe et al. 1998; Wingender et al. 1998). Using FDA assumptions and models for single-use and repeat-use items that come into contact with food, SPI concluded that the dietary concentration was less than 0.25 ppb or 0.75 μg per person per day.

Another product group that has been studied for its potential contribution to BPA exposure is resin-based dental sealants and composites. These products are used to seal and repair teeth. Olea et al. (1996) did a study in which approximately 50 mg of the sealant was applied to the teeth of 18 subjects. One hour later, BPA and other products were measured in the subjects' saliva. BPA was found at 90-931 μg. That 1-hr exposure is 10-100 times greater than the worst-case daily exposures for BPA in food cans (9.6 μg/d). One would expect the exposure in the first hour after application to be fairly extreme because it represents 0.2-2% of the total applied dose in 1 hr. Such release could not continue for long, because the resin would be lost in as little as 2 d.

Alkylphenols are widely used synthetic chemicals. With such large use, releases to the environment and, therefore, exposure to alkylphenol ethoxylates (APEs) could be considerable. Exposure to aquatic organisms in rivers that receive APE-containing effluent is the major area of concern, although exposure is mitigated by wastewater treatment, which removes 92.5-99.8% (Naylor 1995). The upper 95% confidence limit of nonylphenol (NP) in river water for a 30-river

study was 0.35 ppb (Weeks et al. 1996). Assuming no reduction through treatment of drinking water and an average consumption of water at 2 L/d, human exposure to NP in drinking water would be 0.7 μg/d. Although NP is estrogenic (Soto et al. 1991), its activity is approximately 3 orders of magnitude below that of estradiol in vivo (Jobling et al. 1996). Thus, the estrogenicity produced from NP-contaminated drinking water would be equivalent to that produced by a daily estradiol exposure of 0.007 μg/d. Plastics, such as polystyrene and polyethylene, the other major plastic materials in addition to polycarbonate, also contain NP, which is used as an antioxidant to prevent discoloration.

Another class of synthetic chemicals that are putative HAAs are the phthalate esters. Ministry of Agriculture, Fisheries, and Food (MAFF) investigated dibutyl phthlate (DBP) and diethylhexyl phthalate (DEHP) for their presence in paper and board packaging, food, and infant formula (MAFF 1995, 1996a,b). DBP was found in 98% of the packaging materials at concentrations of 5-5,860 ppm. DEHP was present in 95% of the packaging at 5-3,030 ppm. Food samples stored in the packaging were analyzed; DBP was present in 27 of 31 samples at 0.04-62 ppm and DEHP was found in 30 of 31 samples at 0.1-25 ppm (MAFF 1995). The dose concentrations from dietary intake of total phthalates ranged from 0.1 to 0.8 mg per person per day (97.5% upper confidence limit is 0.4-1.6 mg per person per day) (MAFF 1996a). Exposure of infants through formula was 0.10-0.13 mg/kg of body weight per day.

Therapeutic Agents

Women of child-bearing age and postmenopausal women might have substantial voluntary exposure to estrogenic compounds. Current oral contraceptives contain a daily dose of 20-50 μg of a potent orally active estrogen, such as ethinyl estradiol or mestranol (Hardman et al. 1996). Estrogenic compounds are administered by prescription as daily contraceptive preparations, in hormone-replacement therapy, and as postcoital emergency contraceptives (the "morning-after" pill). Birth-control preparations use 20-50 μg of ethinyl estradiol or mestranol per day (Gerstman et al. 1991; Hardman et al. 1996). The postcoital contraceptives use one dose, which is several times greater than the typical daily dose for oral contraceptives, within 3 d of intercourse, followed 12 hr later by the same dose (Trussell et al. 1996).

Oral-contraceptive use might be a potential HAA exposure for the mother and the developing child. Studies have been done on the developmental effects of oral-contraceptive use that occurs before or in early pregnancy (Kallen et al. 1991). That case-control study investigated whether hypospadias in infants were associated with maternal use of oral contraceptives before or in early pregnancy. There was no association demonstrated in that case. Another potential HAA exposure is the use of oral contraceptives during lactation. Such exposure was studied using an exposed group of children whose mothers used ethinyl estradiol

and a control group whose mothers did not (Nilsson et al. 1986). Intellectual and psychologic behavior were studied, and weight and height increases were also investigated. Although the study did not demonstrate changes from maternal exposures to oral contraceptives, such study designs illustrate the importance of evaluating this potential HAA exposure.

Many women receive various forms of postmenopausal estrogen replacement. Estrone and estradiol are absorbed in equal amounts transdermally. Estrone is absorbed 2-4 times more than estradiol when taken orally (Scott et al. 1991). Estrogen exposures of 50-200 mg/d are effective in decreasing postmenopausal bone loss (Lindsay 1987). Shoff et al. (1998) suggest that consumption of foods high in phytoestrogens, particularly dark bread, might inversely affect testosterone concentrations in postmenopausal women.

Each pharmaceutical exposure to estrogen is designed to produce a desired effect. The hormones are administered under the care of a physician, who has control over the dose and can observe the outcome. The use of diethylstilbestrol during a critical period of gestation is now known to have produced adverse effects without evidence of a benefit (Dieckmann et al. 1953). In contrast to environmental exposures, the amount and circumstances of pharmacologic exposures are controlled.

Wildlife

The presence of vitellogenin, a female fish-specific protein, in male fish has been used as a biomarker for exposure of fish to estrogenic compounds. Jobling et al. (1998) found that exposure of roach (*Rutilis rutilis)* to sewage-treatment-plant effluents resulted in a higher than normal incidence of intersex fish (i.e., fish displaying both male and female gonadal characteristics). The number of intersex fish was positively correlated with vitellogenin concentrations in these fish as well as the concentration of sewage effluents in the river water.

In another study, vitellogenin concentrations were shown to be elevated in fish caged downstream from a sewage treatment plant, and the hypothesis was that the cause was an APE release into the environment (Harries et al. 1996). Further study on sewage-treatment-plant effluents indicated that 17β-estradiol and estrone were present in these effluents at concentrations of up to tens of nanograms per liter. Vitellogenin synthesis was observed in male rainbow trout and roach exposed for 3 weeks to estradiol concentrations of 1-10 ng/L and 100 ng/L, respectively. Estrone was approximately 2-5 times less effective than estradiol at eliciting vitellogenin response in the rainbow trout. Exposure of both fish to 4-*tert*-octylphenol also significantly increased vitellogenin production at concentrations of less than 10 μg/L for rainbow trout and 100 μg/L for roach (Routledge et al. 1998).

SUMMARY AND CONCLUSIONS

Exposure to some natural or synthetic HAAs is ubiquitous for humans and wildlife. Although most exposure to synthetic HAAs is involuntary, voluntary exposure can occur as a result of using pharmaceuticals and commercial products containing HAAs and manufacturing and using pesticides containing HAAs.

Many plants, particularly cultivated crops such as soy, contain significant quantities of phytoestrogens. Exposure is dependant on the diet of animals and humans—it might be low for obligatory carnivores and high for herbivores or vegetarians. The amount of phytoestrogens present in plants can also vary from almost nonexistent in most fruits to more the 2,000 μg/g of wet weight in soybeans.

Among the synthetic HAAs to which humans and wildlife might be exposed, some, such as DDT and PCBs, have been banned from commercial production in the United States. However, other HAAs, such as alkylphenols and bisphenol A, continue to be manufactured and used in common commercial products. For many of these chemicals, their persistence in the environment, often for years in covered soils and sediments, means that exposure will continue into the future. PCBs have a half-life in soil of up to 17 yr. Although environmental concentrations of most HAAs have decreased as their use is curtailed, the recycling of contaminated sediments and soils can result in occasional "hot spots" of exposure.

Fish are frequently used as indicators of environmental contamination. Concentrations of PCBs in fish in some parts of the United States exceed 1,000 μg/kg. Furthermore, HAAs are known to bioaccumulate up the aquatic food chain. As a result, concentrations of some HAAs, such as PCBs and DDT, are greatest in organisms at the top of the food chain, particularly in marine mammals, such as whales and seals, and fish-eating birds.

Human dietary intake of synthetic HAAs remains substantial, even intake of HAAs that have not been used commercially for many years. For example, a recent survey of the U.S. diet found detectable residues of DDT in 16% of the food samples. Human exposure is further demonstrated by concentrations of DDT in the adipose (fatty) tissue. Over 95% of adipose tissue samples taken from the U.S. population contained detectable concentrations of some HAA. Although the concentrations were found to be greatest in older individuals, even children were not immune from exposure.

Some populations are known to have extremely high exposure to HAAs. In particular, some aboriginal groups, such as the 32 Inuits of northern Canada and the United States, have diets high in synthetic HAAs as a result of consumption of contaminated marine mammals and fish.

Some infants and young children might also be exposed to high concentrations of HAAs, although in this case, natural phytoestrogens. The use of soy-based formula for infants has been shown to result in high concentrations of phytoestrogens in the blood of these children. Children, because they tend to eat

proportionately greater quantities of fruits than adults do, also might have greater exposure to pesticide residues, including banned pesticides.

The presence of natural estrogens in many plants makes it difficult to establish a baseline for exposure to HAAs, whether natural or synthetic. Given the variety of the diet of most Americans, establishing a background concentration for HAA ingestion is difficult, and little research, particularly research on phytoestrogen exposure, has been done in this area.

RECOMMENDATIONS

Long-term monitoring of known HAA-contaminated media (e.g., sediments) should be conducted to assess the persistence and recycling of HAAs in and between media.

Monitoring of environmental media should be expanded to include fish and other aquatic organisms taken from contaminated surface waters.

Intake of phytoestrogens and synthetic HAAs by humans and other biota should be studied to establish a baseline for typical HAA exposure. Predominant routes of exposure, particularly diet and drinking water, should be assessed to determine background intake concentrations for all HAAs.

Monitoring efforts should include subpopulations that are known or suspected of having high exposures, such as aboriginal populations consuming marine mammals, vegetarians, and infants consuming soy-based formulas.

Exposure assessments should include potential exposure to all possible HAAs, not just individual chemicals.

4

Dosimetry

The risk associated with exposure to hormonally active agents (HAAs) will depend on many factors, such as developmental stage, sex, disease conditions, life-cycle stage, as well as on the potency of the compounds and on the amount of exposure or the dose. A dose can be construed in one or more ways: as the concentration of a toxicant to which organisms are exposed (the external dose); as the concentration in organs, tissues, or cells (the internal dose); or as the amount reaching and binding with a receptor or other target molecule (the biologically significant dose). The extent to which binding occurs will depend on properties of the chemical, properties of the target molecules, the duration of exposure and on the distribution of the chemical in the body. The physiology of the organism and the route to cellular or molecular targets can favor or impede binding or its consequences.

Knowing the shape of the dose-response curve for environmental contaminants is critical for understanding how such contaminants—including HAAs—act on organs and organisms. Understanding the dose-response relationship is also critical for the design of studies to test the effects of contaminants.

If an underlying monotonic dose-response function (i.e., a function where response increases as dose increases or at least does not decrease) and a dose below which there is no effect (a threshold dose) are assumed when designing a toxicologic study, there is a risk of failing to understand or properly test a contaminant that does not display a monotonic dose-response function or a threshold dose.

It is well known that some compounds produce nonlinear and even nonmonotonic dose-response functions in some organisms over certain ranges of dose. Furthermore, some compounds can produce different dose-response functions depending on the target organ and the species exposed. For those reasons, this chapter considers dose-response relationships and the processes that can

determine whether HAAs in the environment accumulate in target organs and cells to concentrations that are biologically significant.

The toxicokinetic processes that determine the accumulation and potency of HAAs are much the same as they are for other xenobiotics, and they are described generally in textbooks (e.g., Klaassen et al. 1996). The accumulation of HAAs is determined by the rate and efficiency of several processes: absorption, or partitioning, of a bioavailable chemical between the external medium and the cell layers first exposed; transport in blood; partitioning between blood and body lipids or target cells; metabolism of the chemical; and excretion and elimination from the body. In this chapter, these processes are summarized, and examples illustrate the complexities as well as the extent of our knowledge in these areas. The processes considered include the effects of HAAs on enzymes that metabolize HAAs or endogenous hormones. In addition to influencing the elimination or persistence of HAAs, metabolism can affect their structure and biologic activity, thereby participating directly in a mechanism of action. Detailed knowledge of these processes is necessary if we are to relate external to internal doses and then to specific outcomes and mechanisms of action. Knowledge of the differences and similarities in uptake, distribution, metabolism, and elimination will determine the degree to which we can extrapolate across species for different chemicals and doses, and it will guide monitoring of humans and wildlife for relevant exposure to HAAs. Such knowledge is essential for assessing risk in species or populations exposed to HAAs in the environment, particularly where exposure concentrations are smaller than those in localized "hot spots," where exposure concentrations are high and effects are clearly seen and reported.

UPTAKE, ELIMINATION, AND ACCUMULATION

Uptake and elimination of xenobiotics by animals have been studied extensively, both under controlled conditions in the laboratory and under natural conditions in the environment. Absorption through epithelial-cell layers is the requisite first event in the uptake of xenobiotics. Electronic, steric, and hydrophobic or hydrophilic properties of the chemical will govern this process. For organic chemicals, including most HAAs, hydrophobicity is particularly important. For the more-hydrophobic (lipophilic) chemicals, uptake appears to be a passive process, involving diffusion of the chemical through the plasma membrane to the interior of the cell (DiFrancesco and Bickel 1985). Among vertebrates, there is probably little difference in the efficiency with which chemicals cross the membrane into epithelial cells (Lin 1995). Rather, the lipid content of the tissues is important. The role of lipids in accumulation of xenobiotics in many types of animals has been recognized for decades (Stegeman and Teal 1973; Cullen and McConnell 1992). Partitioning of chemicals between aqueous and nonaqueous media often is used to characterize the propensity of chemicals to accumulate in tissue. Coefficients, such as the octanol-water partition coefficient (K_{ow}), corre-

late with and predict the degree of accumulation of HAAs and other xenobiotics in organisms (Connell and Hawker 1988; Ikemoto et al. 1992). Species that possess slight capacity to metabolize the xenobiotics, such as molluscs, show equilibrium between the content of organic chemicals, including HAAs, in water and in the organism (Porte and Albaiges 1994), supporting the conclusion that uptake is largely a passive rather than an active transport process (Connell and Hawker 1988). Less hydrophobic chemicals could require facilitated transport, although the mechanisms have not been defined.

Rates of uptake and elimination, hence half-lives, differ greatly for different compounds and in different species. Examples of half-lives of selected HAAs in different organisms are given in Table 4-1. The half-life of a chemical is influenced by the dose and the duration of exposure. The data presented in Table 4-1 are the results of experimental and environmental exposures. In the case of ambient exposure, the external dose and the duration of exposure often are unknown. Table 4-1 nevertheless indicates the large range in estimated half-lives of different HAAs. The half-lives of individual polychlorinated biphenyl (PCB) congeners in trout and in humans vary a great deal (Niimi and Oliver 1983; Ryan et al. 1993). Research conducted with fewer congeners shows that rates of elimination can vary greatly, even for closely related chemical structures. Coristine et al. (1993) reported that PCB congener 77 (3,3′,4,4′-tetrachlorobiphenyl) was eliminated rapidly and congener 126 (3,3′,4,4′,5-pentachlorobiphenyl) was eliminated very slowly from trout. Compounds with long half-lives could be available to act during critical windows of development or hormonal action. Compounds with short half-lives might not be present at those critical windows, depending on the timing and duration of exposure. In many environments, whether point or disseminated sources of contaminants are involved, exposure to HAAs that are slowly degraded is likely to be continuous.

For many compounds, the half-lives and the clearance from blood approximate the rate of metabolism. With chlorinated compounds, such as PCBs, polychlorinated dibenzodioxins (PCDDs), or polychlorinated dibenzodifurans (PCDFs), metabolism and elimination rates depend on the degree and the sites of chlorination (Matthews and Anderson 1975). The rates at which these compounds are metabolized decreases as the degree of chlorination increases (Chen et al. 1982). Compounds that are structural homologues can have markedly different rates of metabolism. For example, the metabolism rates of PCB congeners are strongly influenced by the presence of vicinal, unsubstituted carbons at the *meta* or *para* position (Chen et al. 1982; Matthews and Dedrick 1984). Analysis of residues in blood could suggest species patterns of metabolism (Boon et al. 1994).

ROUTES OF EXPOSURE

The form of the chemical will determine bioavailability (Farrington 1991), and the lifestyle of the free-living organism (i.e., after hatching or parturition)

TABLE 4-1 Half-Lives of Selected HAAs

Chemical	Species	Organ or Excreta	Half-Life	Reference
TCDD[a]	Human	Blood	7.2; 8.5 yr	Flesch-Janys et al. 1996; Michalek et al. 1996
	Rat	Liver	35 d	Nagaoka et al. 1990
	Carp	Body	300-325 d	Kuehl et al. 1986
Total PCBs[b,c]	Rhesus monkey	Blood	0.3-7.6 yr	Mes et al. 1995
	Adelie penguin	Multiple	270 d	Subramanian et al. 1987
	Rainbow trout	Body	219 d	Niimi et al. 1990
p,p′-DDE[d]	Rat	Multiple	80-120 d	Muhlebach et al. 1991
	Adelie penguin	Multiple	580 d	Subramanian et al. 1987
	Herring gull	Multiple	264 d	Norstrom et al. 1978
Genistein	Human	Excreta	1 d[e]	Xu et al. 1995
	Rat	Excreta	1 d[f]	King et al. 1996
Sitosterol	Human	Blood	4-15 d	Bhattacharyya et al. 1991
Zearalenone	Pig	Plasma	87 hr	Biehl et al. 1993
Nonylphenol	Rainbow trout	Multiple	99 hr[g]	Lewis and Lech 1996; Coldham et al. 1998
	Fathead minnow	Whole fish	1.2-1.4 d	Ward and Boeri 1991
Estradiol	Human	Blood	4 hr	Ginsberg et al. 1995
B[*a*]P[h]	Sea bass	Liver	8.2 d	Lemaire et al. 1992

[a] TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.
[b] PCBs, polychlorinated biphenyls.
[c] As Aroclor or the sum of up to 30 congeners.
[d] DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene.
[e] 10-37% recovered in urine after 48 hr.
[f] 42% recovered in urine and feces at 48 hr.
[g] Biphasic elimination.
[h] B[*a*]P, benzo[*a*]pyrene is a polynuclear aromatic hydrocarbon (PAH) included for comparison. PAHs generally are rapidly metabolized and eliminated.

will determine which avenue of ingress—dermal, respiratory, or gastrointestinal—is most important. Hydrophobic compounds, such as dichlorodiphenyltrichloroethane (DDT), and some PCB congeners can pass through the skin of mammals, including humans and can be taken up from the water by fish (Bruggeman et al. 1981; Reifenrath et al. 1991). Xenoestrogens, such as nonylphenol, also can be taken up directly from water by fish (Lewis and Lech 1996). However, dietary exposure and gastrointestinal absorption are the most important routes of exposure for vertebrates (James and Kleinow 1994). Exposure of omnivores or herbivores to natural substances with estrogenic activity produced by plants (phytoestrogens; e.g., genistein) or fungi (e.g., zearalenone) is through the diet (Kuiper-Goodman et al. 1987). Ingestion of milk is the primary route of exposure for neonatal and infant humans and other mammals, because milk is the major if not the sole source of contaminants. Bovine milk also is a source of neonatal human exposure to phytochemicals that are HAAs (Bannwart et al. 1988).

Various factors affect the availability of HAAs in the diet. Thus, the uptake of chemicals in the gut can be influenced by the composition of the diet, the pH in the gut, the rate of digestion, the residence time of food, and the microflora composition in the gut (James and Kleinow 1994). In the case of some phytoestrogens, gut microflora can influence the bioavailability of the chemical for uptake (Xu et al. 1995). Gut flora also have been implicated in formation of methylsulfonyl derivatives of some PCB congeners (Brandt et al. 1982). These variables can differ substantially among species, and they need to be incorporated into pharmacokinetic models. There are, however, few data concerning how these variables apply to specific HAAs.

In mammals, maternal circulation is the source of chemicals to the developing organism. Some nonmammalian species, including live-bearing fish, have a modified placentation, and maternal exposure is also a source of contaminants that can be transported into developing embryos or fetuses (Lombardi and Wourms 1985; Hamlett et al. 1993). However, oviparous species are exposed primarily through maternal deposition of chemicals into the yolk. In either case, the transport of chemicals in the blood, discussed below, is involved in delivery of the compounds to the embryo or fetus and to the ovary and eggs. The major routes of exposure for embryos, larvae, and fetuses differ in different taxa. It also must be emphasized that models based on adult dosing might be inadequate for estimating the distribution to or the effects on target sites in embryos.

The capacity of cells at sites of entry to metabolize HAAs (first-pass metabolism) can determine the form and the amount of the chemical delivered to other parts of the body. If a compound is extensively metabolized by epithelial cells in the gut, for example, then exposure to low doses might not yield an active dose at target sites in the body. This fact could strongly influence interpretations of dose-response relationships, particularly at low doses. Such an effect of metabolism at the site of absorption was clearly demonstrated in studies by Van Veld et al.

(1988) with fish fed benzo[*a*]pyrene (B[*a*]P). At low (environmentally realistic) doses of B[*a*]P in the diet, there was a strong induction of cytochrome P450 1A (CYP1A), which metabolizes B[*a*]P, in the gut. In those same animals, only the metabolites of B[*a*]P were passed to the liver (Van Veld et al. 1988), and there was no induction of CYP1A in the liver. At higher doses in the diet, the capacity for metabolism in the gut apparently was exceeded, and only then did induction of CYP1A begin to appear in the liver. Although B[*a*]P is not generally considered an HAA, the results of the study above demonstrate that first-pass metabolism can affect the systemic distribution of dietary chemicals. Metabolism in the gut wall can be important in the metabolism of steroids, such as ethinylestradiol and fungus-derived estrogens (Olsen et al. 1987; Back et al. 1990). Regulation of drug-metabolizing enzymes in mammalian small intestines show multiple forms of CYP (Kaminsky and Fasco 1991), but the significance to pharmacokinetics "has been studied to only a limited extent" (Kaminsky and Fasco 1991). The ability of dermis, lung, and gill as well as gut to metabolize xenobiotics has long been known (Wiebel et al. 1975; Steinstrasser and Merkle 1995), but the degree to which these epithelial layers in different species differ in their capacity to metabolize or transform HAAs specifically still is not well known.

Milk is a primary source of exposure to contaminants in nursing the young—human and animal. HAAs secreted in milk retain biologic activity. In mice, methoxychlor administered to dams was transferred via the milk to the suckling young, where it stimulated the vagina and uterine horns, indicating that the chemical excreted in milk remained biologically active in the suckling mice (Appel and Eroschenko 1992). Neonatal exposure of rats to *o,p′*-DDT or to the phytoestrogens genistein or coumestrol altered later pituitary responsiveness to gonadotropin-releasing hormone (GnRH) (Faber and Hughes 1991; Whitten et al. 1995), indicating that lactational exposure of neonates can affect reproductive function later in life. Similarly, lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) affects endocrine function in rats later in life (Chaffin et al. 1996).

Concentrations of HAAs and other xenobiotics have been measured in milk from humans around the globe (Skaare 1981; Wickizer et al. 1981; Slorach and Vaz 1985; Skaare and Polder 1990; Dogheim et al. 1991; Dewailly et al. 1993a, 1994; Spicer and Kereu 1993; Larsen et al. 1994; Quinsey et al. 1995; Chikuni et al. 1997). Some of the data are presented in Table 4-2.

Mothers' milk is not the only source of exposure to HAAs in human infants. Sewart and Jones (1996) calculated that PCBs in cows' milk could contribute 11% of the total daily intake of PCBs in the United Kingdom. Bovine milk is a source of HAA exposure in infants, children, and adults. Most of the data on residues in milk are for chlorinated hydrocarbon HAAs. Cows' milk can deliver phytoestrogens to infants. However, soy-based milk substitutes can deliver much greater amounts of phytoestrogens than are found in either bovine or human milk. Plasma concentrations of genistein measured in infants fed soy-based formulas averaged 684 ng/mL, a much greater amount than those found in infants fed

TABLE 4-2 Organochlorines in Human Milk

Location	Year	Compound	Concentration in Lipids	Comment	Reference
Stockholm	1972	$MeSO_2$-CB[b]	9 ng/g	Mainly 4-$MeSO_2$-87 and 4-149	Noren et al. 1996
	1992	$MeSO_2$-CB	2 ng/g		
	1972	$MeSO_2$-*p,p′*-DDE[c]	5 ng/g		
	1992	$MeSO_2$-*p,p′*-DDE	0.4 ng/g		
Faroe Islands	1987	Total PCBs[d]	1.8-3.5 μg/g	Mostly CB congeners 153, 180, 138	Grandjean et al. 1995
Czech Republic	Not reported	Total PCBs	936.7 ng/g	Mostly CB congener 153	Schoula et al. 1996
Norway (Oslo)	1991	Total PCBs (23 congeners)	372 ng/g	Mostly CB congener 153; 126 main TEQs[e]; 81% *p,p′*-DDE; (62-79% decline over 9 yr in total PCBs and DDT[f])	Johansen et al. 1994
		Total DDT	338 ng/g		
		HCB[g]	41 ng/g		
		Oxychlordane	9 ng/g		
		Total HCH[h]	36 ng/g		
Germany	1984-1992	TCDD[i]	3.2 pg/g		Furst et al. 1994
Kenya	1986	Total PCBs	Not detected		Kanja et al. 1992
		Total DDT	4.8 μg/g		
Netherlands Rotterdam	1990-1992				Koopman-Esseboom et al. 1994
		TCDD-TEQ	30.8 pg TEQ/g		
		Planar PCB TEQ	16.4 pg TEQ/g		
		Mono-ortho PCB TEQ	13.7 pg TEQ/g		
		Di-ortho PCB TEQ	4.0 pg TEQ/g		

	Vlaardingen/ Schiedam	TCDD-TEQ Planar PCB TEQ Mono-ortho PCB TEQ Di-ortho PCB TEQ	30.3 pg TEQ/g 15.2 pg TEQ/g 14.1 pg TEQ/g 4.0 Pg TEQ/g		
	Spijkenisse	TCDD-TEQ Planar PCB TEQ Mono-ortho PCB TEQ Di-ortho PCB TEQ	34.3 pg TEQ/g 18.0 pg TEQ/g 15.0 pg TEQ/g 4.3 pg TEQ/g		
	Groningen	TCDD-TEQ Planar PCB TEQ Mono-ortho PCB TEQ Di-ortho PCB TEQ	28.9 pg TEQ/g 15.8 pg TEQ/g 15.5 pg TEQ/g 4.6 pg TEQ/g		
England (bovine)	—	Total PCBs	8.4 ng/g	CB congeners 153, 138, 118, and 180 = 61%	Sewart and Jones 1996

[a] $MeSO_2$, methylsulfonyl.
[b] CB, chlorinated biphenyl.
[c] DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene.
[d] PCBs, polychlorinated biphenyls.
[e] TEQs, toxic equivalents.
[f] DDT, dichlorodiphenyltrichloroethane.
[g] HCB, hexachlorobenzene.
[h] HCH, hexachlorocyclohexane.
[i] TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

cows' milk formulas (3.2 ng/mL) or human breast milk (2.8 ng/mL) (Setchell et al. 1997). The dose of genistein in infants fed soy milk was greater than doses eliciting hormonal effects in adults (Setchell et al. 1997).

The data from most studies on the subject show that 80% or more of DDT measured in human milk is in the form of the *p,p′*-DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) metabolite (Franchi and Focardi 1991; Johansen et al. 1994). Similarly, in most studies, total PCBs in milk represent input from a few congeners, primarily 153, 138, and 180 (Grandjean et al. 1995; Ramos et al. 1997a). In a study in Norway (Johansen et al. 1994), the concentrations of 23 PCB congeners and of DDT and other pesticides were examined in human milk. The researchers determined that congener 153 correlated very closely with the total of the 23 congeners, suggesting that this congener could be an indicator for total PCBs. In cows' milk also, the concentrations of total PCBs represent major input from congeners 153, 138, 118, and 180 (Sewart and Jones 1996). Although the major PCB congeners in milk are ortho-substituted, unsubstituted congeners also occur. Of these, coplanar PCB 77 was found at high concentrations in a sample from the Faroe Islands of Denmark (Grandjean et al. 1995). Johansen et al. (1994) determined that congener 126 was the major contributor to the TCDD toxic equivalents in human milk. Similarly, Ramos et al. (1997b) found that of the coplanar congeners measured, 77 and 126 were most abundant in cows' milk.

Variables that can influence the persistence of HAA residues in milk include maternal age and weight, the number of births, the duration of lactation, and the fat content of milk (Rogan et al. 1986a; Skaare and Polder 1990; Sim and McNeil 1992). In a study in Germany, Beck et al. (1994) found that TCDD concentrations in the mother declined with the number of children born and the duration of breast feeding, reflecting decreases in the content of contaminants in the maternal body. Such decreases also occur in wildlife. In some species of dolphin, it has been estimated that 60-80% of the body burden of organic xenobiotics is eliminated in maternal milk (Cockcroft et al. 1989), with serious implications for the accumulation of these compounds in calves. The shorter lactation period in fin whales is thought to result in less total transfer to the calves than in other cetaceans that have longer lactation periods (Aguilar and Borrell 1994). The milk of different species can differ greatly in lipid content. In seals and whales, milk fat content varies between 10% and 55%, depending on the species and the duration of lactation (King 1983; Trillmich and Lechner 1986; Ridgeway et al. 1995). Those percentages are much higher than those in human milk, in which the fat content typically ranges from 3.5% to 6.6% (Daly et al. 1993). Results showing close correlation between PCB content and milk fat (Schweigert and Stobo 1994) indicate that differences in fat content can be expected to influence the amounts of HAAs in milk.

Analyses of milk have shown important temporal and geographic trends in HAA content and thus in what can be delivered to nursing infants. One Swedish study demonstrated a significant decline in the content of methylsulfonyl me-

tabolites of chlorobiphenyls and *p,p′*-DDE between 1972 and 1992 (Noren et al. 1996). A study by Johansen et al. (1994) noted a 62-79% decline in total PCBs and total DDTs between 1982 and 1991. In general, from the mid-1960s to the 1990s, there has been a consistent and rather sharp decline in concentrations of *p,p′*-DDE in human breast milk in Scandinavia (Ekbom et al. 1996). In a study of worldwide trends in DDT and metabolite residue concentrations in human breast milk between the 1950s and 1990s, Smith (1999) reported that population means have declined in much of the world. Declines from 5,000 to 10,000 μg DDE/kg milk fat to approximately 1,000 μg DDE/kg milk fat have been observed in many countries. Although the means differ among the various regions, the declines in the various countries correspond to their restrictions on DDE use. In regions where DDT use continues, the amounts in milk are high (Bouwman et al. 1992)—often exceeding PCB content (Chikuni et al. 1997). Factors that affect the concentration of HAAs in human and animal milk and the amounts delivered to children who are nursing or who continue to drink cows' milk demand continued close attention (Ramos et al. 1997b).

Because human milk is a biologic indicator of the body burden (the amount measured per unit body weight) of organochlorines (Furst et al. 1994), analysis of milk could indicate HAA residue concentrations in adult females. It is important to establish how HAA concentrations in milk relate to the concentrations in other parts of the body. Kanja et al. (1992) examined 41 samples of maternal blood, milk, subcutaneous fat, and umbilical-cord blood collected from mothers giving birth by Cesarean section at Kenyatta National Hospital in Nairobi, Kenya, in 1986. The mean concentrations of total DDTs (milligrams of DDT per kilogram of fat) were 5.9 in maternal subcutaneous fat, 4.8 in milk, 2.7 in maternal blood and 1.9 in umbilical-cord blood. There was a significant correlation between the concentration of total DDTs in subcutaneous fat and milk fat ($r = 0.963$), in subcutaneous fat and maternal serum fat ($r = 0.843$), and in maternal serum fat and maternal milk fat ($r = 0.868$). Thus, analysis of milk can reveal not only the exposure of infants but also the exposure possible in utero. However, there was no correlation between the concentration of total DDTs in adipose tissue and cord blood, in maternal blood and cord blood, and in maternal milk and cord blood.

FOOD-CHAIN TRANSFER AND BIOACCUMULATION

The literature concerning food-chain transfer and accumulation of chemicals in some trophic levels is abundant. A few studies serve here as examples. Clarkson et al. (1995) reviewed the properties generally required for a contaminant to bioaccumulate in a food chain. These are a high K_{ow}, a chemical and metabolic stability in water and in organisms in the food chain, and a toxicity that is low enough that the chain is not broken by loss of an intermediate species. PCBs, dioxins, and organochlorine pesticides, including DDTs, are among the

organic chemicals that bioaccumulate in aquatic food chains. Top predators in short or long food chains (polar bears, cetaceans, and eagles and other raptors) accumulate higher concentrations of such compounds than do lower-trophic species (Muir et al. 1992).

Consumption of species from the top of food chains results in higher exposure to HAAs in humans. Faroe islanders who consume whale blubber and meat and Inuit who consume marine mammal tissues have unusually high concentrations of PCBs and DDTs in their bodies and breast milk (Dewailly et al. 1993a; Grandjean et al. 1995; Weihe et al. 1996). Such populations are being followed to determine whether elevated concentrations of HAAs in blood and milk are associated with any health effects.

DISTRIBUTION

Blood

Blood is the central medium of distribution of chemicals to target organs and cells in the body. It is often possible to obtain blood in a minimally invasive way, making this a key tissue for monitoring. The significance of blood in monitoring chemical exposures is described in detail in a previous NRC (1991b) report. Knowledge of how the concentrations of HAAs in blood relate to the concentrations and form of the chemicals in target organs is critical for the process of inferring HAA effects. Ideally, one would like to be able to measure HAA concentrations in blood or other biopsy samples and infer the concentration in target organs. However, concentrations of HAAs generally are much greater in tissues than in blood. In one study in rats, the ratio of DDE in tissue to DDE in blood was 6:1 for liver and muscle, 35:1 for skin, and 400:1 for adipose tissue (Muhlebach et al. 1991). Similar distributions for hydrophobic compounds have been observed in several studies in rodents (Table 4-3); at equilibrium, the more-hydrophilic compounds have distribution ratios near unity (NRC 1991b). In a study of free-living herring gulls, the plasma-to-whole-body lipid partition coefficient for DDE was 0.0041 ± 0.0014 (Norstrom et al. 1986). Even before steady-state whole-body burden is reached, there generally is good correlation between blood and tissue concentrations and rates of elimination. Dragnev et al. (1994) described similar time- and dose-dependent changes in the residues of the same PCB congeners in liver, adipose tissue, and blood of rats. In all three organs at all doses, there was a rapid decrease in content of residues after exposure ended. Metabolites of HAAs also are found in blood. Bergman et al. (1994) reported that the PCB metabolites 4-hydroxy-2,3,5,3′,4′-pentachlorobiphenyl and 4-hydroxy-2,3,5,6,2′,4′,5′-heptachlorobiphenyl occurred in the blood of seals and humans at concentrations that were almost the same as those of the most persistent PCB congeners. All identified compounds have a structure with the hydroxy group in a *para* or *meta* position, and they have chlorine atoms on vicinal carbon

TABLE 4-3 Tissue-to-Blood Ratios for Various Halogenated Aromatic Compounds

Chemical	Adipose Tissue	Liver	Kidney	Muscle	Reference
DDT	184-792	2.4-4.4	2.7-3.9	—	Woolley and Talens 1971
DDE	52-412	1.1-5.6	1.1-2.3	—	Woolley and Talens 1971
TCDD	89-135	121-280	—	—	Birnbaum 1986
4-Chlorobiphenyl	30	1	—	1	Lutz et al. 1977
4,4´-Dichlorobiphenyl	70	3	—	2	Lutz et al. 1977
2,4,5,2′,4′,5′-Hexachlorobiphenyl	400	12	—	4	Lutz et al. 1977

SOURCE: NRC 1991b.

atoms. The concentrations of hydroxylated PCBs in the blood were similar for seals and humans.

In some organs, the distribution of specific lipophilic compounds can be influenced by specific proteins. In mammals, the distribution of TCDD, and possibly related planar halogenated compounds, is not fully explained by lipid partitioning. This is the result of induction of a form of CYP (CYP1A2) that can bind TCDD (Voorman and Aust 1987). CYP1A2 sequesters TCDD, and CYP1A2 induction in the liver results in hepatic accumulation that is greater than would be expected on the basis of partitioning. This effect has been confirmed in models that lack CYP1A2 (Diliberto et al. 1997). In mice, a Clara-cell secretory protein binds 4-methylsulfonyl-2,2′,4′,5,5′-pentachlorobiphenyl, causing accumulation in lung and kidney (Stripp et al. 1996).

The validity of inferring tissue concentration from blood concentration is supported by physiologically based pharmacokinetic (PB-PK) modeling, which is modeling the distribution of a chemical within an organism based on knowledge of exposure concentration and route, duration of exposure, partitioning between organs, as well as organ volume, blood-flow rate, binding to receptors, and rate of chemical metabolism and disposition (McKim and Nichols 1994). Parham et al. (1997) reported that the observed partitioning between blood and adipose tissue was similar to the partitioning in PB-PK models and that there was a two- to three-fold difference in the partitioning between different PCB congeners, which was consistent with the model. PB-PK models are being applied to

partitioning of PCB congeners to different organs (Parham et al. 1997; Matthews and Dedrick 1984). That such models support extrapolation among species is indicated by comparisons of the distribution of PCDDs and PCDFs among different organs in different species, including humans (Carrier et al. 1995), but such information is lacking for many HAAs.

Persistence and organ distribution could influence extrapolations of dosage from one species to another—for example, for TCDD—but these have not been adequately determined in enough species (Whysner and Williams 1996). Although knowledge of distribution to different tissues is important to interpretations regarding toxicity, comparing the effects of some compounds on the basis of the total body burden could reveal similarities not evident when concentrations in individual tissues are used to compare responses between species. Thus, DeVito et al. (1995), in comparing the relative sensitivities of human and animal tissues to TCDD, concluded that "it may be more appropriate to compare body burden than tissue concentration." The physiologic rationale for body-burden being predictive across species is not clear.

Plasma-Binding Proteins

Lipophilic chemicals are not free in the serum but associate with proteins, lipoproteins, or circulating cells in the blood, as do endogenous hormones. The degree to which a chemical is free or bound appears to determine the amount available for uptake by a cell. The free-hormone hypothesis posits that the biologically active fraction of total circulating steroid is the fraction that can pass from capillaries into cells and, therefore, is available to bind to intracellular steroid receptors. Only that which reaches a target can exert its activity. Serum proteins that bind hormones include those that are specific (steroid-hormone-binding globulins, SHBGs) and those that are nonspecific (such as albumins). There is evidence that xenoestrogens—including phytoestrogens, other HAAs, and endogenous estrogens such as estradiol—could bind differently to components of serum, resulting in differences in passage into tissues (Raynaud et al. 1971; Skalsky and Guthrie 1978; Sheehan and Young 1979, Akpoviroro and Fotherby 1980; Sheehan and Branham 1987; Harmon et al. 1989; Borlakoglu et al. 1990; vom Saal 1995; Nagel et al. 1998). Thus, the variables that can influence the uptake of steroids into cells include the plasma concentration of albumin and specific glycoproteins, the plasma flow rate, and the rate constant for dissociation of the steroid-protein complex (Ekins et al. 1982; Mendel 1989; Mendel et al. 1989, 1990).

Properties of specific sex-steroid-binding proteins have been described for few nonmammalian species, and not much is known about their ability to bind HAAs. Sex-steroid-binding proteins have been characterized in fish (Thomas and Laidley 1994). There also could be minor components, such as the GnRH-binding protein, that are important in endocrine responses in some species (Huang

et al. 1991). Most vertebrate species possess serum albumins, and although there are species differences in the specific plasma glycoproteins that bind estrogen and other sex steroids with high affinity, albumin likely serves as a low-affinity, high-capacity steroid carrier in vertebrates (Siiteri 1982).

Globulin-bound steroids, such as estradiol bound to α-fetoprotein (AFP), can enter selected cells via receptor-mediated endocytosis (Toran-Allerand 1984). Dohler (1986) proposed that deglycosylation of proteins can facilitate their entry into cells. Vallette et al. (1977) demonstrated that deglycosylated AFP has a high affinity for estrogen and will preferentially enter target cells capable of internalizing AFP. However, in vitro studies showed that the general effect of AFP and SHBG is to decrease the apparent potency of estrogens and testosterone (Damassa et al. 1991), suggesting that trapping of the steroid by the binding protein is their main effect.

Once inside a target cell, a steroid presumably dissociates from the plasma protein and becomes available for binding to specific intracellular receptors because of the lower binding affinity of steroids for plasma proteins relative to intracellular receptors. Siiteri et al. (1982) suggested that binding proteins could help create an intracellular pool of bound steroid, which is protected from metabolism but in equilibrium with the pool of steroid bound to intracellular receptors. Another hypothesis is that binding proteins compete with intracellular receptors in target cells and modulate the cellular response to steroids (Sakly and Koch 1983). For example, neonatal rat testes contain AFP, so it is posited that intracellular AFP binds intracellular estradiol, which is thus rendered biologically unavailable to the receptor (Huhtaniemi 1985).

In addition to either sequestering or delivering HAAs, the binding of HAAs (including phytoestrogens) to human steroid-binding proteins can displace endogenous hormones (Martin et al. 1996), possibly affecting hormone delivery to target cells. Chemicals also associate with multiple lipoprotein fractions and might redistribute among them (Mohammed et al. 1990).

Developmental Stage and Serum Protein

Proteins that transfer chemicals or hormones to or within the fetus include AFP, uteroglobin, albumins, and transferrins. Most published information concerning concentrations of circulating steroids in pregnant females, fetuses, and animals after birth involves measurement only of the total amount of steroid present in the blood. In these studies, steroids are extracted by techniques that do not allow one to discriminate between the fraction bound to plasma proteins and the free fraction. Studies in rats (Montano et al. 1995, Nagel et al. 1998) showed the potential for misinterpreting the bioactive concentration of steroid present during fetal life that is based on information concerning total steroid concentration in blood. In fetal rats, total serum estradiol measured by radioimmunoassay was high (150 pg/mL) relative to that measured in diestrous females (15 pg/mL).

However, the free fraction was much lower during development (0.3%) than adulthood (4%). Thus, the bioactive fraction of estradiol in fetuses is actually similar to that found in adult diestrous females (Montano et al. 1995; Nagel et al. 1998). Similarly, there is a 100-fold difference between human adults and fetuses in the percentage of serum estradiol that is free (Nagel et al. 1998). In humans, total serum estradiol concentration is approximately 100-fold greater in fetuses than it is in adults; the free serum estradiol concentration in human adults and fetuses is similar (Nagel et al. 1996, vom Saal et al. 1997).

The significance of protein transfer to the fetus is suggested by AFP, which might transport estradiol into selected estrogen-responsive regions of the developing brain in rats and mice (Attardi and Ohno 1976; Schachter and Toran-Allerand 1982). Recent work has shown that uteroglobin binds methylsulfone metabolites of DDT and of some PCB congeners (Hard et al. 1995). For example, 3-methlysulfonyl-DDE is transferred across the placenta and accumulates in the adrenal cortex of fetal mice (Jonsson et al. 1995). There is a high-affinity binding of a PCB methylsulfone to uteroglobin (Hard et al. 1995), which can transport these compounds also to sensitive target tissues in the fetus. More must be learned about the role of fetal and maternal serum proteins as targets or vectors of HAAs.

In nonmammalian species, the plasma proteins that can bind steroids or HAAs include SHBG or steroid-binding plasma protein (SBP), albumins, vitellogenin, and other lipoproteins. These binding proteins facilitate uptake of HAAs by eggs during oogenesis in fish and probably in other vertebrates with yolky eggs. PCBs and *o,p′*-DDT bind to fish vitellogenin (Ungerer and Thomas 1996a). In a follow-up study of serum lipoproteins involved in transport of xenobiotics in fish, Ungerer and Thomas (1996b) found that the majority of *o,p′*-DDT in croaker was associated with a triglyceride-rich very-low-density lipoprotein (VLDL). Uptake of the triglyceride-VLDL fraction was identified as the major route of DDT into oocytes, accounting for a majority of the accumulation in the ovary.

Target Organs

The principal target organs for HAAs are those that produce, regulate, or respond to hormones or their action: brain, gonads, liver, uterus, mammary, adrenals, prostate, placenta, and the organs in the developing embryo or fetus. Some studies have established that HAAs are distributed to all of these organs, although information on the concentrations and pharmacokinetics of HAAs in many of these organs in humans or wildlife species is limited. Studies of two key organs illustrate the complex pharmacokinetics.

Brain

In the brain, the pineal and pituitary glands and the hypothalamus are important in the control of reproductive endocrine processes. The blood-brain barrier

prevents many of the more-hydrophilic drugs from passing from the circulatory system into the brain itself. Yet, the uptake or presence of lipophilic HAAs in the brain has been measured in humans and animals (Mes et al. 1995; Corrigan et al. 1996; Jenssen et al. 1996). Moreover, parts of the brain that control reproductive function—the median eminence and other circumventricular organs—are outside the blood-brain barrier (Kandel et al. 1991), affording transfer to these structures. The action of HAAs in the brain is inferred from studies showing, for example, the effects of genistein on pituitary responsiveness to gonadotropin-releasing hormone and on brain structure in postpubertal castrated female rats (Faber and Hughes 1993).

Analysis of residues in environmentally exposed animals provides information about pharmacokinetics. In some avian species, the concentrations of lipophilic xenobiotics, which could be HAAs, are as great in the brain as they are in the liver (Burns and Teal 1979). That result appears not to be the case in some mammals. Measurable concentrations of several pesticides, including DDT (*p,p´*-DDE, *o,p′*-DDD, *p,p,′*-DDD, *o,p′*-DDT, *p,p′*-DDT) and 22 PCB congeners, were found in samples of brain, fat, and liver of gray seal pups (Jenssen et al. 1996). The concentrations of PCBs and DDT in liver were about 75% of those in blubber. Only two PCB congeners were detected in brain tissue, where total PCB content was only about 1% of that measured in the blubber. The basis of tissue-specific accumulation of PCB congeners, with the patterns of PCB congeners in liver and brain differing from those in blood and blubber, is not known.

Studies that directly measure HAA transfer to the brain have shown species and chemical differences in accumulation. Ingebrigtsen et al. (1990) reported different patterns of accumulation of 2,3,3′,4,4′-pentachlorobiphenyl in cod and rainbow trout. In a study in rats, Ness et al. (1994) reported a difference in the accumulation of different PCB congeners. There was little difference in the regional distributions within the brain; however, 3,3′,4,4′-tetrachlorobiphenyl was concentrated in the vicinity of blood vessels, whereas 2,2′,4,4′-tetrachlorobiphenyl was not. That suggests a selective interaction of 3,3′,4,4′-tetrachlorobiphenyl with vascular structures. The relationship between chemical structure and deposition in different regions of the brain should be studied further.

Placenta

In humans and other mammals, the placenta could constitute a barrier to the transfer of HAAs or other xenobiotics from the mother to the developing fetus. However, the rapid transfer of chemicals, including TCDD, across the placenta and the subsequent distribution of toxicants in the fetus have been conclusively demonstrated in the laboratory (Abbott et al. 1996) and are associated with effects in offspring (Gray and Ostby 1997). Transplacental toxicity of 3-methylsulfonyl-DDE in the developing adrenal cortex in mice has been reported (Jonsson

et al. 1995). Prenatal exposure to genistein also affects sexual differentiation (Levy et al. 1995).

In mice, Darnerud et al. (1996) found that 3-4% of 3,3′,4,4′-tetrachlorobiphenyl given to the mother was transferred to the fetus. These investigators also noted that the pharmacokinetics of 3,3′,4,4′-tetrachlorobiphenyl differ from those of other planar PCB congeners. In one species of marine mammal, it was estimated that 4-10% of maternal PCBs could be transferred across the placenta to the fetus (Tanabe et al. 1994). Although transfer across the placenta clearly does occur, the relationship between maternal plasma and serum concentrations and the amounts that cross the placenta is not known. In one study of humans in Oslo (Johansen et al. 1994), concentrations of DDE and PCBs were found to be less in umbilical-cord blood than in maternal blood. The lower cord-blood concentration suggests that the placenta acts as a partial barrier; however, the unique physiologic state of parturition (when cord blood is collected), which is characterized by an increase in serum concentrations of cortisol, could lead to changes relative to earlier times in pregnancy. The degree to which the placenta acts to inhibit maternal-to-fetal transport is not known for most lipophilic HAAs. The placenta should be examined for toxic substances at low doses to determine whether it acts as a barrier. Studies of chemical transfer in isolated, perfused placenta, such as that by Bassily et al. (1995), could help to establish the characteristics of transfer.

Target Cells

Measuring the concentration of HAAs in target organs is one step removed from measuring the concentration that might actually reach the target cells in those organs. Few studies have been published that provide target-cell measurements. However, molecular changes, such as the induction of CYP enzymes in pituitary gonadotrophs, can indicate uptake in particular targets (Andersson et al. 1993). Cellular localization of proteins involved in HAA effects could help to identify sensitive target cells. For example, CYP19 (aromatase) is expressed in Leydig cells in humans, in the seminiferous tubule in mice, and in astrocytes of the brain in rats (Nitta et al. 1993; Inkster et al. 1995; Zwain et al. 1997), indicating that these cells are targets for aromatase-inhibiting xenobiotics.

Some cell types are overlooked as targets of HAAs and other xenobiotics, an example being those in the vasculature. Endothelial cells of blood vessels are the first cells (other then hemocytes) to encounter hormones or chemicals in the blood. Epithelial cells in the vascular system express steroid hormone receptors; estradiol affects vascular function (Rubanyi et al. 1997). The endothelium could work as a physical and metabolic barrier, intercepting molecules in the bloodstream. TCDD and PCBs strongly induce CYP1A in the endothelia of mammals and fish (Dees et al. 1982; Stegeman et al. 1989; Overby et al. 1992). Metabolism of xenobiotics by

enzymes in the endothelium has been demonstrated (Stegeman et al. 1995). Transformation of HAAs in the endothelium has not been shown but probably occurs.

As with the sequestration of TCDD by CYP1A2 in the liver (Diliberto et al. 1997), CYP1A in the endothelium might act in some species to retain or metabolize some compounds preferentially in highly vascularized regions. For example, localization of 3,3´,4,4´-tetrachlorobiphenyl near vascular structures in rat brain (Ness et al. 1994) could involve interaction with CYP induced in the vasculature.

Studies of developmental defects in fish, such as "blue sac" disease in lake trout, suggest that the endothelium or other vascular structures are targets for chemicals that produce developmental abnormalities. Blue sac disease is characterized by vascular dysfunction preceding death. It is associated with induction of CYP1A in the endothelium (Guiney et al. 1997) and with evidence of endothelial cellular damage (Cantrell et al. 1996, 1998).

Depots and Mobilization

Some fatty tissues that accumulate lipophilic chemicals, including many HAAs, can serve as reservoirs or depots for these chemicals. Fatty tissues in vertebrates include adipose tissue, liver, gonads, neural tissue, brain, and, in some fish, muscle. Adipose tissue in most vertebrates acts as a depot for organic xenobiotics. The concentration of HAAs is particularly important in assessing the exposure of some types of animals to HAAs. For example, the concentration of xenobiotics in whales is most often measured in blubber (Muir et al. 1988b; Wade et al. 1997), which can be biopsied as a marker for exposure.

A survey was conducted by the National Human Monitoring Program for the years 1970-1983 to detect and quantify the prevalence of organochlorine pesticides and PCBs in adipose tissue of the general population in the United States (Kutz et al. 1991). This survey showed that mean concentrations of DDT in adipose tissue declined from approximately 8 ppm in 1970 to about 2 ppm in 1983. Similarly, the percentage of individuals having total PCB concentrations greater than 3 ppm steadily declined, although the number of people with detectable amounts of PCBs increased. Aldrin was not detected, but its metabolite, dieldrin, was shown to decline from about 0.18 ppm to 0.06 ppm. The metabolite of chlordane, oxychlordane, was also detected, but the concentrations found in adipose tissue were relatively constant over the survey period. Endrin and toxaphene were not detected. Measurements for chlordecone (Kepone) were also taken, but the population examined was limited to the southeastern United States, because this pesticide is used to control fire ants in this region. Chlordecone was found in less than 1% of the adipose-tissue samples and ranged in concentration from 0.15 to 2.5 ppm (Kutz et al. 1991).

Adipose tissues in some organs (e.g., breast) might be important direct targets of HAA action or important internal reservoirs of HAAs. However, chemi-

cals in these lipid depots can be mobilized during starvation or reproduction. Bigsby et al. (1997), for example, demonstrated that, in fasting mice, some HAAs, such as β-HCH (hexachlorocyclohexane), are released from fat and then produce estrogenic effects, but that is not observed with *o,p′*-DDT, indicating that these compounds are mobilized differently from fat depots. In marine mammals, the amount of blubber can change with the season, with feeding, or with reproduction, resulting in changes in HAA content in the blood of some species (Aguilar and Borrell 1994). Fasting in female polar bears results in increased concentrations of HAAs in milk (Polischuk et al. 1995). Species that lay yolky eggs transport large quantities of egg proteins and lipids to their eggs during oogenesis (Vodicnik and Peterson 1985). In general, that process is associated with mobilization of lipids and other reserves, enhanced synthetic processes in the liver, and transfer of materials from the liver to the egg. Changes in reproductive state also produce increases in vascularization and blood flow to organs (e.g., gonads) that could be targets for the action of HAAs, resulting in transport of greater amounts of chemicals to these organs. At the same time, there is often an increase in the lipid content of the gonads, resulting in a greater degree of chemical deposition there. Similar processes can occur during lactation in mammals, enhancing the transport of chemicals to breast tissue (Ramos et al. 1997a).

Deposition of eggs or release of milk can eliminate some chemicals from the female. Studies in fish document the extensive displacement of contaminants from the maternal body to the ovary and deposition in the eggs. Thirty percent or more of maternal contaminant burden can be lost at the time of spawning, when it is shed with the eggs (Vodicnik and Peterson 1985). Likewise, release of milk can eliminate a substantial fraction of HAA from female mammals. However, that can result in the transfer of chemicals to infants, and the effects of the contaminants can appear in the developing young.

It should be noted that many of the foregoing examples of trend data might not be directly comparable because of differences in methods for detecting clinical residues. As technology advances and becomes standard, more chemicals will be detected and compared in studies (Hill et al. 1995, 1996; Needham et al. 1995; Burse et al. 1996).

METABOLISM

HAAs and endogenous hormones can act though receptor-dependent and receptor-independent (i.e., nonhormonal) pathways. Inferences regarding HAA effects and dose-response relationships can be weakened by the failure to distinguish between these mechanisms. Receptor-dependent pathways are discussed in Chapter 2. This section considers enzymes that metabolize hormones and HAAs and that could be involved in receptor-independent effects.

Enzymes

Enzymes that metabolize steroids and HAAs include members of the CYP family (Nelson et al. 1996), catechol-*O*-methyltransferase, dehydrogenases such as 11β-hydroxysteroid dehydrogenase, sulfotransferases, glutathione(*s*)transferases, glucuronyl transferases, and others. The CYP enzymes figure most prominently in the oxidative metabolism of HAAs and steroids. Many microsomal CYP enzymes have broad substrate specificities and hydroxylate steroids and many HAAs, often catalyzing rate limiting steps (see for reviews, Ioannides and Parke 1993; Ortiz de Montellano 1995). CYP enzymes also catalyze the synthesis and activation of steroids. The consequences of exposure to HAAs could differ, depending on the nature and extent of metabolic transformation of steroids, the metabolic transformation of the HAA, and the interplay between the two. Knowledge of the function and regulation of the enzymes is necessary to any understanding of how metabolism might contribute to the developmental, reproductive, or disease outcomes ascribed to HAAs. The complex ways in which metabolism of HAAs and hormones affects outcomes are illustrated by considering how metabolites of estradiol and HAAs can be involved in hormone-dependent carcinogenesis.

Virtually all HAAs are metabolized through a variety of oxidative and reductive or conjugation reactions. Metabolism can inactivate an HAA or lead to the activation of a hormonally-active metabolite from a non-hormonally active parent compound. Methoxychlor, several PCB congeners, and DDT are metabolized to products that are estrogenic, antiandrogenic, or thyroid mimics (Safe and Zacharewski 1997) (see Chapter 2). Nonestrogenic nonylphenol polyethoxylates are metabolized to the estrogenic nonylphenol in the rat (Knaak et al. 1966). CYP and conjugation enzymes also transform nonylphenol and bisphenol A (Atkinson and Roy 1995; Lewis and Lech 1996; Meldahl et al. 1996), but the reactivity of the products is not known. Methysulfonyl-DDE appears to be further metabolized to toxic derivatives in fetal mice (Jonsson et al. 1995). The metabolism of many phytoestrogens is less well known than is that of the synthetic xenobiotic HAAs. Flavonoids are hydroxylated by CYP enzymes (Silva et al. 1997). Zearalenone also appears to be metabolized to a more estrogenic compound, α-zearalenol (Kuiper-Goodman et al. 1987). In general, nonhalogenated compounds, which include many natural products, are more rapidly metabolized than are chlorinated compounds (Chen et al. 1982).

Metabolism-Modifying Factors

The capacity to metabolize steroids and HAAs in target and nontarget organs can change as a result of exposure to HAAs and to chemicals that affect enzymes that metabolize HAAs or steroids. Induction increases the rate of enzyme synthesis, but chemicals can act on gene products directly. For example, although metabolites of zearalenone can be estrogenic, the estrogenic effects of zearalenone

could result in part from its inhibition of steroid metabolism (Pompa et al. 1986). Tributyltin is thought to cause penis growth in female molluscs by affecting steroid metabolism (Bettin et al. 1996). Methoxychlor can both induce and inhibit CYPs (Li et al. 1993, 1995). Some PCB congeners can induce CYP1As but also inhibit and inactivate them (White et al. 1997a). Interactive effects can involve one HAA affecting the metabolism of another. Thus, lower chlorinated PCBs can be metabolized to reactive products and be eliminated more rapidly than are the more highly chlorinated compounds, but the more highly chlorinated compounds can persist and act as inducers of enzymes that affect HAA and steroid metabolism. The expression and activity of these enzymes can be regulated by hormones as well. For example, the recently described CYP1B1 shows dual regulation by aryl hydrocarbon (Ah) receptor agonists and by hormones (Bhattacharyya et al. 1995). The induction of CYP1A in fish can be suppressed by hormones, ostensibly estradiol, during ovarian maturation (Gray et al. 1991).

Species differences in CYP enzymes and their role in the metabolic disposition of HAAs and hormones can introduce uncertainty in attempts to extrapolate data from one species to another, including to humans. For example, humans create a greater percentage of the more-estrogenic metabolite (α-zearalenol) of zearalenone and dispose of the compounds more slowly than do rodents (Kuiper-Goodman et al. 1987). There are few studies that directly compare rates of metabolism of HAAs in different species or of multiple HAAs in a given species (Borlakoglu and Wilkins 1993; Murk et al. 1994). A few generalizations are possible. For example, fish are much less active metabolizers of PCBs than are mammals (Murk et al. 1994; White et al. 1997b).

Steroid Metabolism and HAAs

The function or rate of action of enzymes involved in steroid synthesis, activation, or degradation can be affected by HAAs. Steroid synthesis involves numerous steps leading to the active hormones. The steroid synthetic enzymes include CYP11 (side-chain-cleavage enzyme), CYP17 (17α-hydroxylase), and CYP19 (aromatase). Functional and cloning studies of the steroidogenic enzymes in nonmammalian vertebrates, including fish (Sakai et al. 1992; Tanaka et al. 1992; Takahashi et al. 1993) and turtle (Jeyasuria et al. 1994), indicate that similar enzymes occur in mammalian and nonmammalian vertebrates. As with other CYP enzymes, the steroid synthetic enzymes are subject to inhibition by substrate analogues that can act as competitive inhibitors or mechanism-based inactivators.

An important example is aromatase, which catalyzes the conversion of testosterone to estradiol. Aromatase is expressed in many organs, where it is a factor in health and disease (Simpson et al. 1997), and it is inhibited by various chemical structures. Those include flavonoids (Ibrahim and Abul-Hajj 1990; Wang et

al. 1994; Pelissero et al. 1996), other natural products (Blanco et al. 1997), and such compounds as fadrazole and aminoglutethimide. The mechanisms of action of those chemicals are under study (Chen et al. 1997). It is possible that exposure to aromatase inhibitors in the environment affects sex determination, as suggested by studies in fish, birds, and reptiles (Lance and Bogart 1992; Monod et al. 1993; Antonopoulou et al. 1995; Richard-Mercier et al. 1995; Abinawanto et al. 1996). There is evidence that nonsteroidal aromatase inhibitors can alter the temperature-dependent sex determination in the terrapin (Jeyasuria et al. 1994) and might be a molecular target in alligators (Crain et al. 1998).

CYP enzymes that hydroxylate steroids have been studied extensively in the liver of mammalian species (Waxman et al. 1991; Ioannides and Parke 1993). A review by Martucci and Fishman (1993) describes several of the pathways of CYP metabolism of estradiol in mammals, including humans. The same enzymes can hydroxylate estradiol (E2) and estrone (E1). Many of these enzymes are involved in the hydroxylation of testosterone.

The degree of expression of the particular enzymes, their affinity for the substrate, and their catalytic efficiency will determine the rate or extent of formation of specific metabolites. Expression of multiple CYP genes in the same cell can lead to formation of multiple products in that cell. Most studies of steroid metabolism, including that of estradiol, have been done with enzyme preparations from the liver. If chemical exposure induces the expression of enzymes that hydroxylate steroids in the liver, elimination of the hormone could be accelerated, thus reducing concentrations in the body. Yet, it is not clear whether altered rates of hepatic steroid metabolism are involved in the effects of HAAs in other target organs. Reactive or protoxic metabolites of HAAs or steroids produced in the liver would need to be transported in the blood to reach other target organs. The effects of HAAs on CYP enzymes in those target organs could be more directly significant.

Extrahepatic Enzymes and HAA Effects

The complement of enzymes that metabolize HAAs can strongly influence the susceptibility of the cell to the effects of these compounds. If an HAA requires metabolism to be estrogenic, then cells lacking the requisite enzymes are less likely to be susceptible to the action of that chemical than are cells that have an active metabolism. Generally, the same enzymes will catalyze similar reactions in different organs and cells. Apart from steroidogenic enzymes in gonads and adrenals, the cellular localization of microsomal CYP enzymes in extrahepatic organs that are targets for HAAs or for hormonally related pathologies is not well known. It cannot be assumed that the steroid-metabolizing enzymes present in the liver are expressed to similar degrees in other organs. Some enzymes expressed only slightly in liver are highly expressed in other organs. A

key example is CYP1B1, which is discussed below. Studies in three target organs (brain, mammary gland, and placenta) provide examples of the complexities in these possible sites of action.

Brain

Some CYP enzymes involved in steroidogenesis, particularly CYP19, have been identified in the brain of most vertebrate groups (Callard et al. 1988). As suggested above, altered aromatase activity has been linked to effects on sex differentiation in reptiles and fish. The brain appears to synthesize steroids, including neurosteroids, completely from cholesterol (Stapleton et al. 1995; Warner and Gustafsson 1995; Rose et al. 1997).

Other hormonally important structures, including the pituitary, are sites of action of some HAAs. Andersson et al. (1993) reported that CYP1A1 was induced in the pituitary of trout exposed to an Ah receptor agonist. Cell types in which induction was strong included gonadotrophs containing gonadotropin II; alterations in the concentrations of gonadotropin were associated with exposure. A prominent site of induction of CYP1A1 by PCBs and TCDD in the brain is the endothelium (Smolowitz et al. 1991). The prominence of induction at this site could contribute to a blood-brain barrier for CYP1A substrates. An increasing number of CYP enzymes are being found to be expressed in brain tissue in humans, other mammals, and lower vertebrates (Strobel et al. 1995; Stegeman et al. 1997). Regional expression of some brain CYPs has been found, for example, in the expression in the hippocampus of a novel CYP identified in rat brain (Stapleton et al. 1995). For most of the known brain CYPs, regional and, more important, cellular localization have yet to be determined.

Mammary Gland

Steroidogenic and hydroxylating CYP enzymes are expressed in mammary tissue. Studies of CYP enzymes in the breast are not extensive. Hellmold et al. (1995) examined the expression of CYP messenger ribonucleic acids (mRNAs) in breast tissue of rats 1, 2, 3, 6, 9, and 15 weeks after birth; 1, 2, or 3 weeks after pregnancy; 2-3 weeks postpartum; and 3 weeks after lactation. The expression of at least 10 CYP genes was detected in the breast tissues. The same CYP enzymes were detected also with antibodies, and there were distinct patterns of expression associated with age, reproductive status, and lactation. Recent studies of the mammary gland have focused on a relatively recently discovered enzyme, CYP1B1, which activates polycyclic aromatic hydrocarbon (PAH) carcinogens (Shimada et al. 1996) and forms catecholestrogens (Hayes et al. 1996). The possible significance of that enzyme in mammary carcinogenesis is considered later in this chapter.

Placenta

Placental CYP enzymes, especially aromatase (CYP19), have been studied for years in animals and humans. In addition to converting testosterone to estradiol, CYP19 appears to form the catecholestrogen 2-OH-E2 (Almadhidi et al. 1996), a possibly important metabolite. Developmental and cellular patterns of expression are important to the consequences of HAA metabolism. Transcripts for 10 CYP enzymes were identified by reverse transcriptase-polymerase chain reaction in at least some samples of first-trimester human placenta (Hakkola et al. 1996a). Similar studies (Hakkola et al. 1996b) of term placenta showed that the patterns of expression had changed, with fewer genes appearing to be expressed. That implies that the capacity of the placenta to metabolize HAAs, or steroids, changes during gestation. For the most part, the cellular sites of expression of these CYP enzymes have not been described. However, involvement of a CYP in arachidonic-acid metabolism in human placental vessels has been suggested (Omar et al. 1992). Also, CYP1A1, induced by and metabolizing planar PCBs and TCDD, is expressed in trophoblasts in human placenta (Slezak et al. 1997).

Placental CYP enzymes might constitute a metabolic barrier, intercepting xenobiotics before they can reach the fetus. That possibility is suggested by studies of placental CYP2E1, which metabolizes alcohol, and fetal alcohol syndrome (Rasheed et al. 1997). Fetal alcohol syndrome might result from acetaldehyde produced from alcohol in the fetus (Holownia et al. 1996). CYP1A, which can metabolize and activate many procarcinogens, was found to be elevated in the placenta of women who were accidentally exposed to high concentrations of PCBs (Lucier et al. 1987) and who smoked during pregnancy (Gallagher et al. 1994; Slezak et al. 1997). In addition to possible involvement of CYP1A, smoking appears to decrease placental aromatase activity (Kitawaki et al. 1993). The significance of placental xenobiotic enzymes to HAA effects in the fetus are not well documented.

Developmental Stage and CYP Enzymes

The expression of CYP enzymes during embryonic, larval, or fetal development might be crucial to the developmental effects of exposure to HAAs or other xenobiotics. Stromstedt et al. (1996) reported that steroidogenic enzymes were not expressed in mouse preimplantation blastocyst, although aromatase is expressed in preimplantation blastocyst of other species (Choi et al. 1997). Toda et al. (1994) documented the presence of steroidogenic CYP enzymes in mammalian fetal tissue. CYP19 is expressed in fetal gonads and brain (Hutchison et al. 1995). Keeney et al. (1995) reported spatial and temporal differences in the patterns of expression of CYP17, aldosterone synthase, and cholesterol side-chain cleavage CYP mRNAs, and they suggested that several factors were required to program cell type and species-specific expression of steroid hydroxylases

during embryonic development. The development of enzymes involved in conjugation and deconjugation of steroids was examined in a study of rats from birth to 50 d of age (Fisher and Weissinger 1972). The clearance time of [^{14}C]diethylstilbestrol (DES) decreased 10-fold between birth and 25 d of age. Intestinal hydrolysis of DES conjugates was minimal at birth (because of the lack of intestinal bacteria) but fully developed by 25 d of age, and there was a deficiency in liver enzymes required for conjugation in newborn rats. Not much is known about such patterns across species.

Whether mammalian blastocysts express xenobiotic-metabolizing CYP enzymes is not clear. However, xenobiotic-metabolizing enzymes detected at the level of mRNA in human fetal liver include CYPs 2C8, 2D6, 3A3/3A4, and 3A7 (Hakkola et al. 1994). The expression of CYP1A1 has been detected during organogenesis in animals (Dey et al. 1989; Yang et al. 1991) and in human fetal liver (Murray et al. 1992). Morse et al. (1995), however, reported that fetal rats had little inducible capacity to metabolize 3,3′,4,4′-tetrachlorobiphenyl, although other CYP1A activity was induced (Sinjari et al. 1993). There might be development-specific CYP enzymes. In mice and humans, there appears to be a fetus-specific member of the CYP3A subfamily (Itoh et al. 1994). CYP3A enxymes are active steroid 6β-hydroxylases, and they are catalysts for most of the drugs metabolized in adult human liver.

Still less is known about the expression of CYP enzymes at different stages of nonmammalian developmental stages. Few homologous CYP enzymes have been identified in many species, and only CYP1A has been well studied. CYP1A1 is induced by TCDD and other Ah receptor agonists at various developmental stages in birds and fish. In fish, that induction is prominent in the endothelium, where it has been associated with developmental toxicity (Guiney et al. 1997; Cantrell et al. 1998). Determination of the forms and functions of CYP enzymes expressed at different stages is an important research need.

Biologically Active Metabolites of Estradiol

As pointed out by Wolff and Toniolo (1995), "different groups of polychlorinated biphenyl . . . congeners evoke different biological responses, and a wide divergence of estrogenic response, CYP enzyme activity, and biological half-life exists within these groups. . . . " A consideration of multiple mechanisms by which HAAs and natural estrogens might act is crucial to evaluating the risk associated with exposure to these compounds. One such consideration is how estradiol metabolism might be related to breast cancer and how HAAs might contribute to the process through multiple mechanisms.

A role for estradiol metabolism in breast cancer has been suggested repeatedly (Yager and Liehr 1996). The formation of 16α-hydroxy-E2, a metabolite of estradiol, has been suggested by some investigators to be a risk factor in human breast cancer (Bradlow et al. 1995). The high estrogenic activity of 16α-OH-E2

and partial antiestrogenic activity of 2-OH-E2 has recently been confirmed (Gupta et al. 1998) and is consistent with the Bradlow hypothesis. However, other data suggest that catecholestrogen formation is a risk factor in nonfamilial breast cancer (Lemon et al. 1992; Adlercruetz et al. 1994) and that catecholestrogen might be causally involved.

Catecholestrogens, both 4-OH-E2 and 2-OH-E2, are estrogen receptor agonists (Schutze et al. 1993). However, data suggest that the action of these products involves generation of reactive oxygen species through redox cycling. Nutter et al. (1993) established that quinone derivatives of catecholestrogens can generate reactive species when incubated with breast-cancer preparations. In particular, 4-OH-E2 has been implicated in such effects (Han and Liehr 1995; Liehr et al. 1995; Liehr and Ricci 1996). Han and Liehr (1995) examined the ability of 4-OH-E2 and 2-OH-E2 to cause oxidative damage of DNA and reported that 4-OH-E2 caused greater damage than did 2-OH-E2, consistent with a more-rapid redox cycling of the 4-OH-E2. Reactive oxygen species can damage DNA, but they also can alter various signal transduction pathways involving cell proliferation, notably in early development (Ozolins and Hales 1997). 2-OH-E2 is more active as an antioxidant (Ruiz-Larrea et al. 1994) and might indeed have some benefit (Bradlow et al. 1996). There is evidence that oxidative damage of DNA is associated with breast cancer (Malins and Haimanot 1991; Malins et al. 1993, 1996), and the evidence is quite consistent with the evidence suggesting involvement of radical formation that could come from redox cycling involving catecholestrogens.

4-OH-E2 has been identified as a risk factor in mammary carcinogenesis (Cavalieri et al. 1997); thus, it is important to discern the regulation of enzymes that form this product. In rodents and humans, CYP1B1 has been established as a primary catalyst responsible for 4-hydroxylation of E2 (Spink et al. 1994). Cloning and expression research shows that human CYP1B1 catalyzes E2-2-OHase activity, but that it is primarily an E2-4-OHase (Hayes et al. 1996). However, in some organs, 4-OHase and 2-OHase appear to be equally important. CYP1B1 also is expressed in breast and breast-cancer tissue (Christou et al. 1995; McKay et al. 1995). CYP1B1 or related proteins are responsible for 4-OH-E2 formation in the uterus, where they might participate in carcinogenesis (Liehr et al. 1995). Other organs where 4-OHase activity is high are targets for estrogen-dependent tumorigenesis.

If the postulated role of catecholestrogens in estradiol-induced carcinogenesis is correct, then exposure to xenobiotic HAAs could influence that process through effects on the formation or persistence of catecholestrogens. For example, TCDD and other Ah receptor agonists, including PCBs, can induce the expression of CYP1B1 (Brake and Jefcoate 1995). The flavonoid quercitin inhibits catechol-*O*-methyltransferase, an enzyme that can inactivate the catecholestrogens. That inhibition can result in accumulation of the catecholestrogens, thereby increasing their availability for redox cycling (Zhu and Liehr 1996). It follows that HAAs contrib-

ute to detrimental effects through their own metabolic generation of reactive oxygen species. Thus, some PCB metabolites form quinones that can undergo redox cycling and damage DNA (Oakley et al. 1996), but those congeners demonstrated to form quinones are not found in the environment.

An additional complexity in carcinogenic effects involves angiogenesis. Growth of vessels to the tumor is essential for the growth of tumors. Notably, 2-methoxyestradiol is an angiogenesis inhibitor that can suppress tumor growth (Fotsis et al. 1994). Inhibition of catechol-*O*-methyltransferase therefore might not only promote accumulation of catecholestrogens but also decrease the formation of a product that can impede tumor growth. Understanding of how estrogen metabolism is affected by HAAs is incomplete. Whether rates of angiogenesis are prognostic indicators for breast cancer is not clear (Hall et al. 1992). The proposed mechanism by which catecholestrogens act in carcinogenesis, involving redox cycling and the generation of free radicals, would not necessarily involve the estrogen receptor but would involve the enzymes of E2 metabolism. It will be necessary to determine whether E2 action on cell proliferation is an independent process that contributes to carcinogenesis in the breast or is related to the radical pathways to carcinogenesis. The balance of these different pathways is not understood. Moreover, estradiol might act as an antioxidant. The diversity of responses could be disclosed when the regulation of the catalysts for estradiol and HAAs is understood.

As with estradiol, HAAs also might act through multiple mechanisms, with detrimental and beneficial effects. Genistein is an excellent example. Genistein is an estrogen receptor agonist, but it inhibits 17β-hydroxysteroid oxidoreductase and thus can limit the conversion of estrone to estradiol (Mäkelä et al. 1995b). Genistein also possesses antioxidant properties and can enhance antioxidant enzymes (Cai and Wei 1996), which can counter the oxidative damage ascribed to catecholestrogens. The compound also inhibits the *p*-glycoproteins (Castro and Altenberg 1997), which transport drugs and some steroids out of the cell. Genistein is an inhibitor of angiogenesis (Fotsis et al. 1993); the mechanism of that effect is not known, but it might confer protection against tumorigenesis, as vessel growth is essential to tumor growth and to metastasis. Inhibition of angiogenesis also can affect fetal development. Moreover, genistein is a potent inhibitor of membrane tyrosine kinases (Spinozzi et al. 1994), which might be involved in effects on cell proliferation independent of effects on estrogen metabolism. Whether different doses of genistein act through different modes is not clear. Prenatal exposure to genistein affects later sexual differentiation (Levy et al. 1995), but it is not clear which mechanisms are involved.

SPECIES DIFFERENCES IN METABOLISM

Species differences in response to xenobiotics, including HAAs, can be enormous (Kennedy et al. 1996). Whether an effect of HAAs observed in one species

occurs in others is the subject of concern when researchers try to extrapolate data for a particular disease, such as breast cancer, from animal models to humans. For procarcinogens, or HAAs that are metabolically activated, the presence of enzymes that activate the chemical in question could be a determining factor. The enzymes involved in the metabolism of hormones in vertebrates are similar in function, but the substrate specificity of xenobiotic-metabolizing and steroid-hydroxylating enzymes can differ substantially for homologous enzymes in different species. There is little information available about CYP enzymes in nonmammals (Hahn and Stegeman 1994). Only now are CYP enzymes that might metabolize HAAs being described in reptiles, including alligators (Ertl and Winston 1997).

Formation of catecholestrogens by hepatic CYP enzymes has been shown in fish (Snowberger and Stegeman 1987) and in adult and fetal marine mammals (Goksoyr et al. 1988; R. White et al. 1994). Fish-liver microsomes also form 6a-OH-E2, 16a-OH-E2 (estriol), and estrone (Snowberger and Stegeman 1987). The major hydroxylated product appears to be 2-OH-E2. Unlike the case in some mammals, CYP1A enzymes in fish appear to have little involvement in forming 2-OH-E2, indicating that HAAs, such as TCDD, might not act through this pathway in fish. There is little evidence for hepatic formation of 4-OH-E2 in fish. Whether fish form this catecholestrogen in endocrine organs or express homologues of CYP1B enzymes has not been established. Studies of homologous CYP enzymes in different groups (e.g., the androgen-hydroxylating CYP3As (Celander and Stegeman 1997)) could show the extent of similarities and differences attendant to HAA exposure in different species. Defining structure-activity relationships for substrate binding to homologous enzymes in different species will help to achieve generalizations.

FACTORS INFLUENCING DOSE-RESPONSE ASSESSMENT

A central issue in evaluating possible consequences of HAA exposure is whether the concentrations accumulating in animals and humans are sufficient to elicit changes in target cells and organs of the adult or the developing organism. Accurately predicting a biologic effect solely on the basis of the chemical concentrations in tissues requires that the dose-response relationship for a particular biologic change be known. To infer an effect from environmental exposure requires extrapolation across dose; that requires a knowledge of the shape of the dose-response curve. Typically high doses used in experimental studies are extrapolated to potentially much lower doses resulting from exposure to concentrations ordinarily found in the environment in contaminated food, water, air, or soil.

Inference also requires extrapolation across species, because with some species (e.g., humans), the possibility for experimental studies is impossible. Extrapolation across species is problematic, because species can differ greatly in their sensitivity to the effects of chemicals. For example, the acute toxicity of

TCDD varies by orders of magnitude between the most- and the least-sensitive vertebrate species studied to date (Peterson et al. 1993; Kennedy et al. 1996). However, at the receptor level, there is little evidence for significant differences among vertebrate species in the affinity of estradiol for estrogen receptors or the protein sequence of the receptors (Katzenellenbogen et al. 1979). Therefore, if estradiol (and potentially xenoestrogens) reaches estrogen receptors in target cells, the capacity to generate responses might not be very different among vertebrate species, although it should be pointed out that the expression of the receptor and its affinity for ligand (e.g., TCDD) binding is not necessarily predictive of responsiveness. Data on the resistant Han/Wistar rat compared with other rats more responsive to Ah clearly illustrate that point in that Ah responsiveness and Ah receptor levels are similar in both responsive and less-responsive rat strains (Pohjanvirta et al. 1994).

Dose-response assessment of HAAs presents a challenge, because HAAs might cause effects not only by acting as reactive molecules that attack biologic macromolecules, but also by acting as stable molecules interacting with the body's natural signaling systems (Barton and Andersen 1997). Based on a small number of in vivo investigations conducted to date involving a relatively small sample of natural hormones and HAAs, data suggest that in some situations a nonmonotonic dose-response relationship can occur (Kociba et al. 1978; Gray et al. 1989; Halling and Forsberg 1993; vom Saal et al. 1995, 1997; Fan et al. 1996; Calabrese 1997). The issue of the shape of the dose-response curve for HAAs could have implications for toxicity-testing protocols and dose-response assessment methods.

In attempting to establish dose-response relationships, the application of molecular probes as biomarkers for changes in cell processes might make it possible to identify changes linked to contamination, even in populations with relatively low concentrations of the contaminants (see discussion in greater detail in Chapter 11).

Dose-Response Functions and HAAs

For the body of experimental studies on estrogens or other biologically active regulators, a typical monotonic dose response or binding curve is observed; as dose increases, response increases or stays the same (Klaassen et al. 1996).

However, there are numerous examples of nonmonotonic inverted U-shaped dose-response curves from in vitro studies. These studies involve a variety of natural and anthropogenic estrogens (e.g., estradiol, estriol, nonylphenol, and DES), end points (e.g., cell proliferation, prolactin synthesis, and induction of specific mRNAs), and cell lines (e.g., Jordan et al. 1985; Soto et al. 1991; Bigazzi et al. 1992; Pilat et al. 1993; Truss and Beato 1993; Tzukerman et al. 1994; Olea et al. 1996). Sonnenschein et al. (1989) also observed a nonmonotonic response curve for androgen-induced cell proliferation in LNCAP cells by using a diverse group of steroidal and nonsteroidal compounds.

Both U-shaped and inverted U-shaped dose-response curves have been reported in in vivo studies of HAAs. For example, an inverted U-shaped dose-response relationship has been reported to occur with respect to prostate size when mouse fetuses are exposed to either estradiol or DES; prostate weight first increases and then decreases with dose (vom Saal et al. 1997). These investigators previously reported an inverted U-shaped dose-response relationship between maternal dose of DES and territorial marking in male offspring (vom Saal et al. 1995). In the former study, pregnant CF-1 female mice were either implanted with Silastic tubing containing increasing doses of estradiol or fed DES once per day from gestation d 11 to 17. A 50% increase in free serum estradiol in male mouse fetuses induced a 40% increase in the number of developing glandular epithelial ducts emanating from the urethra (these form the glandular ducts within the prostate); subsequently in adulthood, the number of developing prostatic androgen receptors per cell was permanently increased by two-fold, and the prostate was enlarged by 30% (due to hyperplasia) relative to unexposed males. However, when free serum estradiol concentration was increased from two- to eight-fold, adult prostate weight decreased relative to males exposed to the 50% increase in estradiol. Maternal ingestion of DES at 0.02, 0.2, or 2 ng/g of body weight significantly increased adult prostate weight in male offspring, and DES at 200 ng/g of body weight (200 ppb) significantly decreased adult prostate weight (see Figure 4-1). However, in another study to address the low-dose response, a single dose DES treatment (0.2 ng/g of body weight) in the same strain of mice failed to cause an increase in prostate weight (Cagen et al. 1999). The decrease in prostate weight reported by vom Saal et al. (1997) is consistent with numerous prior findings that exposure to a high dose of DES during development results in an abnormally small prostate in adulthood as well as a decrease in prostatic androgen receptors (Prins 1992; Newbold et al. 1994; Santti et al. 1994).

In another study of methoxychlor-induced alterations of reproductive development and function in the rat, a U-shaped dose-response curve was reported (Gray et al. 1989). In this study, rats were dosed from weaning, through puberty and gestation, to d 15 of lactation with methoxychlor at 25, 50, 100, or 200 mg/kg/d. The onset of cyclicity in female offspring was accelerated in the low-dose group, normal in the two mid-dose groups, and delayed in the high dose group. Other investigators reported that uterine weight as a fraction of body weight following neonatal dosing displayed a nonmonotonic (inverted-U) dose response with DES but not estradiol (Halling and Forsberg 1993). In addition, there are several examples of inverted U-shaped curves showing protective effects at low doses (Calabrese 1997). A recent report by Fan et al. (1996) showed that TCDD induced an inverted U-shaped dose-response curve for its effects on cell-mediated immunity in the rat; at low doses, TCDD enhanced and at high doses TCDD suppressed the delayed-type hypersensitivity (DTH) reaction. Kociba et al. (1978) demonstrated that low doses of TCDD at 1 ng/kg/d decreased the rate of sponta-

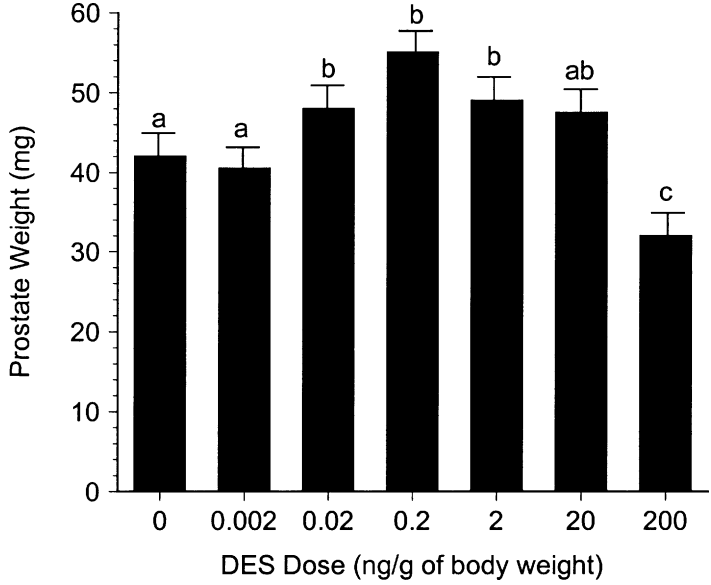

FIGURE 4-1 Mean (+SEM) prostate weight (mg) in 8-mo-old CF-1 male mice produced by females fed different doses of DES from d 11 to d 17 of pregnancy. Group means that differed significantly are indicated by different letters, and group means with the same letter did not differ significantly. Prostate weights presented here were adjusted for significant effects of DES on body weight by analysis of covariance (ANCOVA) using the statistical analysis system (SAS). Source: vom Saal et al. 1997.

neous tumor formation; the rate is then increased at high doses of TCDD (e.g., pituitary adenomas, males; hyperplastic liver nodules, females; and thyroid adenomas, females).

At this time, it is not clear how prevalent or important nonmonotonic dose-response relationships are for different types of end points (e.g., malformations vs. a permanent change in enzyme activity) or what the mechanisms might be. As in any cell assay, high doses might cause toxicity and reduce response rates (Mayr et al. 1992). In vivo nonmonotonic dose-response curves can occur as a result of underlying physiologic and toxicologic processes. Feedback processes typically respond to both excesses and deficits of signals. Thus, untoward responses might occur over a broad dose range, covering interference with natural function at low doses and excess stimulation at high doses (Barton and Andersen 1997). Such factors as down-regulation of receptors in the presence of high doses of a hormone might also partially account for this phenomenon (Gorski and Gannon 1976). Other possibilities might involve the following: stimulation of

response systems that are antagonistic to the initial response as saturation of receptors occurs (Amara and Dannies 1983), induction of metabolism, cross-talk between estrogen and receptors for other steroids as total estrogenic activity in blood exceeds the physiologic range, or a low-dose effect on DNA synthesis increasing cell proliferation, with a secondary effect to increase apoptosis.

In considering the question of U-shaped dose-response curves, it is important to distinguish between nonmonotonic dose-response relationships arising from underlying modes of action and those resulting from difficulties observing an end point at high doses (Barton and Andersen 1997). The latter is particularly important in reproductive and developmental studies (Selevan and Lemasters 1987). For example, developmental toxicity studies often show increases followed by decreases in developmental abnormalities with increasing dose (Lamb 1997). That occurs because fetal mortality increases and masks the developmental effect. Monotonic behavior can also be observed if the time course of the response is altered with dose. Testing over a wider range of doses and using lower doses could address the issue (Martin and Claringbold 1960).

Thresholds

A basic assumption with regard to the dose-response assessment of systemic toxicants is that there is a threshold concentration below which no adverse effect will occur. This default assumption has been challenged for HAAs (vom Saal and Sheehan 1998), because data from the mouse and rat indicate that concentrations of free serum estradiol in fetuses are above threshold for producing responses in estrogen-responsive cells (Nonneman et al. 1992; Montano et al. 1995; vom Saal et al. 1997). In addition, manipulating estrogen (by administering supplemental estrogen, blocking estrogen biosynthesis via aromatase inhibitors, or blocking the binding of estrogen to receptors by antiestrogens) altered fetal development in rodents and birds (Clemens and Gladue 1978; Gladue and Clemens 1980; Adkins-Regan 1983; Takahashi et al. 1989; Lephart et al. 1992; vom Saal et al. 1997). A recent study of sex determination in red-eared slider turtles (Sheehan et al. 1999) provided evidence that regardless of whether a threshold exists for endogenous estrogen, no threshold exists for exogenous estrogen, because endogenous estrogens are at a sufficiently high concentration to exceed the threshold for sex determination. The investigators concluded that "these results provide a simple biologically based dose-response model and suggest that chemicals which act mechanistically like [endogenous estradiol] may also show no threshold dose." Whether the estrogen-response system is already operating above background in all developing animal fetuses and humans is clearly a controversial issue, which has not been unequivocally resolved.

If the threshold for response to estrogen has already been exceeded before exposure to an environmental estrogen, the additional load of an environmental estrogen might cause a significant increase in the occupied receptors required for

the response. For that requirement to occur, one must assume that the exogenous HAA is acting just like the endogenous ligand and, therefore, would be additive to the background effect, and the background dose of an endogenous hormone can be responsible for physiologic and toxicologic responses. However, some compounds have been shown to interact with estrogen receptors differently than estradiol, leading to unique responses (Webb et al. 1995; Yang et al. 1996; Barton and Andersen 1997). The possibility that estrogenic HAAs will show tissue- and life-stage-specific effects that differ from those of natural estrogens within a species will require extensive investigation before this issue is resolved.

Tissue Sensitivity

The issue of sensitivity of tissues to estrogen or other hormones that bind to receptors belonging to the superfamily of intracellular receptors is complex. Tissue sensitivity is influenced by multiple interactions, which include the interaction between the following: the hormone and receptor, the receptor and specific DNA sequences with which it interacts in a tissue (the hormone-responsive elements), the receptor and associated regulatory proteins that modulate responses, and the associated regulatory proteins and genes. It is likely that there are differences among species in each of these interactions that would influence sensitivity to HAAs. Within a species, variability between tissues occurs, and within individual cells, different responses occur at different doses. Finally, the importance of life stage cannot be ignored, particularly with regard to the hypothesis that developing animals show responses to hormones (and thus possibly also to xenobiotics) that are relatively inactive at later times in life (Hajek et al. 1997).

In vitro studies using estrogen-responsive MCF7 breast-cancer cells have investigated the relationship between estradiol concentrations, receptor occupancy, and biologic response (Table 4-4). Estradiol-induced proliferation of MCF7 cells reached half the maximal growth response at approximately 0.54 pg/mL of medium (Welshons and Jordan 1987). Scatchard plots of binding in intact cells show little deviation from linearity, suggesting that significant cooperativity, which has been extensively described in extracted receptors, is not generally detectable. Consistent with these findings, there was no evidence for cooperativity in the experiments that provided the data in Table 4-4.

At the tissue level, it has been shown that the potency of a particular xenoestrogen can vary for different responses in the same tissues (Hyder et al. 1997). An example in the rat uterus is the inhibition of gland genesis and down-regulation of estrogen receptors, which are responses up to an order of magnitude more sensitive to estrogenic chemicals, such as DES, than is uterine weight gain (Branham et al. 1996). Some anthropogenic estrogens, such as DES, have been shown to have increased potency (up to 100-fold) relative to estradiol in the embryo and fetus (Sheehan and Branham 1987) because of their higher availability due to the higher affinity of estradiol for α-fetoprotein as contrasted to DES.

TABLE 4-4 The Relationship Between Estradiol Concentration, Percentage of Estrogen Receptors Occupied, and Percentage of Maximum Proliferation Response in MCF7 Cells

Concentration, Hormone pM	Concentration, Hormone pg/mL	Receptors Occupied, % of Total	Approximate MCF7 Cell Proliferation Response, % Maximum
20,000	5,450	99	
2,000	545	91	100
200	54.5	50	99
20	5.45	9	91
2	0.54	1	50
0.2	0.054	0.1	9

SOURCE: Welshons and Jordan 1987.

The developing rat is unusual in having a serum glycoprotein present that has a high affinity for estradiol and not for other steroids.

Some compounds have been shown to interact with estrogen receptors differently than estradiol, resulting in different responses (Webb et al. 1995; Yang et al. 1996). One of the best-studied examples of tissue specificity of a chemical that interacts with estrogen receptors is tamoxifen, which is an agonist in the mouse uterus, a partial agonist and antagonist in the rat uterus, and an antagonist in the chicken oviduct (Welshons and Jordan 1987; McDonnell et al. 1995). Additionally, a new estrogen receptor (ER_β) was recently discovered that has a different tissue distribution and ligand selectivity from the well-described ER_α (Kuiper et al. 1996). The estrogen receptors are highly expressed in the prostate and, therefore, might play a role in mediating responses to estrogenic compounds as suggested above.

There are potential explanations for the observations described above other than the type and mixture of ERs in tissues, including differences in transcription factors and estrogen-response elements (Truss and Beato 1993; Starr et al. 1996; Uht et al. 1997). Taken together, these findings suggest that the use of a single end point is inadequate to characterize sensitivity of tissues to HAAs. Rather, the most sensitive and relevant responses and adverse effects at different life stages, in different tissues, and in different species need to be defined, recognizing that for wildlife a relevant end point might be population change.

SUMMARY AND CONCLUSIONS

The risks associated with exposure to an HAA depend upon many factors related to pharmacokinetics and pharmacodynamics. The chemical and physical

properties of the HAA and the nature of the species exposed determine whether a chemical will accumulate to a biologically significant dose and cause harm. In the case of HAAs, the hydrophobicity of the compound and the lipid concentration of the tissues are of particular importance. Rates of uptake and elimination, and thus half-lives, vary greatly for different HAAs in different species, and are influenced by the dose and duration of exposure. It is also important to consider the time that exposure occurs during an organism's life span. There are critical periods, such as fetal development, when organisms are more sensitive to xenobiotics.

Organisms can be exposed to environmental HAAs in a variety of ways, usually via the gut, skin, or respiratory surfaces. Contaminant concentrations increase up the levels of the food chain, and longer-lived animals accumulate greater concentrations of contaminants in their tissues. Of particular concern is the developing fetus, which can be particularly sensitive to toxic chemicals transferred transplacentally by the mother. Environmental contaminants have been found in human breast milk and in bovine milk, so these media are also possible routes of exposure.

Blood is the central medium of distribution of chemicals to target organs and cells in the body. Although distribution to different tissues is important for interpreting toxicity, comparing the effects of some compounds on the basis of the total body burden is also important, because it could reveal similarities not evident when concentrations in individual tissues are used to compare responses among species. The bioavailability of an HAA is influenced by its association with plasma-binding proteins, which affect its passage into tissues. Once inside a target cell, a steroid presumably becomes available for binding to specific intracellular receptors. Additionally, binding of HAAs to human steroid-binding proteins can displace endogenous hormones, possibly affecting hormone delivery to target cells. The proteins that transfer chemicals or hormones to or within the fetus are particularly important, because studies have shown that the biologically active concentration of steroid present during fetal life can be misinterpreted if based upon total steroid concentration in the blood.

The principal target organs for HAAs are those that produce, regulate, or respond to hormones or their action. However, there is inadequate information on the concentrations and pharmacokinetics of HAAs in many of these organs in humans and wildlife. There is evidence that biologic barriers, such as the blood-brain barrier and the placenta, which normally protect the brain and developing fetus, respectively, from hydrophilic compounds, do not inhibit the transport of lipophilic HAAs.

There are few studies with target-cell measurements. Molecular changes can indicate uptake in particular targets, and cellular localization of proteins involved in HAA effects could help to identify sensitive target cells. Some fatty tissues accumulate HAAs and can serve as depots for these chemicals. Deposition into eggs or breast milk can occur, thus exposing the fetus or newborn to greater concentrations of HAAs.

Virtually all HAAs are metabolized through a variety of oxidative and reductive or conjugation reactions. The processes involved in metabolism are complex, and a consideration of multiple mechanisms by which HAAs and hormones might act is crucial to evaluating the risk associated with exposure to these compounds. Metabolism can inactivate an HAA or lead to the activation of a hormonally-active metabolite from a non-hormonally active parent compound. The capacity of the enzymes involved in steroid metabolism, such as the cytochrome P450 enzymes, can be modified as a result of exposure to HAAs. The complement of enzymes that metabolize HAAs might strongly influence the susceptibility of the cell to the effects of these compounds. For example, if an HAA requires metabolic action to be estrogenic, then the cells lacking the requisite enzymes are less likely to be susceptible to the estrogenic action of that chemical than are cells that are metabolically active. The expression of cytochrome P450 enzymes during embryonic, larval, or fetal development might be crucial to the developmental effects of exposure to HAAs. However, at this time, there are few data on what forms of these enzymes are expressed at different developmental stages or what their functions are.

There are significant species differences in response to exposure to HAAs, and at this time, it is difficult to extrapolate data from one species to another. Defining structure-activity relationships for substrate binding to homologous enzymes in different species will help to achieve generalization.

Dose-response assessment for HAAs presents a challenge, because HAAs might cause effects not only by acting as reactive molecules that attack biologic macromolecules but also by acting as stable molecules interacting with the body's natural signaling systems. On the basis of a few in vivo investigations conducted to date involving a small number of HAAs, data suggest that in some situations a nonmonotonic dose-response relationship can occur. The issue of the shape of the dose-response curve for HAAs could have implications for toxicity-testing protocols and dose-response assessment methods.

RECOMMENDATIONS

On the basis of its evaluation on the available data on dosimetry, the committee recommends the following:

- For chemicals having certain detrimental effects, studies should be designed to distinguish between direct and indirect effects and between primary and secondary effects of HAAs, and their underlying mechanisms of action should be investigated using both in vitro and in vivo assays that could detect diverse responses.
- Species- and tissue-specific effects of exposure to environmental HAAs need to be investigated further to establish the validity of extrapolation between species.

- In vivo test systems for HAAs should be used to assess the consequences of prenatal and postnatal exposure on developmentally critical or sensitive processes at concentrations commonly found in the environment.
- Dose-response characteristics of recognized actions of various HAAs should be further investigated in in vitro and in vivo studies at concentrations commonly found in the environment.

5

Effects on Reproduction and Development

THE HARMFUL EFFECTS OF exposure to environmental contaminants on reproduction and development in wildlife populations have been reported in the scientific literature for many years. Reported reproductive disorders in wildlife have included morphologic abnormalities, eggshell thinning, population declines, impaired viability of offspring, altered hormone concentrations, and changes in sociosexual behavior.

Laboratory experiments replicating the adverse effects of exposure to the potent synthetic estrogen diethylstilbestrol (DES) during critical periods in development (Newbold 1995; also see Appendix) have focused attention on the potential of chemicals with estrogenic properties to cause developmental and reproductive hazards.

The adverse consequences of prenatal exposure to DES on the female genital tract in humans have been reviewed in detail by Herbst and Bern (1981) and by Mittendorf (1995); they are the subject of continued, intensive investigation. Whether exposure to environmental hormonally active agents (HAAs) affects animals and humans similarly is not clear, but because exposure of animals to DES causes alterations in male and female offspring, the possibility must be considered that there will be adverse effects from exposure to other compounds with estrogenic, antiestrogenic, or antiandrogenic activity. There are also concerns that exposure to low doses of certain chemicals at critical stages in organ development can result in abnormalities that lead to irreversible changes in the functioning of organ systems later in life. Such damage would not occur through genetic mutations, but by processes that regulate genes during development and cell differentiation. The effects of hormones in adults are usually transient, and hormonal effects disappear when the chemical is not present. By contrast, environmental chemicals that alter gene activity during development would produce

effects much harder or impossible to reverse. Evaluating the effects of such chemicals is more difficult than evaluating the effects of chemicals on adults. Effective doses may be lower than effective doses in adults, and the effects are considerably removed in time from the exposure, which can make causal relationships more difficult to establish.

This chapter is a critical analysis of the literature on the link between exposure to HAAs and reproductive and developmental effects observed in laboratory studies, in humans, and in wildlife populations. Only a few (primarily estrogenic) HAAs are covered in this chapter, and evaluations of wildlife are limited to only a few vertebrate species. Although endocrine systems are remarkably well conserved among vertebrate groups, there are significant differences in their operation. While gonadal reversal does not occur in mammals, data from Seveso (Mocarelli et al. 1996) and occupationally exposed cohorts (Goldsmith et al. 1984: Potashnik et al. 1984; Potashnik and Porath 1995) suggest that some selective process may be involved to alter the sex ratio at birth. Hormonal control of sex differentiation is different in birds than it is in mammals, even though the same hormones (for example, estradiol) are involved. There are other important differences in the development of the reproductive systems of various vertebrate groups. Thus, HAAs could affect different vertebrate groups in different ways. For this reason, it is necessary to understand not only the effects of exposure to different HAAs on vertebrate development and reproduction but also the various effects of exposure to single HAAs on reproduction and development in different vertebrate groups.

Laboratory studies are discussed for specific HAAs, including some chemicals known to bind to estrogen receptors: insecticides (dichlorodiphenyl-trichloroethane (*o,p′*-DDT), methoxychlor, and chlordecone); a monomer used in plastic (bisphenol A); an alkylphenol surfactant used in detergents, cosmetics and toiletries, and other household products (octylphenol); and a plasticizer (butyl benzyl phthalate (BBP)). Other compounds are known to bind to androgen receptors: the fungicide vinclozolin and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p′*-DDE), the persistent in vivo metabolite of DDT. Polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) also could disrupt development via several mechanisms. These compounds were selected for review because they are among the most extensively studied HAAs. The work discussed below illustrates the developmental and reproductive effects that can be caused by exposure to these estrogenic, antiestrogenic, and antiandrogenic agents in vivo. The list of compounds discussed in this chapter is by no means complete, and it might not even be representative of all HAAs.

The human studies evaluated here involve exposure to DDE, PCBs, and TCDD. Also evaluated are data on regional and temporal variations in sperm concentration in human populations. There is a discussion of adverse reproductive effects observed in wildlife populations. Where available, laboratory studies related to these findings are presented. The section on wildlife describes effects

of exposure to HAAs in some representative vertebrate species: fish; birds; alligators; turtles; salamanders, frogs, and toads; and the Florida panther.

In evaluating the information, it is essential to examine the data that link reproductive and developmental effects to critical periods of exposure and to concentrations ordinarily found in the environment; however, for the most part this information is not available (see Chapter 1 for more details). In the cases where cause-and-effect relationships can be established or posited, known and suspected toxicologic mechanisms are discussed.

LABORATORY ANIMAL STUDIES OF SELECTED HAAs

Dichlorodiphenyltrichloroethane (DDT)

Immature female rats injected intraperitoneally with a single dose of the estrogenic DDT isomer *o,p′*-DDT at 1 mg/kg had a significant increase in uterine wet weight (Welch et al. 1969), and newborn female rats injected subcutaneously with 1 mg/d *o,p′*-DDT on d 2-4 after birth had early onset of puberty and accelerated loss of fertility, referred to as "delayed anovulatory syndrome" (Heinrichs et al. 1971). Gellert et al. (1974) report that subcutaneous injection of 0.1 mg of *o,p′*-DDT on d 2-4 of life led to marked impairment of fertility and reduced weight of prostate and seminal vesicles in male rats.

Male offspring from pregnant rats fed 100 mg/kg/d *p,p′*-DDE, the antiandrogenic metabolite of DDT, on gestation d 14-18 had reduced anogenital distance and had nipples (androgen normally blocks nipple retention in male rodents) (Kelce et al. 1995). Weanling male rats given daily doses of 100 mg/kg/d *p,p′*-DDE by gavage until d 57 had delayed onset of puberty, and castrated adult rats given daily doses of 200 mg/kg/d *p,p′*-DDE by gavage for 4 d had decreased seminal vesicle and prostate weight (Kelce et al. 1995).

Exposure to DDT during gestation also has been shown to impair locomotor ability in mice (Tilson et al. 1979) and learning in rats (Lilienthal et al. 1990; Lilienthal and Winneke 1991) and monkeys (Schantz and Bowman 1989; Schantz et al. 1989). These studies are discussed in Chapter 8.

The mechanism by which DDT, or DDE, causes structural or functional abnormalities of the reproductive system in laboratory animals is still poorly understood. As discussed in Chapter 2, the DDT isomer *o,p′*-DDT has estrogenic properties; the DDT metabolite *p,p′*-DDE acts as an antiandrogen. Kelce et al. (1995) reported that a concentration of 3.5 μM *p,p′*-DDE occupied 50% of androgen receptors in rat prostate cells. This was approximately 200 times lower than the concentration of *p,p′*-DDE required to occupy 50% of estrogen receptors in rat uterine cells. Soto et al. (1997) also showed that *p,p′*-DDE is a partial agonist of estrogen. They reported that a 10-μM dose of *p,p′*-DDE was required to produce an increase in proliferation of MCF7 human breast-cancer cells, but the increase in proliferation was only 25% of the maximum proliferative response

seen at saturating doses of estradiol. Taken together, these findings show that the capacity for *p,p′*-DDE to bind to and interfere with the functioning of androgen receptors is considerably greater than its capacity to bind to estrogen receptors and stimulate estrogenic responses. The primary activity of *p,p′*-DDE as an HAA is thus as an environmental antiandrogen, not as an environmental estrogen. The antiandrogenic properties of *p,p′*-DDE might be of greater importance than the estrogenic properties of DDT on developing animals. *p,p′*-DDE persists for decades in tissues, whereas estrogenic *o,p′*-DDT is much less commonly detected in human serum (Stehr-Green 1989). However, exposure to *o,p′*-DDT, as well as to other nonpersistent pesticides (methoxychlor, for example), during critical periods in development affects fetal development in mice, and some effects, such as changes in territorial behavior, become apparent only in adulthood (vom Saal et al. 1995).

Methoxychlor

Methoxychlor is an insecticide used in home gardens and on crops and livestock (ATSDR 1994). The effects of methoxychlor on the reproductive systems of female rats and mice have been studied extensively (Cummings 1997). Methoxychlor causes adverse effects on fertility, early pregnancy, and in utero development. Accelerated pubertal ovulation, persistent vaginal cornification, accelerated loss of fertility, and abnormal cell types in the uterus and oviducts have been observed after neonatal administration of doses as low as 0.5 μg/d per neonate (Welch et al. 1969; Gellert et al. 1974; Gray et al. 1989; Eroschenko and Cooke 1990; Gray 1992). When administered to mated female rats during the peri-implantation period, a 300-mg/kg/d (approximately 100 mg/d) dose of methoxychlor completely blocked implantation of embryos (Cummings and Gray 1989; Gray et al. 1989; Cummings 1990).

Exposure to methoxychlor during development also leads to changes in the reproductive system and behavior of male rats and mice. Male offspring of mice fed 20 μg/kg/d methoxychlor in oil during the last third of pregnancy exhibited an increase in territorial marking behavior in adulthood, similar to the effect observed with a 20-μg/kg/d dose of *o,p′*-DDT and a 20-ng/kg/d dose of DES (vom Saal et al. 1995). Daily intraperitoneal injection of 1 mg of methoxychlor to male mice during the first week after birth led to reduced serum testosterone concentrations and to reduced DNA content in prostate and seminal vesicles in adulthood (Cooke and Eroschenko 1990). Administration of 50 mg/kg/d methoxychlor to female rats throughout pregnancy and lactation resulted in smaller testes, epididymides, and reduced sperm count in male offspring (Gray et al. 1989; Gray 1992).

The mechanism by which methoxychlor affects the reproductive system and reproductive behavior of laboratory animals is not understood. Methoxychlor has estrogenic effects in vivo only after demethylation in the liver to mono-

hydroxy-methoxychlor (30% of administered dose) or bis-hydroxy-methoxychlor (23% of administered dose) (Kapoor et al. 1970); the more potent estrogenic metabolite is bis-hydroxy-methoxychlor (Welch et al. 1969; Bitman and Cecil 1970; Bulger et al. 1978; Bulger and Kupfer 1983). Unlike *p,p′*-DDE, methoxychlor is not persistent in vivo (most is cleared within 24 h), although it is relatively persistent (a number of months) in soil (Muir and Yarechewski 1984; ATSDR 1994).

Chlordecone

Chlordecone [decachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta(cd)pentalen-2-one] was used in the 1960s and 1970s to control insect pests of bananas, citrus trees without fruit, tobacco, and ornamental shrubs (ATSDR 1995). The insecticide mirex, which is similar in structure to chlordecone, has been used in much greater quantities to combat fire ants.

When a 15-mg/kg/d dose of chlordecone was fed to pregnant rats on d 14-20 of gestation, 12 of 21 female offspring developed persistent vaginal estrus; the other nine rats were anovulatory at 6 mo of age (Gellert and Wilson 1979). The effects were consistent with those seen after exposure to estrogen, but no estrogenic effects were observed among male offspring. In a study of postnatal exposure, constant vaginal estrus was induced in mature female rats fed 1.5 mg/kg/d chlordecone for 7 d (Hammond et al. 1979). Mature female mice injected with 125 μg/d chlordecone on postnatal d 1-10 developed complete cornification of the vaginal epithelium—this was similar to the effects caused by treatment with 10 μg/d estradiol (Eroschenko and Palmiter 1980). In males, spermatogenesis was completely suppressed by estradiol and was reduced by chlordecone. Injection of either 0.2 or 1 mg/kg/d chlordecone into newborn female rats on d 2 and 3 of life led to early onset of puberty and accelerated loss of cyclicity (Gellert 1978a).

In a study using quail, Eroschenko (1981) fed male quail a diet containing 200 ppm chlordecone for 3 wk and reported a significant increase in testes weight, due to edema, with dilation of the seminiferous tubules and erosion of the germinal epithelium. Abnormal sperm was also observed. With exposure for 6 wk, the testes in some animals began to atrophy.

The binding affinity of chlordecone to the estrogen receptor is approximately 0.02% (5,000-fold) lower relative to estradiol (Eroschenko and Palmiter 1980).

Vinclozolin

Vinclozolin (3-(3,5-dichlorophenyl)-5-methyl-5-vinyl oxazolidine-2,4-dione) is a dicarboximide fungicide widely used to combat damage to a variety of commodities, such as fruits, vegetables, hops, and turf. When vinclozolin was administered via gavage to pregnant and lactating rats from gestation d 14 through

postnatal d 3 at 100 or 200 mg/kg/d, male offspring were indistinguishable from female offspring on external examination at birth, and the males retained nipples (Gray et al. 1994). These findings indicate that the masculinizing effects of androgen on the external genitalia and the defeminizing action of androgen on the development of nipples were blocked by vinclozolin. In adulthood, due to gross abnormalities of the internal and external genitalia, the vinclozolin-treated males were infertile.

Vinclozolin is an androgen receptor antagonist (Kelce et al. 1994). After ingestion, it is degraded to metabolites that compete with endogenous androgen for binding to androgen receptors.

Polychlorinated Biphenyls (PCBs)

PCBs are no longer legally manufactured in the United States, but large quantities were produced for use in such products as electrical transformers and capacitors. Some of the more highly chlorinated of the 209 potential PCB congeners are highly persistent, and they bioaccumulate in the food chain.

Rats exposed to PCB mixtures early in life can develop reproductive effects similar to those caused by DES exposure (Bitman and Cecil 1970; Sager 1983; Sager et al. 1987, Subramanian et al. 1987; Lundkvist 1990; Jansen et al. 1993; Bergeron et al. 1994; Birnbaum 1994; Gray et al. 1995; Li and Hansen 1996). Male offspring of rats fed 32 or 64 mg/kg/d PCBs during lactation had significantly reduced seminal vesicle weight and significantly larger testes (Sager 1983; Sager et al. 1987). Those effects might be due to changes in thyroid hormone levels (Jannini et al. 1993). When treated males were mated with unexposed females, there was a significantly lower incidence of implantation, a significantly lower number of live births, and a significantly greater rate of resorption. Female offspring of dams exposed to 32 or 64 mg/kg/d PCBs during lactation had delays in puberty, vaginal opening, and first estrus (Sager and Girard 1994). At maturity, uterine wet weight was reduced at all stages of the estrous cycle, and fertility was impaired because of reduced success at the pre- or postimplantation stage. Female rats treated intraperitoneally with PCBs also had significant increases in uterine weight (Jansen et al. 1993).

When female guinea pigs were force-fed 2.2 mg/d (1.8-3.2 mg/kg/d) PCBs during gestation, female offspring had delayed vaginal opening and male offspring had significantly reduced absolute and relative testis weight (Lundkvist 1990).

Pre- and postnatal exposure to coplanar PCBs can modulate thyroid hormone concentrations and uptake. When pregnant rats were orally administered 0.2, 0.6, or 1.8 mg/kg/d HCB (3,3′,4,4′,5,5′-hexachlorobiphenyl) or a combination of 1 mg/kg/d TCB (3,3′,4,4′-tetrachlorobiphenyl) and 0.6 mg/kg/d HCB, there were decreases in fetal, neonatal, and weanling plasma total thyroxine (T_4) and free T_4 concentrations, which indicated an increase in peripheral T_4 metabolism (Morse

et al. 1993). An increase in the activity of type II thyroxine 5′-deiod™ase in brain homogenates also was observed, which suggests that local hypothyroidism occurs in the brains of fetal and neonatal rats exposed to these PCBs. The PCB mixture Aroclor 1254, administered orally at doses of 5 or 25 mg/kg/d to pregnant rats on d 10-16 of gestation, caused the selective accumulation of a hydroxylated PCB metabolite (2,3,3′,4,5′-pentachloro-4-biphenylol) in fetal plasma and brain; this was believed to be the cause of the reductions of fetal plasma and brain T_4 concentrations (Morse et al. 1996).

In a reproductive toxicity study of rhesus monkeys, 80 females were fed 5-80 μg/kg/d Aroclor 1254 prior to breeding, during breeding (with untreated males), and after breeding, for 6 yr. There was a significant dose-related decreasing trend in conception rate and a significant dose-related increasing trend in fetal mortality (Arnold et al. 1995). Maternal age was not a confounding factor. It was noted during this study that while some of these animals had endometriosis the incidence or severity of the lesions could not be related to the PCB treatment. Similarly, a recent study in women led to the conclusion that exposure to PCBs and to chlorinated pesticides is not associated with endometriosis in the general population (Lebel et al. 1998).

In addition to reproductive effects, prenatal exposure to PCBs has been shown to cause deficits in neurodevelopment, such as impaired learning and altered activity levels in rats fed PCB-contaminated fish (Tilson et al. 1990; Daly 1992). These studies are described in greater detail in Chapter 6.

As discussed in Chapter 2, there is evidence that PCB mixtures and congeners and hydroxy-PCBs can have estrogenic and antiestrogenic properties. In addition, coplanar PCBs are antiestrogenic through aryl hydrocarbon-estrogen receptor crosstalk (Krishnan and Safe 1993). However, the significance of results from laboratory studies of PCBs are difficult to interpret because most PCB extracts from environmental samples do not resemble commercial PCB mixtures used in laboratory studies (WHO 1993). As PCBs are cycled through the environment, the various congeners are gradually redistributed. The most chlorinated congeners, which are typically the most toxic, accumulate preferentially.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

TCDD is a byproduct of the production of chlorinated products such as herbicides and wood preservatives, the incineration of trash containing papers and plastics, and the burning of fossil fuels (e.g., IARC 1997). A series of studies (Mably et al. 1992a,b,c) has shown effects on male rats whose mothers were given single oral doses of TCDD ranging from 0.064 μg/kg to 1 μg/kg on gestation d 15. At doses as low as 0.16 μg/kg, impaired sexual differentiation was observed in male fetuses, including a decrease in circulating testosterone and in anogenital distance at birth (Mably et al. 1992a). Effects on sexual behavior in male offspring in adulthood included changes in mounting, intromitting, number

of ejaculations, and latency to ejaculation, as well as in the exhibition of the female sexually receptive posture (lordosis) (Mably et al. 1992b). In addition, a dose-related decrease in the weight of the testis and epididymis was observed. There was also a decrease in daily sperm production in male offspring of pregnant rats receiving a single dose of 1 μg/kg, although there was no effect on fertility (Mably et al. 1992c).

Maternal exposure to a single oral dose of 1 μg/kg TCDD on gestation d 8 or 15 caused delayed puberty, partial clefting of the penis, and "thread" tissue development across the opening of the vagina in female offspring of rats (Gray and Ostby 1997). Ovarian weight was significantly reduced. In male offspring, the same treatment on gestation d 15 caused delayed puberty and reductions in ejaculated and epididymal sperm counts and in sex accessory gland size (Gray et al. 1995).

Chronic oral exposure to TCDD caused endometriosis in rhesus monkeys, with incidence and severity related to dose (Rier et al. 1993). Specifically, adult female monkeys were administered 2.5×10^{-7} and 1.25×10^{-6} mg/kg/d in their food over 4 yr. Ten years later, moderate to severe endometriosis was found during laparoscopic examination in three of seven monkeys exposed to 5 ppt TCDD and in five of seven monkeys exposed to 25 ppt TCDD. None of the control monkeys showed severe disease.

The mechanism by which TCDD causes reproductive impairments and affects reproductive behavior is poorly understood. Because outcomes of prenatal TCDD exposure in rats appear similar to effects seen after treatment with DES (Peterson et al. 1993; Eskenazi and Kimmel 1995; Gray et al. 1995), the general assumption that TCDD acts as an estrogen antagonist might not apply to all effects of TCDD. In contrast, studies have shown that TCDD causes antiestrogenic responses in the rodent uterus (Gallo et al. 1986; Romkes et al. 1987; Umbreit et al. 1988, 1989; Astroff and Safe 1990; DeVito et al. 1992) and in breast cancer cells (Biegel and Safe 1990; Harris et al. 1990; Safe et al. 1991; Gierthy et al. 1993; Merchant et al. 1993) and inhibits mammary tumor growth in rodents (Kociba et al. 1978; Gierthy et al. 1993; Holcomb and Safe 1994; Tritscher et al. 1995). TCDD causes antiestrogenic effects via the aryl hydrocarbon (Ah) receptor signaling pathway, which has been characterized at the molecular level (Krishnan et al. 1995; Gillesby et al. 1997).

Bisphenol A

Bisphenol A (4,4′-(1-methylethylidene)bisphenol) is a monomer used in the manufacture of polycarbonate plastic, resins, and some dental sealants; it is an additive in numerous other products. In a developmental toxicity study with pregnant rats and mice (Morrissey et al. 1987), gastric intubation of 1,250 mg/kg/d bisphenol A on d 6-15 of gestation significantly decreased maternal body weight, increased maternal mortality and resorption of fetuses, and decreased the

weight of surviving pups in mice but not in rats. There were no observable malformations in exposed fetuses.

In a subsequent study that used much lower doses of bisphenol A (Nagel et al. 1997), male offspring of pregnant mice fed 2 μg/kg/d bisphenol A on d 11-17 of gestation had significantly increased prostate weight as adults. In addition, 2 μg/kg/d bisphenol A produced significant enlargement of the preputial glands in male offspring, whereas the epididymides were significantly reduced. A dose of 20 μg/kg/d bisphenol A reduced daily sperm production per gram of testis by 20%, while daily sperm production uncorrected for testis weight was not significantly different (vom Saal et al. 1998). The investigators suggested that these doses could be within the range encountered by humans, as evidenced by amounts detected in the saliva of 18 patients (amounts ranged from 90 to 931 μg) treated with dental sealants (Olea et al. 1996) and in a few lacquer-coated cans of vegetables (amounts ranged from 0 to 23 μg/can) (Brotons et al. 1995). Bisphenol A does not bind to plasma-binding proteins or other components of blood to the same degree that estradiol does, and therefore, it passes more readily from blood into cells (Nagel et al. 1998). The inhibition of the uptake of steroids into cells by components of blood is particularly important during fetal life in rats, when the concentrations of gonadal and adrenal steroids are great but the bioactive fraction of steroid that can enter cells is maintained at a low concentration (vom Saal et al. 1992).

Effects of bisphenol A are seen in vitro in human breast-cancer MCF7 cells at 10^{-8} M (10 nM) or 2.3 ppb (molecular weight, 228) (Krishnan et al. 1993; Olea et al. 1996), and in F344 rat prolactin-secreting GH3 pituitary cells at 1 nM (Steinmetz et al. 1997). The finding of increased prostate size in male offspring of pregnant female mice fed 2 μg/kg of bisphenol A (Nagel et al. 1997) is similar to that found in tests with DES at a dose of 0.02 μg/kg (vom Saal et al. 1997), which shows that bisphenol A is approximately 100-times less potent than DES when fed to pregnant mice. The same 2 μg/kg dose of bisphenol A fed to pregnant female mice also advanced the timing of puberty in female offspring, an effect seen with higher doses of other estrogenic chemicals (Howdeshell et al. 1999).

In another study (Steinmetz et al. 1997), estradiol and bisphenol A were administered to F344 and Sprague-Dawley rats via subcutaneous Silastic capsules, and serum prolactin, which is elevated by estrogen treatment, was measured (along with numerous other responses). Bisphenol A induced hyperprolactinemia in F344 rats but not in Sprague-Dawley rats. In the F344 rat, estradiol increased serum prolactin concentrations 10-fold and bisphenol A increased it 7- to 8-fold over controls. These findings are consistent with the findings of vom Saal et al. (1997) comparing bisphenol A and DES in fetal mice. Those findings suggest that bisphenol A is bioactive within the range of human exposure (Walent and Gorski 1990; Krishnan et al. 1993; Brotons et al. 1995; Olea et al. 1996; Takao et al. 1999).

Recently, Cagen et al. (1999) conducted a study to evaluate the effects of low doses of bisphenol A on sexual development in male mice. To the extent possible, the study protocol duplicated the studies of Nagel et al. (1997) and vom Saal et al. (1998) for all factors indicated as critical by those investigators. Some differences between the two studies include the source of the CF_1, mice, the number of doses of bisphenol A, the methods used to determine sperm count, the age of mice at necropsy, and the way the animals were housed. An additional positive control group of mice was dosed with 0.2 µg/kg/d DES. No effects on testes histopathology, daily sperm production or sperm-production efficiency (i.e., daily sperm production per gram of testis), or on prostate, preputial, seminal vesicle, or epididymis weights were observed in the bisphenol A treated groups. In addition, no adverse effects were observed in any of these parameters in the group tested with DES. Thus, this study failed to replicate the results of vom Saal et al. (1997, 1998) and Nagel et al. (1997). The reason for the discrepancies in the findings is a subject of controversy and cannot be resolved at this time.

Octylphenol

Octylphenol (*p*-(1,1,3,3-tetramethylbutyl)phenol) is an alkylphenol used in its ethoxylated form (octylphenol ethoxylate) in a variety of products such as detergents and plastics. In one study (Sharpe et al. 1995), female rats were given drinking water containing 100 or 1,000 µg/L octylphenol before pregnancy, during gestation, and throughout lactation, and the effects on testicular size and spermatogenesis in male offspring in adulthood were investigated. Intake of octylphenol based upon water intake was calculated only for the group exposed to 1,000 µg/L and was reported to range from 129 µg/kg/d in the first 2 d after birth to 367 µg/kg/d just before weaning. There were significant decreases in absolute testis weight, in the ratio of testis-to-kidney size, in relative ventral prostate weight, and in daily sperm production in male offspring exposed to 1,000 µg/L octylphenol, and there was a significant reduction in relative prostate weight at both concentrations. When male offspring were treated postnatally only with 1,000 µg/L octylphenol on d 1-22 after birth, there was a significant reduction in average and relative testis weight (Sharpe et al. 1995). More recently, the authors reported their inability to replicate their findings (Sharpe et al. 1998). While they expressed continued confidence in their original publication, they hypothesized that the inability to replicate the work may have been due to changed biologic factors of which they were unaware and unable to control (Sharpe et al. 1998). In another study, there was no effect on prostate weight of male offspring of pregnant mice fed 2 and 20 µg/kg/d octylphenol on d 11-17 of gestation (Nagel et al. 1997), although there was a significant decrease in daily sperm production (vom Saal et al. 1998).

Butyl Benzyl Phthalate (BBP)

BBP is used as a plasticizer for cellulose resins, polyvinyl acetates, polyurethanes, and polysulfides and in regenerated cellulose films for packaging. When 1,000 μg/L BBP was administered in drinking water to female rats before mating and throughout lactation (nominal intake based on water intake ranged from 126 μg/kg/d in the first 2 d after birth to 366 μg/kg/d just before weaning), there was a small but significant reduction in mean testicular size and a reduction in daily sperm production in male offspring at d 90-95 (Sharpe et al. 1995). Similar findings were reported in rats treated with DES (100 μg/L in drinking water; intake was not determined), which was evaluated in the same study. The researchers note that, although these estrogenic chemicals exert similar effects on testis size and daily sperm production, there is no evidence that the effects are caused by the compounds' estrogenicity.

In a subsequent study, Ashby et al. (1997) failed to find any effects of BBP, despite very similar or identical protocols. Female rats were administered BBP (average intake 182.6 μg/kg/d) in drinking water during gestation and lactation, and their offspring were monitored for 90 d. DES (8.6 μg/kg/d) affected the sexual development of male and female pups, causing changes in anogenital distance; average day of vaginal opening and prepuce separation; weight of the uterus, testis, and accessory sex glands; and caudal epididymis sperm count and homogenization-resistant testicular sperm count. BBP had no effect other than to cause a slight advance in the average day of vaginal opening and a small increase in male anogenital distance, but those effects were attributed to the increased weight of the pups treated with BBP.

The reason for the discrepancy in findings between the study by Sharpe et al. (1995) and that of Ashby et al. (1997) is unknown.

Di-*n*-Butyl Phthalate

Di–*n*-butyl phthalate (DBP) has been characterized as a reproductive and developmental toxicant in several studies (e.g., Cater et al. 1977; Ema et al. 1994, 1995), causing fetal death and skeletal malformations (predominantly cleft palate) in rats. In addition, DBP has been shown to cause testicular atrophy, early sloughing of germ cells, and vacuolization of Sertoli cell cytoplasm (Cater et al. 1977; Fukuoka et al. 1989). Immature rats appear to be more susceptible to these effects than adult rats (Gray and Gangolli 1986; Creasy et al. 1987). Recent studies have investigated the effects in more depth.

DBP was tested in the National Toxicology Program's Reproductive Assessment by Continuous Breeding protocol using Sprague-Dawley rats (Wine et al. 1997). DBP was administered in the diet to male and female rats continuously, with an average daily intake of 52, 256, and 509 mg/kg for males and 80, 385, and 794 mg/kg for females, respectively. Breeding pairs (F_0 generation) were mated

for an extended period, sufficient to produce five litters (F_1 generation). The last litter was raised to adulthood and allowed to mate and produce offspring (F_2 generation). In the F_0 generation, the only adverse reproductive effects observed were a 5-17% reduction in the number of live pups per litter and a decrease (<13%) in live pup weights. When crossover matings were conducted to determine the affected sex, the number of offspring was unchanged, but pups from treated females weighed significantly less, whereas offspring from treated males were unchanged. At necropsy, the high-dose females had a 14% reduction in body weight, and both males and females had a 10-15% increase in kidney and liver to body weight ratios compared with controls.

In the F_1 generation, indices of mating, pregnancy, and fertility in the high-dose group were all significantly affected. Specifically, only one live litter was delivered from 20 breeding pairs. In all dose groups, weights of the F_2 pups were 6-8% lower than controls. At necropsy, the high-dose F_1 males were found to have significantly reduced epididymal sperm counts and testicular spermatid head counts. Eight of the 10 males had degenerated seminiferous tubules and five had underdeveloped or otherwise defective epididymides. No adverse effects on ovarian or uterine development were observed in the F_1 females. The investigators concluded that DBP is a reproductive and developmental toxicant to both adult and developing rats and that DBP had greater effects on the second generation than the first generation.

Mylchreest et al. (1998) showed that similar effects on the male reproductive tract could be produced with much shorter gestational and lactational exposure. Pregnant CD rats were given DBP at 250, 500, or 750 mg/kg/d throughout pregnancy and lactation until their offspring were 20 d old. Anogenital distance was decreased in the male offspring of the mid- and high-dose groups. In adulthood, the epididymis was underdeveloped or absent in 9%, 50%, and 71% of the males of the low-, mid-, and high-dose groups, respectively. Testicular atrophy and widespread germ-cell loss were also found. Hypospadias was observed 3%, 21%, and 43% of males, and the testes were abnormally positioned or absent in 3%, 6%, and 29% of the males in the low-, mid-, and high-dose groups, respectively. In addition, small testes and seminal vesicles were found, and in some cases, the prostate glands and seminal vesicles were missing. The investigators concluded that DBP specifically impaired the androgen-dependent development of the male reproductive tract, suggesting that DBP is antiandrogenic rather than estrogenic. The investigators caution that it will be important to determine whether the reproductive toxicity of DBP is metabolite-mediated, because marked species differences in metabolism exist.

Earlier studies with DBP (Ema et al. 1993), in which exposures were conducted during organogenesis, revealed no evidence of selective effects on the developing reproductive system. Foster (1997) suggests that the difference in developmental outcomes was that the developmental toxicity study (Ema et al. 1993) did not include exposure during the critical period of sexual differentiation,

which occurs after gestation d 15. Recently, Ema et al. (1998) reported data that support this. They showed that when pregnant rats were fed DBP on d 11-21 of pregnancy at an average daily intake of 555 and 661 mg/kg, there was a significant incidence of undescended testes and a significant decrease in anogenital distance in male fetuses.

HUMAN STUDIES

DDT and its Metabolites

In a study of 722 women from North Carolina, Rogan et al. (1987) reported significantly shortened duration of lactation in relation to increasing breast milk concentration of the antiandrogenic DDT metabolite *p,p′*-DDE. Median duration of lactation decreased from 7.8 mo in the lowest exposed group (0-2.5 ppm *p,p′*-DDE in milk fat) to 3.8 mo in the most exposed group (10.0-12.5 ppm *p,p′*-DDE in milk fat). Adjustment for possible confounders (i.e., mother's age, race, education, occupation, smoking, and drinking) did not change these findings. Another study examined 229 women in Tlahualilo, Mexico, where DDT is used extensively for agricultural purposes (Gladen and Rogan 1995). Exposure measures were based on *p,p′*-DDE concentrations in breast-milk samples collected at the time of birth. As in the North Carolina study, median duration of lactation was 7.5 mo in the group with the least contaminated milk (0-2.5 ppm *p,p′*-DDE in milk fat) and 3 mo in the group with the most heavily contaminated milk (≥12.5 ppm *p,p′*-DDE in milk fat). The trend was more marked among women who had breast fed children from previous pregnancies. Median duration of lactation was 8.8 mo in the least contaminated group and 2.8 mo in the most contaminated group. This cohort is being studied to determine whether there is any relationship between prenatal exposure to DDEs and adverse effects on lactation and possibly on reproductive development.

Wasserman et al. (1982) compared serum levels of DDT and its metabolites in 10 women from normal term pregnancies and 17 with premature delivery. The mean DDT serum level in women with premature deliveries (71.1 ppb) was significantly higher than that for normal controls (26.5 ppb, $p < 0.005$). The percentage of *o,p′*-DDT was unusually high in the case group.

PCBs

Several studies of the effects of consuming PCB-contaminated fish have been conducted on a variety of reproductive parameters. One of the best studied populations has been the New York State Angler Cohort, a population-based cohort of angler families from 16 counties in New York near Lake Ontario (Vena et al. 1996). Questionnaires regarding duration (number of years) and monthly frequency of consuming sport fish were completed by this cohort in 1991, and a

PCB-exposure index was calculated (a crude estimate of potential lifetime PCB exposure through fish consumption). In one study (Mendola et al. 1997), the effects of fish consumption on menstrual-cycle length was evaluated using telephone interviews conducted in 1993 with 2,223 women from the cohort. Multiple regression analyses revealed a significant reduction in the menstrual cycle length of 1.11 d with consumption of more than one fish per meal per month and with moderate to high (>1 mg) estimated PCB index. Frequency of consumption and PCB index appeared to have a stronger relation with cycle length than the number of years of consumption. Those data must be interpreted cautiously because of the limitations of secondary data analysis, including the lack of information on potential confounders. The researchers concluded that "While the small decreases in menstrual cycle length observed are not likely to be clinically relevant or of major public health concern per se, they may indicate potential endocrine effects on a population level."

Another study of the New York State Angler Cohort investigated the effects on fish consumption on time-to-pregnancy (TTP) (Buck et al. 1997). Telephone interviews were conducted in 1993 with 2,445 women who stated upon enrollment in the cohort that they were considering pregnancy. Among the 1,234 women who reported being pregnant, 874 had a known TTP. Multiple regression analyses of the data indicated that duration of fish consumption explained virtually none (0.5%) of the observed variance in TTP among all women with a known TTP, even after the analysis was restricted to women who reported eating fish. Thus, consumption of contaminated sport fish did not appear to have a detrimental effect on TTP.

The risk of spontaneous fetal death has also been studied in 1,820 multigravid women from the New York State Angler Cohort in relation to sport-fish consumption (Mendola et al. 1995). The reproductive histories of the women were obtained from the most recent New York State live-birth certificates found on a computerized registry. No significant increases in risk for fetal death were found in regard to lifetime estimates of PCB exposure based on species-specific PCB levels, the number of years of fish consumption, kilograms of sport fish consumed between 1990-1991, and a lifetime estimate of kilograms eaten.

Studies are being conducted to determine the effects of fish consumption on the reproductive health of a cohort of Michigan anglers (Courval et al. 1996a). In one study, the effects of fish consumption on conception is being evaluated in married couples, with conception failure being defined as inability to conceive after 12 mo. Preliminary results from 626 couples show that 15% of both men and women reported conception failure (Courval et al. 1996b). The unadjusted odds ratio for conception failure across three increasing levels of fish consumption compared with no fish consumption was 1.2, 1.3, and 2.0 for men (trend test $p = 0.06$) and 0.9, 1.0, and 1.4 for women (trend test $p = 0.35$). After adjusting for covariates, the odd ratios for conception were 1.4, 1.8, and 2.8 for men and 0.8, 0.8, and 1.0 for women. The investigators report that these data suggest a modest

association between sports-fish consumption and the risk of conception failure for men only but these preliminary results are consistent with the frequency of infertility in the population in general (OTA 1988).

Other studies have been conducted to determine whether body burdens of PCBs could be linked to adverse effects on reproduction. For example, in a study in Rome, Italy, between 1983-1984, concentrations of PCBs in blood were measured in 120 women who had miscarried and compared with 120 controls (Leoni et al. 1989). Concentrations of tetra- and penta-isomers of PCBs, measured as Fenclor 54, were significantly greater in the blood of women who had miscarried than in controls: 8.65 ppb vs. 6.89 ppb ($p < 0.05$). Although this study shows a significant correlation between PCBs and miscarriage, it is only the first study to have examined this association, and further work is needed on the subject.

In a study of 170 men in which semen samples were analyzed for 74 PCB congeners (Bush et al. 1986), no association was found between PCB congener concentration and sperm count, motility, or percent normal forms. However, in samples with a low sperm count (<20 mil/mL), sperm motility was inversely associated with 3 PCB congeners (2,4,5,2′,4′,5′-hexachlorobiphenyl, $p = 0.002$, SE = 26; 2,4,5,3′,4′- pentachlorobiphenyl, $p = 0.002$, SE = 20; and 2,4,5,2′,3′,4′-hexachlorobiphenyl, $p < 0.01$, SE = not provided).

Two episodes of accidental exposure to PCB-contaminated rice oil occurred in Yusho, Japan (1968), and Yu-Cheng, Taiwan (1978-1979). In Yusho, pregnant women exposed to contaminated oil in 1968 delivered infants with "fetal PCB syndrome." The signs included dark pigmentation of the skin and mucus membranes, gingival hyperplasia, exophthalmic edematous eye, dentition at birth, abnormal calcification of the skull, rocker bottom heel, and low birth weight (Yamashita and Hayashi 1985). A similar accident with PCBs and polychlorinated dibenzofurans (PCDFs) occurred in Yu-Cheng, Taiwan, in 1978-1979, and fetal PCB syndrome and an increased mortality rate were observed among infants of exposed women (Hsu et al. 1985; Rogan et al. 1988). Most of the affected infants were found to be shorter and had less total lean mass and soft-tissue mass than did children in a matched control group. The effects were observed only in the first children born after maternal exposure and not in subsequent children (Guo et al. 1994, 1995a). In addition, a preliminary study on sexual development was conducted on a matched cohort of 55 pairs of Yu-Cheng exposed and unexposed boys in 1993 (Guo et al. 1995b). The authors reported that exposed boys 11-14 yr had significantly shorter penis length. However, no data were provided to support this conclusion nor is it known if they corrected for the smaller body size of the children. Neurodevelopmental effects, such as delays in cognitive, psychomotor, and behavioral development, have been observed. The effects of pre- and postnatal exposure to PCBs on neurodevelopment are discussed in detail in Chapter 6.

Several developmental studies have also been conducted in the Great Lakes region of the United States of prenatal exposure to PCBs as a result of maternal

consumption of contaminated sport fish. In the Michigan/Maternal Infant Cohort Study, Fein et al. (1984) evaluated the birth size and gestational age of 242 infants and found that maternal consumption of fish and concentrations of PCBs in cord serum were correlated with lowered birth weight, shortened gestation, and smaller head circumference. Lower weight was also observed in children from this cohort at 4 yr in a dose-dependent fashion (Jacobson et al. 1990). Children with cord serum PCB levels of 5.0 ng/mL or more weighed 1.8 kg less on average than the lowest exposed children. Prenatal exposure was also associated with deficits in neurologic development in follow-up studies of these children at up to 11 yr (Jacobson et al. 1992; Jacobson and Jacobson 1996). The details of these neurodevelopmental studies are presented in Chapter 6.

In a study of 94 Inuit infants from Hudson Bay whose mothers had high concentrations of PCBs in their breast milk, no significant differences were found between male and female newborns for birth weight, head circumference, or thyroid-stimulating hormone level. There was a negative association between male height at birth and concentration of PCBs (r values ranged from –0.23 to –0.41, p values ranged from nonsignificant to 0.04), hexachlorobenzene ($r = –0.41$, $p = 0.006$), mirex ($r = –0.34$, $p = 0.02$), and toxicity equivalency factors (TEQs) of chlorinated dibenzodioxins/chlorinated dibenzofurans ($r = –0.48$, $p = 0.04$) in milk fat. On the other hand, there was a positive association between female birth height and TEQs coplanar PCBs ($r = 0.47$, $p = 0.05$) and TEQs polychlorinated dibenzo-*p*-dioxins/polychlorinated dibenzofurans ($r = 0.49$, $p = 0.04$) (Dewailly et al. 1993b).

Occupational studies in New York of offspring of women exposed to PCBs during the manufacture of capacitors found a significant relation between increased maternal serum PCB levels and decreased birth weight and gestational age (Taylor et al. 1984, 1989). The decrease in birth weight was found to be at least partially related to the shorter gestational age. The investigators concluded that the magnitude of the effects was small compared with other known determinants of gestational age and birth weight, and the biologic importance of these effects was likely to be negligible.

In the North Carolina Breast Milk and Formula project, 912 children were followed to investigate the effects of prenatal exposure to environmental levels of PCBs and DDE from maternal breast milk (Rogan et al. 1986a). No relationship was found between birth weight, head circumference, and neonatal jaundice with PCB or DDE levels in milk fat. Follow-up studies of the neurodevelopment of these children have found evidence of neurobehavioral deficits that persist up to 2 yr (Gladen et al. 1988; Rogan and Gladen 1991). See Chapter 6 for the details of these neurobehavioral studies.

In an ongoing study of perinatal exposure to PCBs in the Netherlands, the birth weight and growth of 105 breast-fed infants was compared with that of 102 formula-fed infants. The investigators reported that prenatal exposure to PCBs, as measured in cord plasma, had a significant negative effect on birth weight and

on growth from birth to 3 mo of age, but not thereafter. Postnatal exposure to PCBs and dioxin, as measured in breast milk, had no effect on growth (Patandin et al. 1998). Neurologic development in this cohort of children was found to be adversely affected in some studies (Huisman et al. 1995a; Pantandin et al. 1999) but not in others (Koopman-Esseboom et al. 1996; Lanting et al. 1998). The varied results of these studies are presented in more detail in Chapter 6.

Collectively, the data on prenatal exposure to high levels of PCBs from the Yusho and Yu-Cheng incidents and studies from the United States and the Netherlands of prenatal exposure to PCBs from maternal diet indicate that PCBs can affect birth weight and growth. Lower birth weights were not observed in the North Carolina cohort, but that might be explained by the lower exposure level of this cohort compared with the others. In addition, the Yu-Cheng children were also exposed to PCDFs, and exposure to those compounds was not noted in the other cohorts.

TCDD

In 1976, an industrial accident in Seveso, Italy, released kilograms of TCDD, the most toxic of the dioxins. Investigators are conducting studies to determine whether exposure to TCDD has caused reproductive effects. Mocarelli et al. (1996) evaluated the sex ratio among children born to heavily exposed residents from 9 mo after the accident until the end of 1984 (corresponding to one TCDD half-life in adults). Among 74 births, there was a significant deficit in the number of males (26 males vs. 48 females; χ^2 test; $p < .001$). Serum samples collected and stored from families who resided in the most heavily exposed area were analyzed after 1988. There were no males among the 12 births to the 9 parents with the greatest TCDD exposure (104-2,340 ppt in serum lipid). The exposed population in Seveso is still under study. This TCDD-associated altered sex ratio may or may not be related to recent reports of similar declines in sex ratio between the 1950s and the 1960s from the Netherlands (van der Pal-de Bruin et al. 1997) and Denmark (Moller 1996), and in Canada since 1970 (Allan et al. 1997). However, the causes of the declines in sex ratio is yet unknown.

A toxicokinetic analysis (Bois and Eskenazi 1994) using TCDD concentrations in blood from 19 highly exposed residents of Seveso showed that exposures for some of the residents were greater than were the exposures shown to cause endometriosis (5 and 25 ppt) in rhesus monkeys (Rier et al. 1993). Whether the exposures encountered in Seveso will result in an increased incidence of endometriosis is under study.

A recent epidemiology study on TCDD exposure and cancer risk in Seveso women concluded that there was a very low incidence of breast and endometrial cancers (Bertazzi et al. 1993). That study is described in more detail in Chapter 9.

Because alterations in circulating thyroid hormones in newborns might influence the maturation of the central nervous system and could thus have conse-

quences for psychomotor development (Birrell et al. 1983), thyroid hormone concentrations were measured in the blood of Dutch infants exposed to elevated concentrations of dioxin in breast milk (Pluim et al. 1993). Breast-milk contamination is the result of maternal consumption of contaminated meat, dairy products, and fish oils. The total dioxin concentration in milk fat from 38 healthy mother-infant pairs was calculated as the sum of the toxic equivalence (TEQ) relative to TCDD of the 17 most toxic congeners (7 dioxins and 10 dibenzofurans). The mothers were divided into two groups based on the median dioxin concentration: low exposure (8.7-28.0 ng TEQ/kg milk fat; mean 18.6 ng TEQ/kg milk fat) and high exposure (29.2-62.7 ng TEQ/kg milk fat; mean 37.5 ng TEQ/kg milk fat). At 1 and 11 wk of age, significantly greater concentrations of total T_4 (tT_4) (3,3′,5,5′-tetraiodo-L-thyronine) and tT_4/TBG (thyroxine-binding globulin) ratios were found in the high-exposure group. This was attributed to intrauterine exposure to TCDDs, although the investigators note that some exposure from contaminated breast milk could not be ruled out (Pluim et al. 1993). But in another study, the thyroid hormone status of 105 Dutch women and their infants was evaluated in relation to exposure to dioxins and PCBs. Higher levels of chlorinated dibenzo-*p*-dioxins, chlorodibenzofurans, and PCBs in breast milk were significantly correlated with lower plasma levels of maternal total triiodothyronine and total thyroxine, and with higher plasma levels of thyroid-stimulating hormone in infants during the second week and third month after birth. Lower plasma free thyroxine and total thyroxine levels were also observed in the second week after birth (Koopman-Esseboom et al. 1994). In another recent study of plasma thyroxin (T4) levels in 93 Dutch newborns showed that exposure to organochlorine pesticides, PCBs, dibenzodioxin, and dibenzofuran levels as measured in breast milk was correlated with lower T4 levels (whether measurements were of total or free T4 was not specified). However, multivariate analyses of the data suggest that the body-mass index of the mother and smoking during pregnancy are possible confounding factors (Fiolet et al. 1997).

PCBs and dioxin can alter binding of thyroid hormone to plasma proteins, thus resulting in a decrease in plasma thyroid hormone levels. Such binding is principally to transthyretin, which is involved in transporting thyroid and retinol in humans, rodents, and other species. Experimental evidence in rats suggests that transthyretin is particularly important with regard to transport of thyroid hormone from mother to fetus across the placenta (Brouwer et al. 1998). But Brouwer et al. (1998) did point out that humans and rats differ in major plasma thyroid hormone transporters (e.g., transthyretin (TTR) is greater than thyroxine binding globulin (TBG) in rodents and TBG is greater than TTR in humans). These data imply that thyroid hormone levels in plasma may be less effected in humans than rodents. In any case, whether this disruption of thyroid hormone homeostasis mediates the developmental effects associated with exposure to these HAAs remains to be determined.

Serum concentrations of testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) and sperm concentration in men exposed to TCDD have been examined. Henriksen and Michalek (1996) studied veterans of the Vietnam War (Operation Ranch Hand) exposed to Agent Orange and its major contaminant, TCDD. Using regression models, the investigators found that testosterone concentrations in serum decreased with increasing TCDD exposure (slope = –0.4276, standard error = 0.0950). When adjustments were made for possible confounders, the slopes changed with military occupation. Negative slopes were observed for the three occupational strata evaluated, with the strongest among officers (slope = –1.2381, standard error = 0.2895) and weakest among enlisted ground personnel (slope = –0.3485, standard error = 0.1441). Without adjustment, FSH and LH decreased with TCDD exposure (FSH: slope = –0.0121, standard error = 0.0153; LH: slope = –0.0276, standard error = 0.0111). After adjustment, FSH increased with TCDD (slope = 0.0073, standard error = 0.0171) and LH decreased with TCDD (slope = –0.0242, standard error = 0.0127). The investigators note that these findings are inconsistent with earlier work (Michalek et al. 1995) showing that exposure to TCDD is greatest in enlisted ground personnel and least in officers.

An ongoing cross-sectional study of the long-term health effects of occupational exposure to chemicals contaminated with TCDD is being conducted by the National Institute for Occupational Safety and Health (Egeland et al. 1994; Sweeney et al. 1997-98). A variety of health end points have been analyzed and continue to be analyzed (Sweeney et al. 1997-98). In linear regression analyses, current serum dioxin was positively and significantly related to LH and FSH levels, and inversely related to total testosterone, after adjustment for potential confounders ($p < 0.05$). This suggests that as TCDD serum levels increase, testosterone decreases, and the consequence is an appropriate increase in LH and FHS. This further suggests that TCDD is operating to decrease testosterone synthesis, and that this is not occurring through an effect on the neuro-endocrine system.

Chlordecone

Chlordecone is a highly stable chlorinated hydrocarbon pesticide that was produced between 1958 and 1975 as an insecticide and fungicide, primarily for control of the banana root borer and tobacco wireworm and as bait for control of ants and cockroaches. The two principal manufacturers of chlordecone, Allied Chemical and Life Science Product Company (LSPC), were located in Hopewell, Virginia, and operated between 1968 and 1975. Allied Chemical closed in 1974, and LSPC was closed in July 1975 after a state health department inspection found severe chlordecone-related illness and contamination. An investigation by the Centers for Disease Control (CDC) followed. Of the 110 individuals who worked at the plant, more than 50% showed high blood levels of chlordecone.

Semen analysis of 14 chlordecone-exposed workers showed abnormal sperm morphology, decreased sperm mobility, and oligospermia (Guzelian 1976). A recent evaluation of chlordecone by the Agency for Toxic Substances and Disease Registry (Faroon et al. 1995) concluded: "The available human data on chlordecone provide qualitative evidence to support the conclusion that intermediate or chronic-duration exposure to high concentrations of chlordecone in the workplace cause oligospermia and decrease sperm motility among male workers." That finding was consistent with reproductive toxicity demonstrated in multiple toxicologic studies, including testicular atrophy in rats, testicular abnormalities, and reduction of germinal epithelium and number of spermatozoa (Eroschenko and Wilson 1975) and a strong estrogenic effect on the oviduct of female quail (McFarland and Lacy 1969).

Agricultural Exposures

Several ecologic studies have investigated associations between exposure to agricultural chemicals, such as pesticides (insecticides, herbicides, and fungicides), and effects on fertility and development. Positive correlations have been found in some studies, and these are presented below to illustrate some of the reproductive and developmental effects observed among people exposed to agricultural chemicals. However, specific causative agents have not been identified in many of these studies, so it is not know whether they might be HAAs.

Fuortes et al. (1997) examined the industrial and occupational histories of 281 infertile women and compared them with those from 216 postpartum women. Only agriculturally related occupations were associated with infertility. However, the reliability of this association is uncertain because the sample sizes were small. Decreases in fertility were reported in a study of fruit growers exposed to pesticides, with a greater time-to-pregnancy occurring during the months when pesticides were applied (de Cock et al. 1994). Mosher and Pratt (1987) reported a higher probability of infertility among male farmers in a National Center for Health Statistics survey. A study of 32 male farm sprayers who were exposed to the herbicide 2,4-D found statistically significant levels of abnormal, motionless, and dead spermatozoa compared to 25 unexposed controls (Lerda and Rizzi 1991). Urine measurements of 2,4-D confirmed exposure status, at least for that chemical. However, the significance of this study is unclear, because the number of subjects was small. Furthermore, studies of Vietnam veterans exposed to much higher concentrations of Agent Orange, which includes 2,4-D (CDC 1989; Wolfe et al. 1992; IOM 1994), and laboratory studies with 2,4-D (Lamb et al. 1981) show no such effects.

Some studies have found no association between exposure to agricultural chemicals and adverse reproductive outcomes. For example, in a study of low-level exposure to malathion, Thomas et al. (1992) found no substantial or significant associations between maternal exposure and the occurrence of spontaneous

abortion, intrauterine growth retardation (IUGR), or other anomalies as a group. McDonald et al. (1987) found no relationship between maternal occupation in agriculture/horticulture and spontaneous abortion, although an excess of stillbirths was noted. In another study, no significant difference was found in the incidence of abnormal semen in farmers (Gerber et al. 1988).

Garry et al. (1996) reported significant increases in circulatory/respiratory, urogenital, and musculoskeletal/integumental birth defects in 4,935 children born between 1989 and 1992 to 34,772 state-licensed male pesticide applicators in Minnesota. Those effects were more pronounced for children conceived in the spring, and was most marked in western Minnesota, where phenoxy herbicide/fungicide use is highest. The authors noted that there is a possibility that the chemicals were contaminated with dioxin. Elevated levels of the herbicide atrazine found in municipal water supplies in Iowa were associated with excess rates of cardiovascular, urogenital, and limb-reduction deficits (Munger et al. 1992). Preliminary results from a study by Munger et al. (1992) found that communities with herbicide-contaminated water supplies had elevated IUGR rates compared with neighboring communities with different water supplies. Contaminants included atrazine, metolachlor, and cyanazine, but the authors concluded that a strong causal relationship between any specific contaminant and risk of IUGR could not be inferred.

Exposures in the studies above are to a broad range of chemically diverse pesticides. Which pesticides are responsible for the reproductive and developmental effects is unknown. Furthermore, it is difficult to isolate a single type of exposure from the wide variety of other exposures to chemicals and environmental factors to which agricultural workers are subject. Nonetheless, such ecologic studies are useful for identifying potential effects. Nurminen (1995) evaluated a number of the ecologic studies and concluded that "the published studies have given some indications of elevated reproductive risk and exposure to pesticides but, altogether, the collective epidemiologic evidence did not allow any clear inference to be drawn."

Adverse Effects on the Male Reproductive System: Regional and Temporal Variations

Some authors suggest that the incidence of several male reproductive disorders is increasing (Giwercman et al. 1993; Sharpe and Skakkabaek 1993; Toppari et al. 1996). The disorders include testicular cancer, hypospadias, cryptorchidism, and poor sperm concentration. Studies with laboratory animals have shown that prenatal exposure to some HAAs, such as methoxychlor (Gray et al. 1989; Gray 1992), TCDD (Mably et al. 1992c), and octylphenol and bisphenol A (vom Saal et al. 1998) can reduce sperm production. Other authors have questioned the validity of the trend analyses (Farrow 1994; Nieschlag and Lerchl 1996; Paulozzi 1999). Because hypotheses linking the trends to human exposure to HAAs have

generated considerable discussion, and because such links are biologically plausible given the reported effects of exposure to HAAs in laboratory animals and wildlife species, they are discussed here.

Testicular cancer rates have been reported to be increasing in the United States (Ries et al. 1997), Canada (Weir et al. 1999), and in six European countries, particularly among men born after 1950 (Bergstrom et al. 1996). The trend has been particularly marked in Denmark, where the incidence of seminomas and nonseminomas has been rising for several decades (Forman and Moller 1994). The risk factors for testicular cancer are increased with cryptorchidism (RR = 5.2) and hypospadias (RR = 4.2), suggesting a prenatal etiology (Prener et al. 1996). An increase in the incidence of hypospadias has been observed in England and Wales (Matlai and Beral 1985), Hungary (Czeizel 1985; Czeizel et al. 1986), Sweden (Kallen and Winberg 1982), Norway (WHO 1991), and Denmark (WHO 1991). One study (Paulozzi et al. 1997) in the United States shows a doubling of hypospadias rates between 1968 and 1993, from 40/10,000 to 80/10,000 male births. Some of those changes in incidence may be due to the reporting criteria for the disorders and other aspects of diagnosis. However, the increase is most marked among the most severe cases, which are least likely to be subject to changes in diagnosis. Hypospadias has been associated with defects in testosterone receptors and testosterone metabolism, suggesting a possible link to endocrine factors (Allen and Griffin 1984; Glatzl 1984). Genetic and other factors have also been implicated in hypospadias (Bauer et al. 1981; Harris 1990; Weidner et al. 1999). Several authors present data suggesting that cryptorchidism rates have risen in England, Wales, and Scandinavia, although the trends are less consistent, and they could be related to changes in diagnosis or treatment (Giwercman and Skakkeback 1992). Berkowitz et al. (1993) reported a rate of cryptorchidism among New York City births in 1990 that was similar to the rate reported by Scorer (1964) in London in the 1950s. Berkowitz et al. (1993) conclude there is no evidence of an increase in the cryptorchidism rate. However, their data do not directly address the question of a change in rate of the defect; 1990 New York births were not compared with births in New York in the 1950s, when rates might well have been lower. With regard to hypospadias, cryptorchidism, testicular cancer, and sperm count, there is considerable evidence for geographic variation. Studies to assess the factors contributing to this variation will be required to determine whether HAAs, in addition to or in conjunction with other factors, are related to this variability. Paulozzi (1999) reported that international trend rates of hypospadias and cryptorchidism increased in some geographic locations, such as the United States, Japan, and Scandinavia, while changes did not occur in other regions, most notably Canada. Since 1985, incidence rates for hypospadias have leveled off in regions which showed an increase. Incidence rates for cryptorchidism have actually declined in most regions since 1985. In any case, the relations of these trends in testicular cancer, hypospadias, or cryptorchidism to HAAs has not been examined.

Regional and Temporal Variation in Sperm Concentration

Sperm concentration is the best-studied male reproductive end point, and it is perhaps the measure of male reproductive function that has generated the greatest controversy. The possibility of declining sperm concentration and the environmental causes of such a decline are not new concerns.

After noting sperm concentrations in 1970-1973 that were markedly below those reported by MacLeod and Gold in 1951 (48×10^6/mL vs. 107×10^6/mL), Nelson and Bunge (1974) wrote, "The overall decrease in the sperm concentration and the semen volumes would tend to incriminate an environmental factor to which the entire population has been exposed." In 1979, Macleod and Wang noted that several studies on fertile males found a "marked reduction in spermatogenesis since 1951." These authors' analysis of their own historical data of infertile populations, however, did not support a decline. In 1980, James conducted a multinational analysis of 29 studies of sperm concentration published over 45-yr and concluded, "There can be no reasonable doubt that these reported mean sperm counts show a decline with time of publication, at least since 1960." Dougherty et al. (1981) found a correlation between sperm concentrations taken from a sample group of university students and the presence of toxic substances, which they identified by negative-chemical-ionization screening procedures. They note that "PCB uniformly gave negative slope correlations with sperm." Murature et al. (1987) analyzed sperm concentrations listed in 45 studies published between 1929 and 1981 and found a decline in sperm concentration between 1949 and 1981. These authors argue that mean sperm concentration was correlated with several environmental exposures, including the total production of synthetic compounds within the United States. Feichtinger (1991) discusses several possible etiologies for a decline, in particular, exposure to organochlorines, such as PCBs, DDT, and hexachlorobenzene (HCB). Although there was no significant correlation between exposure to these compounds and sperm count, samples of semen that led to pregnancy after in vitro fertilization and embryo transfer (IVF/ET) contained lower average concentrations of PCBs and HCB than did samples that did not produce pregnancy (Feichtinger 1991).

Several distinct questions underlie the above controversy. It is important to separate them: (1) Has semen quality (as measured by sperm concentration, morphology, or motility) declined? (2) Does sperm concentration vary significantly according to geographic location? (3) If the answer to either of the first two questions is yes, is exposure to HAAs or other environmental toxicants causing the changes?

The question of a possible decline in sperm concentration over time has most recently been raised by an analysis (Carlsen et al. 1992) of 61 studies conducted throughout the world and published between 1938 and 1990. Carlsen's group used simple linear regression to model the changes in sperm concentration over time, concluding, "reports published worldwide indicate clearly that sperm con-

centration . . . declined appreciably during 1938-1990." The analysis was followed by considerable debate, and several articles challenged its conclusions. Analyses proposing alternative, and increasingly complex, statistical models of the same data set followed (Olsen et al. 1995; Bahadur et al. 1996; Becker and Berhane 1997; Swan et al. 1997). This group of publications, the "multinational trend studies," all analyzed basically the same historical data set. "Local trend studies," on the other hand, examine data within a single country or state to assess trends in sperm concentration. While many local and multinational trend studies include data on multiple parameters; all provide data on trends in sperm concentration, the parameter for which methods have remained most comparable over time.

Carlsen et al. (1992) reviewed the literature published between 1930 and 1990 to identify studies that include data on sperm concentration in normal males. Studies that included men from infertile couples, studies that selected men on the basis of a high (or low) sperm count, and studies that used nonmanual methods for counting sperm were excluded. The authors found that mean sperm concentration had decreased from 113×10^6/mL in 1940 to 66×10^6/mL in 1990, with a slope of minus 0.93×10^6/mL/yr ($p < .0001$).

In response, several authors noted that mean sperm concentration in the data set appears to decline until some point in the early 1970s and then to level off or even increase. This suggested the need to use alternative (nonlinear) regression models. Olsen et al. (1995) fit three such models: a spline function (or "hockey stick"), which corresponds to a decline in sperm concentration until 1970, when the decline levels off, ceases, or is reversed; a quadratic function, which corresponds to a smoothed form of the spline with no abrupt change in sperm concentration but a gradual upturn late in the study period; or a step function. The first and second alternative models suggest that the change in sperm concentration was smooth; the step function assumes that mean sperm concentration remained constant until it dropped abruptly around 1970. Olsen et al. (1995) maintained that their three nonlinear analyses explain somewhat more of the variability in the data, as measured by the adjusted R^2, than does the simple linear regression, although the incremental increase in R^2 was minimal (approximately 1-3%). Bahadur et al. (1996) recognized the need to control for geographic region, and they did so by fitting separate curves for the United States, Europe, and elsewhere. These authors reported that data from the United States fit the linear and quadratic functions equally well, although the quadratic term was not statistically significant. In their analysis, data from Europe and other countries fit neither model well. Becker and Berhane (1997) were the first to present the data set using multiple-regression analysis. Their method controlled for region and type of study population (whether men were of proven fertility or not), as categorized by Carlsen et al. (1992). Their final model, which controlled for two regions (United States and non-United States), fit the data somewhat better than had the previous models (adjusted $R^2 = .51$, compared with .36 for Carlsen et al. (1992)).

Swan et al. (1997) abstracted data from 56 of the 61 underlying studies to control for additional variables, such as age, abstinence time, method of specimen collection, and percentage of men with proven fertility. In their analysis, studies were grouped into three regions: United States (27 studies, 1938-1988), Europe and Australia (16 studies, 1971-1990), and other (non-Western) countries (13 studies, 1978-1989). Multiple-regression analyses were used to fit linear and nonlinear models. Swan et al. (1997) included data from all regions in a single model, which fit separate lines (for the linear, spline, and step models) or curves (for the quadratic model) in each region. Their multiple-regression analyses considerably improved model fit. For example, the adjusted R^2 was .80 for the linear multiple-regression model compared with .36 for the simple linear model. Studies from non-Western countries did not fit any of these models, perhaps reflecting the heterogeneity of the areas included, the small number of studies (13), and the short time during which these studies were published (12 yr). Nonlinear multiple-regression models fit the United States and European data nearly as well as did the linear multiple-regression model. All models show a decline, on average, in the United States and Europe, and none identifies any post-1970 increase in sperm concentration in men from these countries.

Of the studies included in these analyses, 88% were published after 1970. Because a possible decline during the last 20 yr of the study period was of particular interest, and because studies from all regions could be directly compared only after 1970, Swan and Elkin (1999) fit a multiple linear-regression model restricted to post-1970 studies. The results were comparable to those seen for the entire study period.

Becker and Berhane (1998) argued that the exclusion of five studies by Swan et al. (1997) had altered the statistical outcome. Two of the excluded studies did not meet the criteria for inclusion established in the original study by Carlsen et al. (1992). The other excluded studies (from Peru, Denmark, and Germany) were not written in English. The effect of these exclusions on the global average decline is minimal (overall slope = -0.95×10^6/mL, $p < 0.001$ vs. -0.93×10^6/mL, $p < 0.001$) (Swan et al. 1997). With respect to the more rigorous stratified analysis, after excluding these non-English studies the European trend was no longer statistically significant. The U.S. slope was, however, unchanged. Since the method of selecting these three non-English studies from the large non-English literature on semen quality is not known, the possible bias in the inclusion of these three studies cannot be evaluated.

These semen analysis studies were conducted in multiple laboratories using a variety of techniques, leading to concerns about the reliability of these data and possible biases that might have contributed to the observed decline. A recent analysis (Swan and Elkin 1999) examined these techniques and found no trend in variability or systematic changes in methods that would explain the observed decline. Nevertheless, controversy over the possibility of bias and the quality of the data remains that can only be resolved using carefully controlled prospective studies.

Although multinational trend studies are consistent with a downward trend in mean sperm concentration, this pattern may be confounded by local geographic variation (Fisch and Goluboff 1996; Saidi et al. 1999). Younglai et al. (1998) noted large differences in sperm counts in 11 centers across Canada. Eight local trend studies, published between 1979 and 1996, described trends in sperm concentration in normal men living within a single city, state, or country (Table 5-1). Only one study (Vierula et al. 1996) includes data collected before 1970, so these local trend studies, which included about one-third the number of men in the multinational trend studies (5,122 vs. 14,947), provide data only on post-1970 trends in sperm concentrations. There is considerable variability among these studies in design, population, and size; some measure mean sperm concentration at only a few time points and include a few hundred men, others include mean sperm concentration from 20 to 30 time points and more than 1,000 men. Some provide estimates of slope adjusted for age, abstinence time, and season; some are unadjusted for any covariates. Therefore, apparent geographic differences could in part be the result of these differences in method. Significant declines in sperm concentration were reported in Belgium (Van Waeleghem et al. 1996), Scotland (Irvine et al. 1996), and Paris, France (Auger et al. 1995). In the United States, significant increases were reported in Washington (Paulsen et al. 1996), as well as in Los Angeles, Minneapolis, and New York (Fisch et al. 1996). Other authors report no significant change in France (Bujan et al. 1996), Finland (Vierula et al. 1996), and Wisconsin (Wittmaack and Shapiro 1992). Differences such as those reported Fisch (114.0×10^6/mL in New York vs. 72.7×10^6/mL in California) are as large as the change in sperm concentration across the entire period studied by Carlsen et al. (1992) (113×10^6/mL to 66×10^6/mL). More recently, a study of regional differences in sperm concentration among fertile French men found that mean counts from eight regions within France ranged from 82×10^6/mL to 102×10^6/mL (Auger and Jouannet 1997).

Rasmussen et al. (1997) reported that sperm concentration had not changed in Denmark during the past 20-30 yr. Although it was noted that the method used to determine sperm counts overestimated sperm concentrations, the authors judged that it did not affect the conclusion that there was no change over time.[1] In a study conducted in Australia using potential sperm donors, Handelsman (1997) found no significant difference in sperm concentration over time (1980-1995) or between years or according to year of birth. The overall median sperm concentration was 69×10^6 mL. Five studies of median sperm concentration were also conducted on potential participants in a male contraceptive study. The median sperm concentrations for two of the studies were significantly higher (103×10^6/mL and 142×10^6/mL; $p < 0.05$) than for the other three (63, 67, and 84×10^6/mL, respectively),

[1] One committee member believes this finding may reflect possible selection bias as evidenced by under representation of men with low sperm counts.

TABLE 5-1 Studies of Trends in Sperm Concentration of Normal Men

Reference	Country (City or State)	Population Source[a]	Start Yr	End Yr	Count 1 (10^6/mL)[b]	Count 2 (10^6/mL)[b]	Slope (10^6/mL)[c]	Probability	*n*	Variables Controlled[d]
Auger et al. (1995)	France (Paris)	*a,b*	1973	1992	89	60	−2.1%/yr	<0.001	1,351	w,y
Bujan et al. (1996)	France (Toulouse)	*a,b*	1977	1992	88.4	92.3	+0.3/yr	0.09	302	w
Fisch et al. (1996)	U.S. (CA, NY, MN)	*c*	1970	1994	77	89	+0.8/yr	0.04	1,283	w,y
Irvine et al. (1996)	Scotland (Edinburgh)	*a*	1984	1995	117.9	93.9	−2.1%/yr	0.002	577	
Paulsen et al. (1996)	U.S. (WA)	*a*	1972	1993	49.2[e]	52.0[e]	+0.13/yr	0.014	510	
Van Waeleghem et al. (1996)	Belgium (Ghent)	*a*	1977–1980	1990–1995	71.0	58.6	−0.83/yr	0.035	416	x
Vierula et al. (1996)	Finland (Turku)	*a,b*	1967	1994	79.4	87.8	+0.30/yr	0.454	238	w,y,v
Wittmaack and Shapiro (1992)	U.S. (WI)	*a*	1978	1987	55.6	87.1	+2.62/yr	NS[f]	446	

[a] Population source: a, healthy, normal volunteers and/or potential sperm donors; b, prevasectomy patients; c, proven fertility.
[b] Count 1 and Count 2, unadjusted mean sperm concentration for Yr 1 and Yr 2.
[c] Slope over all study years (adjusted if possible, for covariates in last column); if not provided by authors, slope = (Count 2 – Count 1)/(Yr 2 – Yr 1); total is total number of men studied.
[d] Covariates controlled: w, age controlled in analysis; x, authors state no change in age; y, abstinence controlled in analysis; v, season controlled in analysis.
[e] Median sperm concentration given instead of arithmetic mean.
[f] NS, not significant.

as well as for potential sperm donors (median 69×10^6/mL). The authors suggest that this substantial variation between donor groups reflects bias from self-referred volunteers. Collectively, the studies described above suggest considerable geographic variation in sperm concentration; however, the degree to which the differences are ascribable to population selection is still not known. One of the reasons for the controversy regarding temporal trends in sperm concentration is that it is impossible to control for all confounding factors in retrospective studies due to limitations in the original data sets, and only prospective studies can clearly address the issue of the factors affecting sperm concentration. The question of whether sperm concentration has declined over the past 50 yr may not be the appropriate question for study. No analysis to date can prove or disprove a uniform global trend in sperm concentration. In fact, within single study centers and populations, considerable local variation has been demonstrated, with some studies suggesting a decline, and others no change, or even a possible increase in sperm concentration over the past 20 yr.

However, given the limited information available, one cannot assume an environmental etiology for the variability observed in human populations. Cross-sectional comparisons of sperm concentration now under way in comparably selected populations in several countries could identify areas of low (and high) sperm concentrations. Careful exposure assessments in these areas will be required to identify etiologic factors.

Joffe (1996) indirectly examined the relationship between sperm concentration and fertility by comparing time to pregnancy between Finland (high to normal sperm counts) and Britain (low sperm counts) in a pair of prenatal studies and a pair of cross-sectional studies. In both comparisons, time-to-pregnancy distributions were significantly shorter in Finnish couples ($p < .05$ and $p < .025$). The authors conclude that this supports the hypothesis that the difference in sperm counts between Finland and Britain is not artefactual. They also point out, however, that the lower smoking rates among Finnish women may be a factor. Zukerman et al. (1977) demonstrated that no statistically significant decline in fertility is observed until sperm counts reach a level of 10×10^6/mL, and even then sterility is not assured. However, a recent study of 430 couples planning their first pregnancy in which semen quality was measured at the onset of the study found that the probability of conception increased with increasing sperm concentration up to 40×10^6/mL. With higher sperm concentration, little association was seen (Bonde et al. 1998).

WILDLIFE STUDIES

Data on the reproductive and developmental effects in wild populations of fish, birds, alligators, turtles, amphibians, and panthers are summarized and evaluated below. A discussion of how these effects could influence the population dynamics of some species is presented in Chapter 10.

Fish

Three well-studied polluted waters have been associated with endocrine modification and altered reproductive physiology of fish: (1) waters that receive sewage treatment plant effluent (STPE), (2) contaminated waters of the North American Great Lakes, and (3) waters that receive bleached paper mill effluent (BPME).

Sewage Treatment Plant Effluent

In the United Kingdom, intersex adult roach (*Rutilus rutilus*) with testes containing oocyte have been observed in wild populations near sewage treatment plants. These observations led some researchers (Purdom et al. 1994) to hypothesize that sewage effluents might contain estrogenic substances. Immature male rainbow trout and common carp were placed in cages in the effluent stream of 15 sewage treatment plants throughout southern England and Wales (Purdom et al. 1994), and the fish were evaluated for estrogen-induced vitellogenin synthesis. These fish exhibited a site-dependent, 500- to 100,000-fold increase in plasma vitellogenin concentration over fish in control groups. Subsequent work has also shown that rivers downstream of STPE inputs are estrogenic to caged rainbow trout and that up to 100% of the male roach exhibit intersex conditions (Harries et al. 1997; Jobling et al. 1998). High levels of vitellogenin induction and up to 20% ovotestis have been observed in male flounder (*Platichthys flesus*) from industrialized estuaries in the United Kingdom (Allen et al. 1998).

Estrogenic compounds excreted by humans into wastewater, such as ethinylestradiol (the synthetic estrogen used in birth control pill formulations), have been postulated to be the active ingredients in the effluents (Purdom et al. 1994). To test this hypothesis, male rainbow trout were injected intramuscularly with ethinylestradiol at varying dosages (Purdom et al. 1994). Ethinylestradiol was shown to be more potent in trout than is naturally occurring estradiol, based on the magnitude of vitellogenin induction. Other groups of trout placed in water containing 1-10 ng/L ethinylestradiol exhibited plasma vitellogenin concentrations similar to those produced by the fish caged in effluent streams (Purdom et al. 1994). Some investigators have not detected ethinylestradiol in STPE in the United Kingdom (Purdom et al. 1994); others have detected it at <20 ng/L (Aherne and Briggs 1989). However, recent work by Desbrow et al. (1998) showed that ethinylestradiol and the natural steroids estradiol and estrone are the major contributors to estrogenicity of several STPEs.

Alkylphenol ethoxylates (APEs) and their degradation products have also been hypothesized to be responsible for observed estrogenic effects. Concentrations of APEs have been measured at hundreds of micrograms per liter in domestic sewage effluent (Ahel and Giger 1985) and at even greater concentrations in industrial effluent (Naylor et al. 1992). The nonethoxylated breakdown products

of the APEs 4-*tert*-octyl-phenol ethoxylate and nonylphenol ethoxylate, octylphenol and nonylphenol, have been shown to be estrogenic in fish cells in vitro (Jobling and Sumpter 1993; Jobling et al. 1995). To examine the effects of those compounds on testicular function, male rainbow trout undergoing sexual maturation were placed in water containing 30 µg/L octylphenol, nonylphenol, or two other alkylphenols (the concentration is representative of those found in STPE of some rivers) for 3 wk. All of the alkylphenols caused a significant increase in plasma vitellogenin concentration, accompanied by a concomitant and significant decrease in the rate of testicular growth (Jobling et al. 1996). Dose-response studies, in which trout were exposed to various concentrations of alkylphenols for 3 wk at three distinct stages of testicular development, showed that the concentration of vitellogenin produced depended on the estrogenicity of the compound, the dosage, and the stage of testicular development. When testicular growth rates of alkylphenol-exposed fish were compared with those for control fish, testicular growth rate was inhibited by as much as 50% after exposure to octylphenol, the most estrogenic alkylphenol. Inhibition of testicular growth was directly correlated with the estrogenic potency of the various experimental treatments (Jobling et al. 1996). Field studies on the River Aire in Yorkshire, United Kingdom, have shown that the strong estrogenicity found below one STPE input could be largely attributed to alkylphenols derived from textile mill effluent (Harries et al. 1997). The voluntary cessation of the use of cleaning products containing the estrogenic surfactant nonylphenol by the textile industry in England led to the amelioration of this problem in male fish in the Arne river (Matthiessen and Sumpter 1998).

Studies at other locations outside of England have confirmed the observation that male fish exposed to STPE exhibit elevated plasma concentrations of vitellogenin. In a study of male common carp from five riverine locations in Minnesota, elevated plasma vitellogenin and depressed plasma testosterone concentrations were observed in fish collected from 1 location, an effluent channel below a metropolitan sewage treatment plant on the Mississippi River (Folmar et al. 1996). In another study, Nichols et al. (1999) measured concentrations of estradiol and vitellogenin in plasma of fathead minnows and the common goldfish in central Michigan. Caged fish were placed directly in the effluents of seven treatment plants with a range of treatment types and sources of waste. The variability in vitellogenin measurements was great, making it difficult to demonstrate any statistically significant differences among locations. Elevated vitellogenin was observed in only one of the seven locations. Even though induction of vitellogenin was observed in both species, the degree of induction was only about 6-fold over background. The investigators concluded that the estrogenic potency of STPE was slight.

Overall, the expression of vitellogenin in male fish exposed to STPE in observational and experimental studies indicates that some STPEs have estrogenic properties that could cause the observed reproductive alterations in the wild.

North American Great Lakes

For nearly 2 centuries the Great Lakes have received industrial and municipal wastewater. The fish of the Great Lakes have been found to contain literally hundreds of synthetic and natural compounds (Fitchko 1986; Allan et al. 1991; IJC 1993). However, chemical contaminants such as DDE and PCBs have decreased markedly in recent years with improvements in reproductive success of several species of predatory birds (Tremblay and Gilman 1995). Two of the best-studied fish of the Great Lakes have been the introduced salmon and the native lake trout.

Salmon

Pacific salmon (*Oncorhynchus* spp.) were introduced into the Great Lakes, mainly in the 1960s, and soon became established there (Becker 1983), although still are largely maintained through stocking. In 1980 and 1981, the rearing mortality of chinook salmon increased to 7.4% and 19.5%, respectively, from 2-3% before 1980 (Eadie 1983; Allan et al. 1991). Although a normal hatch rate was observed, hatched fry failed to feed, swam in circles, lost equilibrium, and died within several weeks. This upward trend in rearing mortality was alarming, especially during a period when the concentrations of many of the routinely monitored residues (PCBs, DDT, and TCDD) were decreasing in fish, and it raised the question of whether other synthetic residues might be responsible for the observed effects (Mac and Seeyle 1981a; Mac et al. 1985, 1993). Because toxic substances concentrate in the lipids of fish eggs and become more concentrated in the remaining egg yolk as the fry absorb the yolk, it is believed that the fry receive a large dose of xenobiotics at the "swim-up" stage when the last of the yolk is absorbed (Atchison 1976). A cursory investigation of the concentrations of 14 organochlorine compounds in chinook salmon fry revealed that normal fry contained 2.5 mg/kg total PCBs, whereas sick and dead fry contained 3.9 and 5.2 mg/kg total PCBs, respectively (Flagg 1982). Concentrations of *p,p′*-DDE were 8.0, 13.0, and 17.0 mg/kg for normal, sick, and dead fish, respectively. It should be noted that a syndrome similar to that observed in the chinook salmon was found in coho salmon fry reared in Michigan during the late 1960s and was attributed to residues of DDT in coho salmon eggs (Johnson and Pecor 1969).

Based on laboratory studies of different fish species, the concentration of DDT that is lethal to salmonid eggs is estimated at 1.0-10 mg/kg, wet weight (ww) (Macek 1968; Hopkins et al. 1969; Burdick et al. 1972). Current concentrations of DDT in salmonid eggs from the Great Lakes are in the range of 0.1-2 mg/kg, ww (Giesy in press), depending on species and location. In 1984, the concentration of DDT in chinook salmon eggs from Lake Michigan was 1.2 mg/kg, ww (Giesy et al. 1986). Historically, concentrations of DDT were as great as 10 mg/kg, ww, in eggs of fish from the Great Lakes (Burdick et al. 1964; Hopkins et al.

1969; Johnson and Pecor 1969; Atchison 1976). Thus, it is plausible that DDT could have caused reproductive impairment of salmon in the Great Lakes. Attempts to correlate rearing mortality in the field with DDT concentrations in the environment have been inconclusive: Some researchers have found a positive correlation (Giesy et al. 1986); others have not (Mason et al. 1967; Giesy et al. 1986).

Another major class of contaminants of the Great Lakes—PCBs—has been found to affect early and adult life stages of fish in laboratory studies (Delzell et al. 1994). When total concentrations of PCBs in fish and fish eggs from the Great Lakes were examined and compared with the results from controlled laboratory studies, the calculated hazard indices indicated that concentrations of these residues were probably sufficient to have caused egg and fry mortality (Willford et al. 1969; Halter and Johnson 1974; Lidman et al. 1975, Mauck et al. 1978; Guiney et al. 1980; FWS 1981, Binder and Lech 1984; Fisher et al. 1994). However, it has been difficult to correlate concentrations of PCBs with observed adverse effects in field studies (Gilbertson et al. 1990; Mac and Gilbertson 1990; Gilbertson 1992; Delzell et al. 1994). This is probably due to two factors: The exposure of fish to several compounds simultaneously and the great deal of variation in exposure and responses has made relating the effects to a single compound impossible (Stauffer 1979; Giesy et al. 1986).

Calculation of hazard indices indicates that currently, and possibly historically, TCDD-like compounds are the critical toxicants in the Great Lakes fish (Giesy et al. 1994a). Coho salmon exposed to 5.4 ng TCDD/g for 12 h had reduced growth and survival (Miller et al. 1979), and the threshold concentration in 24-96 h exposures was 0.54-5.4 ng TCDD/g. Dioxins and dioxin equivalents from all dioxinlike chemicals (TCDD-EQ) also have been shown to be toxic to fish at concentrations that currently occur in the eggs of salmonid fish in the Great Lakes (Giesy et al. 1986; Ankley et al. 1991; Newsted and Giesy 1992, 1993; Williams and Giesy 1992). Comprehensive reviews of the effects of TCDD on fish of the Great Lakes are given by Walker and Peterson (1994) and by Cook et al. (1993).

Since the early 1970s, pink, chinook, and coho salmon introduced into the Great Lakes have exhibited thyroid enlargement (goiters). These enlargements, due to both hypertrophy and hyperplasia, have ranged in size from gross lesions to histologic enlargements (Leatherland 1992, 1998). Because PCBs have been shown to be goitrogenic in various vertebrates (Brouwer et al. 1989; Leatherland 1992), it was hypothesized that these contaminants could be the cause. However, coho salmon collected from Lakes Erie, Ontario, Superior, and Michigan exhibited no correlation between the concentration of PCBs in the body and thyroid enlargement (Sonstegard and Leatherland 1976). It has been noted that rodents fed PCB-contaminated salmon (30-100% of the diet) from the Great Lakes for 2 mo showed significant thyroid enlargement, hepatomegaly, increased mixed-function oxydase activity, and a reduction in plasma thyroxine concentrations

(Leatherland 1992). Why a relationship between PCB contamination and goiter formation is not apparent in coho salmon is not known. Other studies indicate that goiter formation in fish might be caused by bacterial agents rather than by PCBs (Gaitan et al. 1980; Leatherland 1992, 1993).

In Lake Erie, some coho salmon (Fairview stock) have displayed a series of reproductive dysfunctions (Leatherland 1993). These include reduced egg-survival-to-hatch rates, depressed steroidogenesis, lowered plasma concentrations of steroids and pituitary hormones in males and females, and lowered egg thyroid hormone concentrations. Another reproductive abnormality is the increased incidence of embryonic teratogenesis. In some years, 90% of male salmon exhibited early onset of sexual maturation and reduced development of secondary sex characteristics (Leatherland 1993). At this time, there is no evidence that goiter or any of the reproductive dysfunctions observed in coho salmon are caused by exogenous-hormone-modulating compounds.

Lake Trout

Annual rearing mortalities in lake trout fry of as much as 97% were described for hatchery-reared fish between 1978 and 1981 (Mac et al. 1985). Mortality could not be attributed to disease or nutrition and was characterized by erratic swimming behavior and loss of equilibrium before death. Poor survival was significantly correlated with the source of eggs and sperm and not to the residue concentrations or chemistry of the water in which the eggs were reared (Mac et al. 1985). In addition, a number of fry developed blue sac disease, an edematous condition that results in fluid filling the yolk sac, which takes on a bluish color, and leads to the death of the egg. Early efforts to correlate the degree of fry mortality in lake trout with concentrations of PCBs and DDT were unsuccessful (FWS 1981; Seelye and Mac 1981).

DDT and PCBs have been shown to cause mortality in lake trout fry and eggs in laboratory studies (Berlin et al. 1981). However, the concentrations required to cause 30-50% mortality were as much as 25 times greater than were the concentrations actually found in the eggs in the Great Lakes. In addition, the concentrations of DDT and PCBs were higher during the period of normal rates of rearing success (1972-1975) than they were during the period when abnormally low rates of fry survival were observed (1978-1981). Subsequent studies have identified the total concentrations of TCDD-equivalents (EQ) as a more plausible causative agent (Symula et al. 1990; Walker and Peterson 1994), and TCDD and structurally similar compounds have been found to induce blue sac disease in the laboratory (Walter and Peterson 1994). Current concentrations of TCDD-EQ in fry are near the threshold for mortality in lake trout fry (Walter and Peterson 1994), thus it is likely that the concentrations of TCDD-EQ in the lake trout eggs were well above the threshold in the recent past, and that current concentrations might still impede survival.

Bleached Paper Mill Effluent

Since the late 1980s, a series of studies has investigated the effects of BPME on the reproductive physiology of the white sucker in the Great Lakes. BPME-exposed white suckers collected from Jackfish Bay, Lake Superior, showed decreased concentrations of plasma sex steroids, decreased egg and gonadal size, and delayed sexual maturity (Van Der Kraak et al. 1992). To examine those effects further, white suckers were collected (Munkittrick et al. 1994) from the receiving areas of 12 paper mills (excluding Jackfish Bay). BPME from paper mills that used chlorine-based and sulfite-based processes were included, as was effluent from mills that used primary or secondary treatment facilities. Fish exposed to BPME exhibited increased hepatic mixed-function oxydase (ethoxyresorufin-*O*-deethylase) induction, and the greatest concentrations of dioxin were found in the liver. Fish collected from sites where sulfite-based processes was used had elevated enzyme activity and decreased steroid concentrations that were not correlated with concentrations of hepatic mixed-function oxydase activity (Munkittrick et al. 1994). BPME-exposed white suckers exhibited effects on steroid synthesis pathways other than those of the sex steroids.

Male and female BPME-exposed white suckers exhibited a 30- to 50-fold decrease in gonadotropin hormone-II (GtH-II) (Van Der Kraak et al. 1992). To investigate this observation further, BPME-exposed fish were collected and given a single injection of a synthetic-gonadotropin-releasing hormone (sGnRH-A). This treatment induced an increase in plasma GtH-II, although the magnitude of response was significantly lower in the exposed population when compared with the control population (Van Der Kraak et al. 1992). In the same study, ovulation in BPME-exposed females did not occur after injection of the GnRH analogue, yet 10/10 control females ovulated within 6 h. Not only were concentrations of 17-20-progesterone lower for BPME-exposed fish, the fish did not exhibit an increase in the plasma concentrations of this hormone after sGnRH-A treatment. Plasma testosterone in females and males and 11-keto-testosterone in males were elevated in fish from the control site compared with the BPME site, but they were not increased after the GnRH injection. In contrast, BPME-exposed fish showed a transient increase in testosterone in response to the sGnRH-A injection (Van Der Kraak et al. 1992). These findings suggest an inhibitory effect on the pituitary gland.

As a follow-up to the in vivo plasma hormone studies, in vitro cultures of follicles obtained from the BPME-exposed fish and control fish were incubated with and without human chorionic gonadotropin (hCG) or forskolin, a stimulator of adenylate cyclase activity. Follicles from fish exposed to BPME had a decrease in basal secretion of testosterone and 17-20-progesterone and a reduced response to either hCG or Forskolin (Van Der Kraak et al. 1992). There was no significant difference in follicular production of prostaglandin E, either basally or after stimulation with phorbol ester or calcium ionophore a23187, when BPME

and control follicles were compared. Together, these data suggest that exposure to BPME affects the ovary selectively rather than generally (Van Der Kraak et al. 1992). This analysis also provides evidence that the exposure to BPME affects reproduction in the white sucker by acting at different points in the pituitary-gonadal axis.

BPME is known to be a complex mixture of artificial and naturally occurring compounds, that have been shown to affect the reproductive physiology of fish. For example, BPME contains numerous chlorinated byproducts of the bleaching process (Peterman et al. 1980) and one phytoestrogen, β-sitosterol, is present at some mill sites at 1,200 μg/L in primary treated BPME and at 280 μg/L after secondary treatment. Experimental study has shown that injections of β-sitosterol alter the reproductive physiology of the domesticated goldfish (MacLatchy and Van Der Kraak 1995). Testosterone and 11-keto-testosterone in male goldfish and testosterone and estradiol in female goldfish were significantly depressed after treatment; GtH-II concentrations were increased at the same time. As with virtually all toxicologic experiments, the doses used were higher than those monitored in the environment, making direct comparisons difficult to determine (see Chapter 4). In vitro cultures of testes removed from β-sitosterol-treated male goldfish produced decreased testosterone and pregnenolone concentrations both basally and after hCG stimulation. In vitro cultures of follicles removed from β-sitosterol-treated females produced decreased pregnenolone concentrations basally. After hCG stimulation, testosterone and pregnenolone remained depressed. However, basal testosterone was not significantly different from controls (MacLatchy and Van Der Kraak 1995).

Those data indicate that synthetic and natural HAAs released from paper mills that process raw wood products can alter the reproductive physiology of fish. How these chemicals interact with each other is not known and continues to be investigated.

Amphibians

There are many reports that salamander, toad, and frog populations are declining. Disappearances of these amphibians have been reported in areas of North America, Central and South America, Europe, Asia, Africa, and Australia (Blaustein and Wake 1990; Wake 1991; Wake and Morowitz 1990). Although there is debate about whether such declines are occurring multinationally, local declines have been documented (Blaustein and Wake 1990; Phillips 1990). For example, 80% of the Cascade frogs that have been monitored in Oregon since the mid-1970s have disappeared (Blaustein and Wake 1990), and the gastric-brooding frog of Queensland, Australia, is believed to be extinct (Tyler 1991). Similarly, the golden toad of the Monteverde Cloud Forest Preserve in Costa Rica has not been found in its traditional breeding sites since 1987 (Wake and Morowitz

1990). The hypotheses proposed to explain local declines include disease and the introduction of exotic predators (Hayes and Jennings 1986; Carey and Bryant 1995), modification in exposure of eggs to ultraviolet light (Blaustein et al. 1994), and pathogens (Morell 1999). There also have been numerous reports of amphibian kills after chemical spills or agricultural spraying. Environmental contamination also might contribute to continuing declines by affecting the growth and development of young amphibians (Carey and Bryant 1995).

There have been reports of deformities, such as extra, missing, or malformed limbs, in wild populations of frogs in the United States and Canada (Schmidt 1997; Ouellet et al. 1997; Tietge 1997). The deformities could be caused by exposure to chemical pollutants, increased exposure to ultraviolet light, parasite infestations, or some combination of the three (Schmidt 1997). However, more recent studies indicate that the physical presence of parasitic trematode worms, which form cysts in the developing hind-limb regions of frogs, might be primarily responsible for supernumerary limbs in frogs (Sessions and Ruth 1990; Sessions 1997; Johnson et al. 1999; Sessions et al. 1999). The phenomenon seems to be caused by physical disturbances, because similar effects can be induced by implanting inert resin beads, similar in size to trematode cysts, into the developing limb buds of frogs (Sessions and Ruth 1990). However, results from studies conducted by the Minnesota Pollution Control Agency and the National Institutes of Environmental Health Sciences have linked gross deformities observed in frogs in northwestern Minnesota to biologically active agents in the water they inhabit (Burkhart et al. 1998). Specifically, water samples taken from ponds with high incidences of frog malformations (affected sites) were tested in FETAX assays, in which *X. laevis* embryos were exposed to pond water for 96 h and observed for mortality and malformations. The results of these assays were compared with those conducted with water samples taken from ponds with unaffected frog populations (reference sites). The water from affected sites induced mortality and malformations, whereas the water from reference sites did not. The observed malformations were dose dependent and reproducible. Research projects are under way to try to establish a plausible link between exposure to specific chemical contaminants and the appearance of specific deformities under laboratory conditions.

The role of hormones, such as estradiol, testosterone, and corticosterone, in the growth and metamorphosis of toads has been studied in the laboratory (Richards and Nace 1978; Gray and Janssens 1990; Hayes et al. 1993; Hayes 1995; Hayes and Wu 1995a,b). For example, the action of exogenous corticosterone on toads has been shown to closely resemble the effects of exogenous thyroid hormones, suggesting that steroids might interact with endogenous thyroid hormones (Hayes et al. 1993; Hayes 1997). In a study of DDT, male African clawed frogs were induced to synthesize the female yolk protein vitellogenin in the liver after intraperitoneal injection of 1.0 or 250.0 μg *o,p′*-DDT/g body weight for 7 d (Palmer and Palmer 1995). Vitellogenin synthesis is considered a hallmark of exposure to estrogens, but other studies involving DDT suggest that DDT might act as a

corticoid mimic or stressor (Hayes et al. 1997). However, studies are needed of altered hormone concentrations or modifications of the endocrine system of free-living amphibians to explore whether deformities observed in the wild are related to hormonal modification.

Reptiles

Alligators

The American alligator of Lake Apopka, Florida, is one of the most cited examples of a wildlife population affected by environmental toxicants, including HAAs. The work on the alligator in Florida's wetlands began as the population rebounded as a result of protection under the terms of the Endangered Species Act. Studies of the reproductive biology of alligator populations began in the late 1970s to determine whether this animal could sustain annual harvests for their hides. During this work, most of the study lakes showed reduced egg viability and elevated embryonic mortality (Masson 1995). Lake Apopka exhibited a massive reduction in neonatal and juvenile populations and extremely high embryonic and neonatal mortality in the early 1980s (Woodward et al. 1989, 1993). Chapter 10 discusses the population effects in detail.

Lake Apopka is Florida's most polluted lake (EPA 1979a; Schelske and Brezonik 1992). Contamination of the lake has come from extensive agricultural activities around the lake and from sewage and runoff from several municipalities. In addition, there was an accidental release of the pesticide dicofol (contaminated with up to 15% DDT and its metabolites) (Clark 1990) and sulfuric acid (EPA, unpublished report) in 1980 from the Tower Chemical Company. Water samples obtained after the spill showed DDT concentrations ranging from nondetectable to 433 μg/L and dicofol concentrations of 66-150 μg/L (EPA, unpublished report). Sediment sample concentrations of DDT were 31-1611 μg/kg, dry weight, and of dicofol were 6,400-31,000 μg/kg, dry weight (EPA, unpublished report). One report from sampling conducted in 1993 (EPA 1994b) indicates that DDT and its breakdown products were still elevated in sediment samples, as was the pesticide toxaphene.

A study by Heinz et al. (1991) identified high concentrations of various persistent pesticides and their metabolites in alligator eggs collected between 1984 and 1985 from Lake Apopka (Table 5-2). With the exception of toxaphene, dieldrin, and chlordane, the pesticides and metabolites bind to the alligator estrogen receptor (Vonier et al. 1996). No correlation was found between elevated concentrations of organochlorine compounds and poor egg viability (Heinz et al. 1991). However, the mean concentrations of *p,p′*-DDE observed, 5.8 ppm wet weight (1984; range, 3.4-7.6 ppm) and 3.5 ppm wet weight (1985; range, 0.89-29 ppm), are above the concentrations known to reduce hatching success and cause deformities (Cooper 1991). Studies by EPA (1994b) have shown that juvenile

TABLE 5-2 Concentration of Pesticides in 23 Lake Apopka Alligator Eggs

Contaminant	Mean, ppm	Range, ppm
trans-Nonachlor	0.15	0.01–0.68
Dieldrin	0.11	0.02–1.0
Oxychlordane	0.03	ND[a]–0.21
cis-Chlordane	0.06	ND–0.25
trans-Chlordane	0.006	ND–0.05
Toxaphene	2.4	0.05–13
p,p′-DDE	3.5	0.89–29
o,p′-DDE	0.007	ND–0.06
p,p′-DDT	0.02	ND–1.3
p,p′-DDD	0.37	ND–1.8

[a] ND, not detectable.

SOURCE: Heinz et al. 1991. Reprinted with kind permission from *Environmental Monitoring and Assessment;* copyright 1991, Kluwer Academic Publishers, Dordrecht, The Netherlands.

and hatchling alligators from Lake Apopka have elevated lipid (1.6-8.5 ppm) and hepatic (0.013-0.17 ppm) concentrations of *p,p′*-DDE. Those concentrations were significantly higher in hatchlings with developmental abnormalities. Specific reproductive anomalies found in the alligators of Lake Apopka are discussed below.

Morphologic Abnormalities of the Gonad

Alligators hatched from eggs collected from Lake Apopka have exhibited abnormal gonadal morphology. Male juvenile alligators had poorly organized testes with unique, aberrant structures within the seminiferous tubules (Guillette et al. 1994). Some of the germ cells had clear mitotic figures, suggesting that premature spermatogenesis had begun. No mitotic or meiotic activity was seen in the testes obtained from males of the less contaminated reference lake, Lake Woodruff. Female alligators examined at 6 mo had prominent polyovular follicles, and many of the oocytes were multinucleated (Guillette et al. 1994). A normal ovarian follicle contains a single oocyte with a single nucleus. Polyovular follicles contain more than one oocyte, and in the case of the neonatal and juvenile alligators, as many as six discrete oocytes were counted in a follicle. All females hatched from the Lake Apopka eggs exhibited this condition; none of the reference females did (Guillette et al. 1994). Follow-up studies of juvenile alligators from Lake Apopka and the reference lake indicate that some oocytes are multinucleated in females from the control group, but polyovular follicles were not seen in any of the control females (Pickford 1995).

The abnormal morphologies of the ovarian follicles and oocytes in female alligators from Lake Apopka are similar to those observed in mice treated with

DES (Iguchi et al. 1990; Guillette et al. 1994). Mouse polyovular follicles can be stimulated to ovulate and can be fertilized in vitro and in vivo, although the number of eggs fertilized is significantly less than for uniovular follicles (Iguchi and Takasugi 1986; Iguchi et al. 1986, 1990). Fertilized mouse ova from DES-induced polyovular follicles develop to implantation-stage embryos, but the frequency of embryos reaching that stage is significantly reduced compared with embryos derived from uniovular follicles (Iguchi et al. 1991). It has been hypothesized that the low egg viability observed in Lake Apopka could in part result from the greater frequency of polyovular follicles in females from this population (Guillette and Crain 1996). Egg viability on Lake Apopka has averaged less than 20% since the mid-1980s, and embryonic death occurs mainly at the zygote to late gastrula stage (Masson 1995). Additional work is needed to determine the causes and the significance of polyovular follicles in wild populations of alligators. Since Sager et al. (1991) has shown that developmental exposure of male rats to PCBs results in loss of embryos (at the blastocyst stage) they sire as adults, the importance of sperm abnormalities in embryonic loss also has to be considered.

Hormonal Abnormalities

In addition to morphologic abnormalities of the gonad, Lake Apopka male and female neonatal (6 mo old) alligators exhibited abnormal plasma sex steroid concentrations (Guillette et al. 1994, 1995a). Male alligators had greatly reduced plasma testosterone concentrations compared with males from the less-contaminated reference lake. The females from Lake Apopka had plasma estradiol concentrations that were higher than those found in reference females.

In vitro synthesis of estradiol was significantly different from that predicted by plasma concentrations: Ovaries from Lake Apopka females synthesized less estradiol than did ovaries of females from the reference lake (Guillette et al. 1995a). Testes from Lake Apopka males synthesized significantly greater concentrations of estradiol than did those of reference males. Testosterone synthesis from the testes was the same for animals from both lakes. Abnormalities in plasma hormone concentrations observed in hatchlings persisted into later prepubertal stages (Guillette et al. 1997). The current hypothesis is that modifications in gonadal synthesis, hepatic degradation, and plasma-steroid-binding protein concentrations appear to act in concert to modify plasma hormone concentrations in the Lake Apopka alligators (Guillette et al. 1997; Arnold et al. 1996).

Genital Abnormalities

Male alligators from Lake Apopka had significantly smaller penis size than did males from the reference lake (Guillette et al. 1997). Because penis development and size depend on elevated androgen concentrations in alligators (Raynaud and Pieau 1985), the researchers suspect that abnormal androgen concentrations

or functioning were the cause. Although there was a positive relationship between plasma androgen concentrations and penis size in the males from the reference lake, no relationship was observed among male alligators from Lake Apopka, although penis dimensions did exhibit allometric relationships with body size. Differences between collection sites were noted on Lake Apopka. Juveniles in the area of Gourd Neck Spring near the drainage site of a pesticide spill exhibited markedly smaller penises than did juvenile males found along the northwestern shore (Guillette et al. 1996a). One hypothesis is that androgen function was modified during embryonic development and early life (Guillette et al. 1996b). Recent evidence suggests that Lake Apopka males with small penises do not have reduced plasma concentrations of 5a-dihydrotestosterone but do have reduced plasma concentrations of testosterone. Most female alligators exhibited normal secondary sex characteristics at the gross morphologic level. However, a few individuals on Lake Apopka had hypertrophied clitorises (Guillette et al. 1997).

Alligator populations on Lake Apopka exhibited a dramatic decline in numbers and recruitment in the 1980s after the pesticide spill described above. Recruitment problems at first were thought to be associated with habitat modifications—nests and nesting materials were abnormal—but this was not borne out (Masson 1995). The age of the female also can affect egg quality, and although no significant differences in female morphometrics have been noted, females from Lake Apopka are slightly larger (Rice et al. 1996). The isolation of populations also can contribute to inbreeding depression of reproduction, but alligator populations in Florida do not appear inbred. The major watersheds are extensively connected, and adult alligators move over extensive ranges (Abercrombie 1989).

Given the above observations, it has been proposed that the abnormalities observed in the alligators of Lake Apopka can be explained, in part, by hormonal disruption caused by pesticides (dicofol, DDT, and toxaphene) or their metabolites (*p,p′*-DDE) (Guillette et al. 1997). Experimental studies on this species (Crain et al. 1998), other reptiles (Bergeron et al. 1994), and birds (Eroschenko and Palmiter 1980; Biessmann 1982; Rattner et al. 1984; Elliott et al. 1994; Fry 1995) suggest a relationship between the developmental abnormalities observed and exposure to xenobiotics, such as *p,p′*-DDE, is likely.

Turtles

Snapping turtles of the Laurentian Great Lakes region of North America feed near the top of the food chain and are exposed to persistent environmental contaminants that bioaccumulate and biomagnify in the food chain. The turtles have been studied to determine whether there is a connection between environmental contamination and developmental abnormalities (Bishop et al. 1991). Eggs collected from various localities on Lake Ontario, Lake Erie, and the upper St. Lawrence River exhibit elevated concentrations of PCBs, dibenzo-*p*-dioxins,

dibenzofurans, and various organochlorine pesticides (DDT, dieldrin) or their metabolites (*p,p′*-DDE) (Bishop et al. 1991; Hebert et al. 1993; Struger et al. 1993). Eggs containing the greatest concentrations exhibited significantly greater rates of embryonic mortality and deformity (deformed tails and deformed and stunted legs) when compared with eggs from reference sites. Bishop et al. (1996) reported significant increases in PCBs, p,p′-DDE, and dieldrin between 1984 and 1991 in Lake Ontario eggs, and present geographic and temporal patterns demonstrate a large variation in the contaminant load among individual turtles. PCBs and pesticides have been found in turtle embryos throughout organogenesis and muscle and skeletal development (Bishop et al. 1995). As described for the alligator eggs collected from Lake Apopka (Masson 1995), the greater rates of embryonic mortality in the more contaminated snapping turtle eggs occurred early in development (Bishop et al. 1991). Although gross anatomical deformities were noted in many hatchlings from eggs collected at the most contaminated sites, histologic examinations were not performed. Likewise, no hormone data are available. Thus, it is unknown whether the reproductive organs of these animals were abnormal or whether the observed anatomical deformities might have been hormonally mediated. Also, the sex ratio of the offspring was not recorded, which could have had important implications.

Studies of turtles in temperature-shift experiments have revealed that the duration and magnitude of incubation temperature affect gonadal development during roughly the second third of egg incubation (Bull et al. 1990; Wibbels et al. 1991). The experiments indicate that sexual determination is extremely labile during the temperature-sensitive window. Exogenous estradiol can cause gonadal feminization of turtle embryos that are incubated at male-producing temperatures. Higher doses of estradiol result in the production of significantly more female turtles (Crews et al. 1991). Exposure to exogenous estradiol alters gonadal differentiation during the same developmental period (Wibbels et al. 1991), which suggests that temperature and estradiol act in a common pathway. However, the effects of estradiol are not completely understood.

The effects of exposure to various hydroxylated PCB congeners on gonadal development of turtles also have been studied (Guillette and Crain 1996). These congeners induce sex reversal in turtles when applied to the outside of the eggshell; the effects observed are similar to those described above for estradiol. Bergeron et al. (1994) observed that 100 μg 2′,4′,6′-trichloro-4-biphenylol induced 100% sex reversal (based on histologic examination of gonads and internal ducts) in the red-eared turtle, whereas treatment with 100 μg 2′,3′,4′,5′-tetrachloro-4-biphenylol stimulated total sex reversal in 50% of the embryos and partial sex reversal (intersex) in 21% of the embryos. The hydroxy-PCBs used in this study are not found in the environment. It is still not known whether HAAs or any other contaminants might be responsible for the effects observed in wild populations of snapping turtles in the Great Lakes region.

Birds

Studies examining the birds of the Laurentian Great Lakes provided much of the initial data that led to the concept of hormonally active environmental pollutants (Colborn 1990). Contaminant-related effects on the reproductive health of several avian species appear to involve modifications during embryonic development (Colborn et al. 1993; Fox 1992). The studies of interest have shown alterations in sexual behavior, abnormal reproductive morphology, severe developmental abnormalities associated with growth and metabolism, and eggshell thinning.

An important aspect of understanding the potential effects of exposure to HAAs on the development of avian reproductive systems is that estrogen plays an important role in regulating the course of development and adult functioning of the gonads and accessory reproductive structures (Fry 1995). Estradiol in conjunction with other endocrine and paracrine factors, is implicated in the unilateral development of the left ovary and regression of the right ovary. Estradiol also influences whether the embryonic tissues that differentiate into oviducts and shell glands persist or regress, via the interaction of estradiol with Müllerian inhibiting hormone; the action of Müllerian inhibiting hormone is inhibited by estradiol. The active differentiation of female reproductive structures under the action of estrogen is in direct contrast to mammals, in which males are the heterogametic and differentiating sex, and estradiol is not required for differentiation of the ovary; although in mammals, estrogen and functioning estrogen receptors are required for subsequent normal functioning of the ovary (Lubahn et al. 1993). Thus, embryonic exposure to environmental estrogens or other HAAs can generate end points in birds that are different from what might be expected in mammals or other vertebrate species that do not rely on estrogen-induced sexual differentiation. This concept of varying end points among species is essential to explaining the effects observed in wildlife. Such variations do not preclude the use of data from birds or other wildlife to predict abnormalities or effects in other species. The information can be used to help determine which end points are the most likely for a given species and to explain why effects are seen in some groups and not others. Thus, an understanding of the role of estrogen in avian development leads to the prediction that exposure to environmental estrogens could alter sex ratios and feminize male birds even though different outcomes would be predicted in other vertebrates.

Sex Ratios

During the 1950s and 1960s, when organochlorine (e.g., *o,p′*-DDT, PCBs, dioxin) contamination was at its greatest in the United States, an increased incidence of female-female pairings in gull populations was observed in colonies in California and the Great Lakes (Schreiber 1970; Harper 1971; Hunt and Hunt

1973; Gress 1974; Hand 1980; Shugart 1980; Fitch and Shugart 1983; Fry et al. 1987). This phenomenon is usually estimated by documenting the number of nests that contain five or more eggs (supernormal clutch); a single female gull typically lays one to three eggs.

The most dramatic and well-documented example of sex skew occurred in the western gull population on Santa Barbara Island in California from 1968 to 1978 (Hunt et al. 1980). The adult sex ratio in that population was measured by laparotomy of 856 captured birds to be 0.26 males to females. The investigators also calculated the male-to-female ratio by estimating the number of nests on the island (896), the number of nonbreeding birds (200), and, based upon the number of nests with more than three eggs, the percentage of female-female pairs (15%). Using those estimates, the male-to-female ratio was 0.67. Because many birds laid fewer than normal numbers of eggs in 1978, the investigators believe that the estimate for female-female pairs might been too low. Therefore, the ratio of males to females was probably between 0.26 and 0.67.

A supernormal clutch incidence of 0.6% to 1% was documented in northeastern Lake Michigan herring gulls from 1978 to 1981 (Shugart 1980; Fitch and Shugart 1983). Both the California population and the Great Lakes gulls were exposed to great levels of organochlorine contamination, including DDT, during the 1950s to the 1970s (Fry and Toone 1981). Several historical studies have been done to investigate the occurrence of supernormal clutches in gulls using literature sources and museum specimens to determine whether incidences have actually changed in the pre- and post-DDT era. The incidence of supernormal clutches has decreased significantly for many species of terns throughout the United States (Conover 1984a). Supernormal-clutch incidence had only increased significantly in western gulls, herring gulls nesting in the Great Lakes, and Caspian terns breeding in the United States since 1950. Supernormal clutches were a regular occurrence in ring-billed and California gulls before the DDT era, and their occurrence has not changed over time (Conover and Hunt 1984a). In contrast, supernormal clutches were not found regularly in western or herring gulls until after 1950, and the sex ratio for their populations as a whole has changed dramatically for both species toward an excess of females since then. Sex skew and its effects on the population dynamics of gulls are discussed in detail in Chapter 10.

There are a number of hypotheses concerning sex skew. In general, female-female associations in gull colonies are believed to occur when there is a relative shortage of breeding male gulls available. Experimental manipulation of sex ratios in gull colonies by selectively removing males from stable colonies has demonstrated that sex ratio skew alone is sufficient to cause a proportion of the excess females to pair (Conover and Hunt 1984b). Sex skew toward females in western and herring gulls could be due to a differential mortality between males and females; however, such a differential mortality has not been well documented. It is possible that male gulls could be more susceptible to poisoning

from persistent organochlorine contaminants. Male western gulls weigh about 25% more than females on average, and they feed higher up on the food chain (Pierotti 1981). Also, male gulls do not have the ability to excrete lipophilic contaminants by egg-laying. For these reasons, it is expected that male gulls might accumulate greater body burdens of toxicants throughout their lifetimes than females gulls do. It has also been suggested that the skewed sex ratios observed in western gulls in California and in Great Lakes herring gulls might have been caused by estrogenic contaminants, such as DDT, in the environment, either due to a differential male mortality or a feminization of male embryos which resulted in chemical sterilization and a failed recruitment into the breeding population (Fry et al. 1987). This is a plausible hypothesis, but there is no direct evidence to support it. Another hypothesis is that some females might have paired with the wrong sex due to chemically induced masculinization. However, a behavioral study of western gulls in Santa Barbara did not find significant differences in behavior between females mated with other females and those paired with males (Hunt et al. 1984).

In conclusion, there is good evidence to suggest that there has been a fundamental change in the sex ratio of several North American gull populations in the post-DDT era, such that there is an overabundance of females in some breeding colonies. The observations that the colonies most affected were in areas of great DDT contamination and that a few DDT congeners have produced abnormal gonadal development in laboratory studies support the hypothesis that environmental contaminants may have played a role in the sex ratio skew.

Alterations in Behavior

Behavioral abnormalities observed in the wild include aberrant parental behaviors, such as less inclination to sit on eggs or to defend nests, which was observed in herring gulls in Lake Ontario (Fox et al. 1978). Those alterations were sufficient to account for the high incidence of egg loss observed in this population. Because high levels of chemical contamination were found in the gulls, it was suggested that HAAs might be responsible for the behavioral alterations. Laboratory experiments with birds exposed to hormones and some environmental pollutants suggest that this hypothesis is plausible.

For example, male Japanese quail embryos injected with 1 μg estradiol or 500 μg testosterone were completely demasculinized and were behaviorally indistinguishable from females (Adkins 1979; Adkins-Regan 1987). As adults, they failed to mount, crow, or strut. This effect occurred only if treatment was given before d 12 of the 18-d incubation period (Adkins 1979), and it is believed to result from a fundamental change in the neural substrate underlying behavior that confers a differential responsiveness to the activating effects of testosterone in adulthood. Testosterone treatment restores copulation in castrated adult males but is without effect in females. Female quail treated with an antiestrogen before

hatching can be masculinized and as adults will mount other females (Adkins-Regan 1987).

Sexual differentiation of behavior has been extensively studied in the zebra finch, which exhibits sexual behavioral dimorphism; that is, normally only males sing, dance, and mount. The brain of this finch is sexually dimorphic. The telencephalic nuclei greater vocal center, nucleus robustus archistriatalis, nucleus magnocellularis of the anterior neostriatum, and area X of the *lobus* paraolfactorius are larger and more extensively connected in males than in females, and are essential for learning and production of the complex vocalizations of this species (Simpson and Vicario 1991a). The administration of estradiol to female zebra finches during the first week after hatching results in a profound organizational masculinization of brain and behavior (Gurney and Konishi 1980; Simpson and Vicario 1991a; Adkins-Regan et al. 1994), including neural masculinization of telencephalic nuclei that sets up a functional circuit in females similar to that in males, which enables them to learn and produce complex vocalizations (Simpson and Vicario 1991b). When the treated females are stimulated as adults with testosterone, they engage in male behavior of singing and dancing (Adkins-Regan et al. 1994). Males treated with estradiol during the first week after hatching are demasculinized, and they fail to mount as adults (Adkins-Regan et al. 1994). Thus, the pattern of sexual-behavioral differentiation in the zebra finch is quite complex. It is clear from these studies and those involving quail that the process of sexual behavioral differentiation in birds is sensitive to exogenous hormones, and that hormonal manipulation can result in a profound and permanent change in reproductive behavior in both sexes.

In laboratory studies with contaminants, ring doves were fed mixtures of DDE, PCBs, mirex, and photomirex (contaminants found in salmon and gulls of Lake Ontario) during mating. The feed of the low-dose group contained 8 ppm Aroclor 1254, 1.67 ppm DDE, 0.297 ppm mirex, and 0.0954 ppm photomirex; the high-dose diet contained 29.03 ppm Aroclor 1254, 4.61 ppm DDE, 0.897 ppm mirex, and 0.324 ppm photomirex. The doves had reduced or delayed behaviorally induced increases of sex hormones, females failed to respond normally to male courtship behavior, pairs spent less time building their nests, and pairs receiving the greatest dosage spent less time feeding their young (McArthur et al. 1983). There was a marked dose-related decrease in fledgling success, and the breeding cycle was greatly asynchronous. In other studies, adult breeder doves fed PCBs exhibited aberrant incubation (Peakall and Peakall 1973) and courtship (Tori and Peterle 1983). Thus, there is evidence from laboratory studies that environmental contaminants in the Great Lakes region could cause behavioral anomalies in breeding synchrony, nest construction, incubation attentiveness, and parental care at ambient concentrations but these effects are not necessarily attributable to their hormonal activities.

Abnormal Reproductive Morphology

Most studies of abnormal breeding in gull populations were conducted during the mid-1970s. Fifty-seven percent of the male gull embryos collected from Scotch Bonnet Island, Canada, in 1975 and 1976 had testicular feminization (Fox 1992). Eggs at that site were contaminated with dioxins, PCBs, and mirex (Gilman et al. 1979; Fox 1992). One study of a tern colony also showed a high incidence of abnormal tests indicative of estrogenic exposure in ovo (Calambokidis et al. 1985; Nisbet et al. 1996), but the contaminants that could mediate these effects have not been identified. However, the significance of these findings is unclear because there are reports (going back to the 1800s) of apparently abnormal testes in terms as embryos or hatchlings, and this might be a normal condition that disappears as the birds age (Hart 1998).

Some studies have evaluated the reproductive morphology of adult birds. For example, 31 adult female glaucous-winged gulls collected in 1984 from Tacoma, Washington, adjacent to the Commencement Bay, Puget Sound (a PCB- and heavy-metal-contaminated Superfund site), were trapped on their nests and killed for gonadal inspection (Calambokidis et al. 1985). The right oviducts of these gulls were found to be persistent and large. The length of the right oviduct was correlated with the estimated chemical contamination (Fry et al. 1987). However, the significance of these data are unclear, as all birds were successfully incubating clutches. Furthermore, the most severe category of oviduct enlargement was rated as greater than 10 mm long; the literature indicates that a vestigial right oviduct of 9-10 mm is normal in the herring gull (Boss and Witschi 1947). An attempt to correlate alterations in testes in male birds with organochlorine contamination in this gull population was inconclusive (Fry et al. 1987).

Ovotestis formation in male embryos and retention of the right oviduct in female embryos also were observed in experimental studies of gull eggs injected with hormones such as estradiol (Fry and Toone 1981) and DES (Boss and Witschi 1947) and with environmental contaminants such as methoxychlor and DDT (Fry and Toone 1981). Chicken and quail eggs injected with DDT showed similar effects (Lutz-Ostertag and David 1973). Because concentrations of DDT (2-100 ppm) found in the eggs of wild gulls caused effects consistent with those induced by estradiol and DES (Fry and Toone 1981), it is plausible that DDT or other estrogenic contaminants could be responsible for the effects observed in the wild.

Most morphologic abnormalities in the wild have been found near areas identified as hot spots of organochlorine contamination. Residues of PCBs, TCDD-EQ, and DDT are approximately 10-fold greater than those in other locations (Giesy et al. 1994b). Concentrations of many of the residues are declining in the Great Lakes, but are still among the highest. Green Bay and Saginaw Bay also have hot spots, where concentrations of organohalogen compounds are significantly greater than the Great Lakes as a whole (Giesy et al. 1994b).

Growth and Development Abnormalities

A group of embryonic abnormalities directly related to contaminant exposure in some fish-eating birds has been defined as a specific syndrome, GLEMEDS (Great Lakes embryo mortality, edema, and deformity syndrome) (Gilbertson and Fox 1977; Gilbertson et al. 1991). GLEMEDS involves a consistent pattern of subcutaneous edema, beak malformations, cardiac edema, and skeletal malformations. The expression of this syndrome in bald eagle, cormorant, gull, and tern chicks is correlated with dioxin toxic equivalents of some PCB congeners that are primarily the result of maternal bioaccumulation from eating contaminated fish and resultant deposition of coplanar PCB congeners in the eggs (Gilbertson et al. 1991). Adults from populations in which chicks have GLEMEDS have shown abnormal plasma thyroid hormone concentrations and thyroid morphology (Fox 1992), but no relationship between thyroid hormonal disfunction and GLEMEDS has been found. Several sources of organochlorines have been controlled in response to regulatory action in the early 1970s, and concentrations of DDT and PCBs in fish tissues decreased approximately 20-fold in the late 1970s and early 1980s. However, no change was found between 1985 and 1992 in chinook salmon (Miller 1994), and these contaminants continue to persist in tissues and the environment today.

Eggshell Thinning

During the 1960s and 1970s, when the pesticide DDT and its metabolite DDE were present at higher concentrations than today in North America, it was observed that populations of several bird species declined when individuals were unable to successfully incubate eggs because of abnormally thin eggshells (Cooke 1973). Many of these species, such as the double-crested cormorant, have experienced dramatic population increases since DDT was banned from use in the United States (Ludwig 1984; Weseloh and Ewins 1994). It is now well established that the DDT metabolite, DDE, and to a lesser extent other organochlorines, causes eggshell thinning (for a review, see Cooke 1973). Research into the mechanism of DDE-induced eggshell thinning has been extensive (Gould 1972; Peakall et al. 1975; Cooke et al. 1976; Miller et al. 1976; Eastin and Spaziani 1978; Cooke 1979; Lundholm 1980, 1982, 1984a,b,c, 1985, 1987, 1988, 1993, 1994; Lundholm and Mathson 1983; Lundholm and Bartonek 1991, 1992; Haynes and Murad 1985). Some of the postulated mechanisms include premature termination of shell formation, premature oviposition, effects on the protein matrix of the shell, effects on initiation sites of shell formation, enhancement of shell-growth inhibitors, decrease in carbonate availability for shell formation, effects on progesterone binding in the shell-gland mucosa, and alteration in calcium metabolism of the shell gland. The current hypothesis regarding the mechanism of DDE-induced eggshell thinning is an inhibition of prostaglandins by the shell-

gland mucosa (Lundholm and Bartonek 1992). Many of the biochemical end points described above are interrelated, and it has been difficult to determine which end points are the direct targets of DDE and which are merely coinfluenced by its action. However, it does not appear that eggshell thinning is a result of DDE acting as a hormone-receptor agonist or antagonist. The situation is complicated further because sensitivities to DDE-induced eggshell thinning vary among avian species, suggesting that different mechanisms cause eggshell thinning in different species.

Florida Panther

The possibility that exposure to HAAs affects reproduction in the endangered Florida panther has generated considerable interest. The population size is estimated at 30-50 animals of two genetic strains (O'Brien et al. 1990). Most of the panthers exhibit developmental abnormalities (including congenital heart defects) and defects of the reproductive system (cryptorchidism, low sperm density, and sperm defects) (Barone et al. 1994). Reproductive abnormalities had been attributed to genetic inbreeding (Miththapala et al. 1991; Roelke et al. 1993), but a study by Facemire et al. (1995) examining contaminant loading in female panthers has led investigators to conclude that persistent, bioaccumulated contaminants, such as organochlorines, also could contribute to the problems observed. Because the Florida panther is an endangered species, tissue samples for analysis are rare. Three females were examined after death for concentrations of mercury and several bioaccumulated organochlorine compounds. Concentration ranges of various contaminants found in the muscle (μg/g lipid fresh weight) of the animals were 5.45-57.65 μg/g *p,p′*-DDE; 7.32- 27.06 μg/g Aroclor-1254; <0.0098-2.00 μg/g oxychlordane; and <0.0098-4.82 μg/g *trans*-nonachlor.

The most frequent developmental abnormality in the Florida panther population is cryptorchidism or testicular nondescent. Cryptorchidism has increased exponentially in male cubs since 1975 (Roelke 1990), and 70% of wild Florida panthers are at least unilaterally cryptorchid, as compared with 20% in the mid-1980s (Roelke et al. 1993). Most of the male panthers exhibiting cryptorchidism have the testis in an inguinal location. A comparative study of free-ranging and captive panthers from Florida, Texas, Colorado, Latin America, and North America indicates that the incidence of cryptorchidism in the Florida panther is more than ten times that found in other populations (Barone et al. 1994). Only two cases of cryptorchidism have been reported in captive populations of North American panthers; the condition has never been reported in any other large felid (Roelke et al. 1993). Cryptorchidism is a heritable trait in some inbred domesticated species (McPhee and Buckley 1934; Claxton and Yeates 1972) that could be a response to an abnormal hormonal environment during embryonic development (Hezmall and Lipshultz 1982; Sharpe and Skakkebaek 1993; Hutson et al. 1994).

Müllerian-inhibiting hormone has been implicated in testicular descent (Hutson et al. 1994), but no data are available on the influence of HAAs on the synthesis of that hormone during gonadal development. In studies with rats, prenatal exposure to the antiandrogenic metabolites of the fungicide vinclozolin caused hypospadias, cleft penis, and suprainguinal (cryptorchid) ectopic testes (Gray et al. 1993; Kelce et al. 1994); exogenous treatment of rats with the androgen dihydrotestosterone stimulates testicular descent (Frey et al. 1983). Treatment of male mice with DES on d 9-16 in utero led to an increase in the incidence of cryptorchidism (McLachlan et al. 1975). Male mammals lacking either adequate concentrations of androgen or androgen receptors exhibit a high incidence of cryptorchidism (Wilson and Foster 1985). Those data indicate that compromised (or altered) androgen receptors—because of receptor abnormalities or because of the presence of an antiandrogen or potent estrogen—preclude normal testicular descent during development in mammals. The elevated concentrations of *p,p′*-DDE present in the tissue of three female Florida panthers (Facemire et al. 1995) and the knowledge that this metabolite of DDT is known to exhibit antiandrogenic activity (Kelce et al. 1995) suggest a relationship between contaminant exposure and cryptorchidism in Florida panther cubs. However, the tissue of panthers has been shown to be contaminated with a variety of toxic substances, including mercury (Roelke et al. 1991).

Electroejaculation studies of 12 male Florida panthers have shown low sperm density, poor sperm motility, and elevated numbers of sperm defects (Facemire et al. 1995). Sperm density (concentration/milliliter) averaged $4.8 \pm 1.4 \times 10^6$ sperm in Florida panthers compared with $15.4 \pm 4.4 \times 10^6$ for males from a Texas population and $22.5 \pm 9.2 \times 10^6$ in Latin American populations (Barone et al. 1994). The male panthers studied had 24-50% more sperm abnormalities than were found in Texas panthers and significantly smaller testicular volumes (Barone et al. 1994). Furthermore, males had abnormal sex-steroid ratios, exhibiting higher concentrations of estradiol-17β than testosterone in plasma (Facemire et al. 1995). As yet, there are no exposure data for other populations against which the contaminant concentrations in Florida panthers can be compared.

Although the available evidence suggests that the reproductive anomalies could be the consequence of environmental contaminants, the role of extensive inbreeding in this small population cannot be discounted.

SUMMARY AND CONCLUSIONS

Several reproductive and developmental disorders have been observed in wildlife and human populations exposed to environmental contaminants, including HAAs. Laboratory studies using male and female rats, mice, and guinea pigs, and female rhesus monkeys have shown that exposure of these animals during development to certain HAAs (e.g., DDT, methoxychlor, PCBs, dioxin, bisphenol A, octylphenol, BBP, DBP, chlordecone, and vinclozolin) can produce structural

and functional abnormalities of the reproductive tract. Some of these studies, according to the investigators, were conducted using doses at or near levels encountered in the environment, but in most instances the environmental relevance of the dose used is unknown, because of lack of data concerning the level of environmental contamination.

With the exception of PCBs, TCDD, and DDT and its metabolite DDE, there are few human studies on the reproductive and developmental effects of exposure to HAAs. The effects of prenatal exposure to PCBs, DDE, and other contaminants from maternal consumption of contaminated fish or other food products has been studied in several populations in the United States and abroad. Collectively, these studies indicate that prenatal exposure to PCBs can cause lower birth weight and shorter gestation, and have also been correlated with IQ and memory deficits as well as delayed neuromuscular development. Pre- and post-natal exposure to PCBs and PCDFs from accidental contamination of rice oil in Yusho, Japan and Yu-Cheng, Taiwan have resulted in various developmental defects.

Exposure of men to environmental HAAs has been suggested as the cause of worldwide increases in hypospadias, cryptorchidism, testicular cancer, and declines in sperm concentration. Studies examining these trends show considerable variation, both temporally and geographically. The degree to which the results reflect differences in the populations selected for study (fewer men of proven fertility, or men with concerns about their sperm concentration), diagnostic practice, or other methodologic differences, is the subject of continued controversy. With respect to the end point most closely studied, sperm concentration, retrospective analyses of trends over the past half-century remain controversial. When the data from large regions are combined together and analyzed, some data sets indicate a statistically significant trend consistent with declining sperm concentrations. However, aggregation of data over larger geographic regions may not be an appropriate spatial scale for this analysis given significant geographic heterogeneity in genetic and environmental factors. The current data are inadequate to assess the possibility of trends within more appropriately defined small regions. Acquiring data at smaller regional scales is critical to assessing the significant geographic variation in sperm concentration which is the subject of collaborative studies currently being conducted in the United States (funded by NIEHS), Europe (funded by the European Union), and Japan (funded by the Japanese EPA).

Many wildlife studies show associations between reproductive and developmental defects and exposure to environmental contaminants, some of which are HAAs. One of the best established linkages between exposure to an environmental contaminant and reproductive effects in birds has been the correlation of DDT, and its metabolite DDE, with eggshell thinning. Many potential mechanisms for DDE-induced eggshell thinning have been described. The most current hypothesis is that the mechanism involves an inhibition of prostaglandin by the shell-

gland mucosa. However it does not appear likely that this is a result of DDE acting as a hormone receptor agonist or antagonist.

Reproductive and developmental abnormalities have also been observed in several populations of fish exposed to effluents from sewage treatment plants and paper mills and polluted waters of the Great Lakes. Effects observed include intersexes in trout exposed to sewage treatment plant effluent (STPE); increased egg and fry mortality in Great Lakes trout and salmon; thyroid enlargement in Great Lakes salmon; and changes in plasma sex-steroid concentrations, decreased egg and gonad size, and delayed sexual maturity in whiter suckers exposed to effluents from paper mills along Lake Superior.

Laboratory experiments with specific HAAs found in those effluents and polluted waters have produced effects consistent with these wildlife observations. For example, certain HAAs found in STPEs induce estrogenic responses in male trout. Specifically, ethinylestradiol and alkylphenol ethoxylates have been shown to induce vitellogenin synthesis, a hallmark of estrogen exposure, and to decrease the rate of testicular growth in male fish in tests that duplicate concentrations found in some effluents. Dioxin and structurally related compounds have been shown to induce blue sac disease in trout and reduced growth and survival of salmon. Thyroid enlargement in salmon of the Great Lakes is hypothesized to be caused by exposure to PCBs, which also have been shown to induce goiter formation in laboratory rodents fed PCB-contaminated salmon. Finally, B-sitosterol found in paper-mill effluent has been shown to alter the reproductive physiology of goldfish under experimental conditions.

Laboratory studies are also consistent with some reproductive and developmental abnormalities (e.g., skewed sex ratios, behavioral modifications, and morphologic abnormalities of the gonads) observed among North American gull populations. Specifically, it has been shown that gull eggs injected with DDT at concentrations found in wild gull eggs induce gonadal abnormalities that are similar to those observed in contaminated gulls. Also, doves fed mixtures containing DDE and PCBs exhibit abnormal breeding behavior.

Similarly, defects seen in alligators from Lake Apopka (the site of a chemical spill containing dicofol and DDT) including small penis size and abnormal testes in males and abnormal ovaries in females, are consistent with structural and functional reproductive abnormalities that occur following perinatal exposure of laboratory rodents to estrogenic and antiandrogenic chemicals.

It has also been suggested that cryptorchidism, the most common reproductive anomaly found in male Florida panthers, is the result of exposure to p,p′-DDE. Because testicular descent is in part androgen dependent, and because antiandrogens and potent estrogens have induced cryptorchidism in rats and mice, it is plausible that exposure to contaminants with antiandrogenic or estrogenic properties could be causing the effects in male panthers. However, the Florida panther population is exposed to many other contaminants, including methoxy-

chlor, PCBs, and mercury, and the role of extensive inbreeding in this small population cannot be discounted.

RECOMMENDATIONS

Based on evaluation of reproductive and developmental effects observed in humans, laboratory animals, and wildlife exposed to HAAs, the committee recommends that wildlife and human populations continue to be monitored for adverse developmental and reproductive effects. Specifically, the committee recommends the following:

—Studies of wildlife that exhibit population declines, abnormal sociosexual behavior, or deformities should be designed to investigate those phenomena in light of specific environmental factors, including chemical contamination and environmental degradation.

—Prospective and cross-sectional studies, using common protocols and strict quality control, be conducted in human populations suspected of being affected by HAAs. Serum hormone concentrations, body burdens of HAAs, and sperm concentration in seminal fluid should be measured, especially in relation to any adverse effects, or banked for later exposure assessment. Prospective and cross-sectional studies are particularly needed on cohorts tracked from conception through adulthood on female and male reproductive end points such as sperm concentration, cryptochidism, and hypospadias.

—Regional differences in male reproductive end points such as sperm count and rates of hypospadias, cryptorchidism, and testicular cancer should be examined prospectively to determine whether the differences can be associated with genetic and environmental factors. Such prospective analyses should be accompanied by quantitative sensitivity analyses.

—Free range farm animals should be studied for potential effects of environmental contaminants on fertility.

6

Neurologic Effects

MOST OF THE AVAILABLE DATA on the neurologic effects of hormonally active agents (HAAs), particularly in humans, are limited to polychlorinated biphenyls (PCBs) and dioxins. A connection has been hypothesized between prenatal and postnatal exposure to HAAs and disturbances in neurological and behavioral development. Possible mechanisms underlying such interactions have been suggested. In humans particularly, a number of difficulties are associated with the investigation of potential neurological and behavioral effects of exposure to HAAs in utero, including the recognition of such effects that may be quite subtle, and the possible long delay between exposure and outcome. The objective of this chapter is to review and analyze the available information in this case.

ANIMAL STUDIES

Studies with laboratory animals indicate that PCBs can cause impaired locomotor ability in rodents, impaired learning in rodents and monkeys, and impaired cognition in monkeys. For example, offspring of female mice treated with 32 mg/kg body weight (BW) 3,4,3′,4′-tetrachlorobiphenyl on d 10-16 of gestation showed signs of neurotoxicity (intermittent stereotypic circling, head bobbing, and hyperactivity) in adulthood (Tilson et al. 1979). In a prenatal study with rats, the offspring of female rats fed 30 mg/kg Clophen A30 (a mixture of PCBs) in their diet 60 d prior to mating until postnatal d 21 had significant changes in active avoidance learning, operant conditioning, and open-field ambulations (Lilienthal et al. 1990; Lilienthal and Winneke 1991). Impaired learning and altered activity levels, such as increases in the reactivity to aversive events, were observed in rats fed a 30% diet of Lake Ontario salmon, which are contaminated

with PCBs, DDT, DDE, mercury, and dioxin (Daly et al. 1989; Daly 1991). The offspring of monkeys fed 1.0 ppm Aroclor 1016 (a mixture of PCB congeners) during gestation and lactation were impaired in their ability to perform spatial discrimination-reversal learning tasks, but this was not observed in offspring of monkeys exposed to 0.25 ppm Aroclor 1016 or 1.0 ppm of Aroclor 1248 (Schantz et al. 1989). These varied effects with the amount of exposure and PCB congener type, suggest the importance of PCB congener identification in the measures of human exposure. In addition, significant deficits in cognitive ability were observed in infant and juvenile monkeys born 3 yr after maternal exposure to 2.5 ppm Aroclor 1248 had ended (Schantz et al. 1989). Possible mechanisms that have been suggested include alterations in neurotransmitter function (Rosin and Martin 1981), intracellular signaling mechanisms (Kodavanti et al. 1993, 1994, 1996), gonadal hormones (Tilson et al. 1979), and thyroid hormone function (Bastomsky and Murthy 1976; van den Berg et al. 1988). The structure of the PCB congener and the age of the animals at the time of exposure were shown to influence the effects (Seegal 1996). Furthermore, it is possible that some congeners influence the development of neurochemicals indirectly by altering the concentrations of thyroid hormones and neuroactive steroids (Seegal 1996). Schantz et al. (1995) reported on spatial learning behavior that was altered in adult rats by exposure to three PCB congeners, but thyroid hormone function was altered by only two of those. This highlights the complexity of the problem of teasing apart neurobehavioral and neurodevelopmental effects and mechanisms for cognitive functioning.

HUMAN STUDIES

Neurobehavioral effects of exposure to PCBs and other chemicals that might act as HAAs have been studied extensively in four human populations, and studies are continuing in three other populations (Table 6-1). All but one of the studies focus on neurodevelopment, and most of the studies involve PCBs, polychlorinated dibenzofurans (PCDFs), DDE, and their metabolites.

Yusho, Japan

In 1968, an industrial accident in Yusho, Japan, caused PCBs that were being used to clarify rice cooking oil to leak into the product. The leak went undetected for 9 mo, and it is estimated that 1,700 people were exposed (Kuratsune et al. 1972). Data on the developmental neurotoxicity from this incident are sparse, but reports described prenatally exposed children as dull, apathetic, and hypotonic and having subnormal intelligence (Harada 1976; Urabe et al. 1979). Little data on exposure concentrations were available, so no dose-response relationship could be demonstrated. However, this incident did serve to warn epidemiologists of the possible neurologic effects of PCB exposure.

Taiwan, China

In May 1979, an unusual cluster of symptoms, characterized by chloracne and hyperpigmentation of the skin and nails, was reported in Taiwan (Hsu et al. 1985). By October 1979, PCBs and their heat-degraded products in samples of rice cooking oil were identified as the cause of the outbreak. In addition to PCBs, the contaminated oil contained an unusually high concentration of PCDFs, which can be more toxic than PCBs and could explain some of the reported effects (Schantz 1996). The resulting symptom complex, Yu-Cheng disease, was similar to that described in Yusho, Japan, ascribed to PCB-contaminated rice cooking oil. By the end of 1980, 1,843 cases had been reported in four central-Taiwan counties. Most were classified as mild (40.0%) or moderate (26.2%). Most of the affected population were between the ages of 11 and 20, and most were students or factory workers. It was estimated that PCB intake was 0.7-1.84 g, and the onset of clinical signs occurred 3-4 mo after exposure (Hsu et al. 1985).

Between 1979 and 1983, 39 babies with hyperpigmentation were born to exposed mothers. Their mortality rate was high; eight infants died of a variety of causes. The registry of Yu-Cheng, maintained by the Taiwanese Bureau of Disease Control, identified 128 surviving children born to 74 exposed women between June 1978 and March 1985. Over the next 2 decades, 118 of the children (born to 69 mothers) were followed to identify the cognitive-development effects of prenatal (transplacental) and postnatal (breast milk) exposure to PCBs (Hsu et al. 1985; Rogan et al. 1988; Yu et al. 1991). Exposure data were incomplete: Serum PCB concentrations were available from 61 of 69 exposed mothers (mean: 49.3 ppb; maximum: 456 ppb), serum concentrations were listed for 21 of 118 children (mean: 1 ppb; maximum: 77.8 ppb), and only fragmentary dietary data were available for the mothers. All of the exposed mothers in the study, however, had exposure-related symptoms at the time of enrollment. Control children were matched for neighborhood, age, sex, mother's age, educational level, and occupation of both parents. Testers were unaware of the child's exposure status (unless revealed by the parents' chloracne). Between the ages of 4 and 7 yr, there was a consistent, statistically significant, 5-point difference in the Chinese versions of the Stanford Binet test and Wechsler Intelligence Scale for Children, Revised (WISC-R) between exposed and control children. However, there was no relationship between either the mother's or the child's PCB serum concentration and developmental outcome. When the children's scores were arrayed by year of birth, there was some moderation in the effect among the 4- and 5-yr-olds born the longest after exposure. However, data from the 6- and 7-yr-olds indicated that children born up to 6 yr after the mothers' exposure and those born within 1 yr of exposure were equally affected (Chen et al. 1992).

The Japanese and Taiwanese studies demonstrate developmental delays and cognitive defects in young children prenatally exposed to PCBs and other products of heat-degraded PCBs. Because the studies were designed to assess the

TABLE 6-1 Neurologic Effects Associated with Exposure to PCBs[a] and Other Compounds

Location	Exposure	Route of Exposure	Biomarker of Exposure	Test	Age	Findings	Reference
Yusho, Japan	PCBs	Contaminated cooking oil	None	NS[b]	NS	Prenatally exposed children described as dull, apathetic, hypotonic, and as having subnormal intelligence	Harada 1976; Urabe et al. 1979
Taiwan, China	PCBs, PCDFs[c]	Contaminated cooking oil	Maternal and child serum	Cognitive development	4-7 yr	Consistent 5-point difference in IQ; no dose response	Rogan et al. 1988; Chen et al. 1992
Lake Michigan	PCBs	Contaminated fish	Maternal and umbilical cord serum (PCBs not detected in 70% of cord serum and 22% of maternal serum), breast milk	Visual recognition	7 mo	Dose-dependent decrease in score for preference for novelty in prenatally exposed children; no effects from postnatal exposure	Jacobson et al. 1985
				Cognitive development	4 yr	Dose-dependent decrease in short-term memory function in verbal and quantitative tests of prenatally exposed children; no effects from postnatal exposure	Jacobson et al. 1992
				Cognitive processing	4 yr	Prenatal exposure associated with less efficient visual discrimination and errors in short-term memory; no effects from postnatal exposure	Jacobson et al. 1992

				Cognitive development	11 yr	Prenatal exposure associated with significantly lower full-scale and verbal IQ scores; no effects from postnatal exposure	Jacobson and Jacobson 1996
North Carolina	PCBs, DDE[d]	Environmental	Maternal serum, umbilical cord blood, placenta, breast milk	Behavioral development	<1 mo	Prenatal exposure to PCBs and DDE significantly related to tonicity, reflex cluster scores	Rogan et al. 1986b
				Mental and psychomotor development	6, 12 mo	Transplacental exposure to PCBs associated with lower psychomotor scores at both ages, but not mental scores; postnatal exposures to PCBs had no effect on either test score; no consistent effects from DDE exposure	Gladen et al. 1988
				Mental and psychomotor development	18, 24 mo	Delay in motor maturation up to 24 mo associated with prenatal exposure to PCBs; no effects from postnatal exposure; no consistent effects from DDE exposure	Rogan and Gladen 1991
				Mental and psychomotor development	3-5 yr	Developmental changes observed at younger ages in children prenatally exposed were no longer observed	Gladen and Rogan 1991

(table continues)

TABLE 6-1 Continued

Location	Exposure	Route of Exposure	Biomarker of Exposure	Test	Age	Findings	Reference
Oswego, New York	PCBs, HCB,[e] PCDDs,[f] dieldrin, lindane, chlordane, cadmium, mercury, mirex	Contaminated fish	None	Neurobehavioral development	12-48 hr	Highly exposed infants had poorer reflex functioning and greater autonomic immaturity	Lonky et al. 1996
Groningen and Rotterdam, Netherlands	PCBs, PCDDs, PCDFs	Contaminated dairy products, industrial oils	Maternal and umbilical cord blood, breast milk	Neurobehavioral development	10-21 d	Concentrations of PCBs in maternal and cord sera not related to neurologic dysfunction, but higher concentrations of PCBs, PCDDs, and PCDFs in breast milk associated with hypotonia; no severe neurologic effects observed	Huisman et al. 1995a
				Neurobehavioral development	18 mo	Significant reduction in neurologic function in prenatally exposed children; little association seen with lactational exposure	Huisman et al. 1995b
				Mental and psychomotor development	3,7, and 18 mo	Prenatal PCB exposure had a small negative effect on psychomotor score at 3 mo. PCB and dioxin exposure from breast feeding had an adverse	Koopman-Esseboom et al. 1996

						effect on psychomotor outcome at 7mo. Mental outcome at 7 mo was positively influenced by breast feeding, but prenatal exposure to PCBs and dioxins does not. At 18 mo, no effect on mental or psychomotor development found.	
				Cognitive development	3.5 yr	Prenatal exposure to PCBs was significantly associated with lower cognitive scores, but postnatal exposure and current exposure at 3.5 yr was not.	Patandin et al. 1999
				Neurobehavioral development	3.5 yr	No effect from prenatal, postnatal, or current exposure was found.	Lanting et al. 1998
Germany	PCBs	Environmental	Umbilical cord blood, breast milk	Neurologic optimality	10-20 d	No effect on neurologic development	Winneke et al. 1998
				Cognitive and motor development	7 mo	No significant effects on cognitive or motor development	Winneke et al. 1998
Great Lakes	PCBs, DDE	Contaminated fish	Blood	Neuropsychologic performance	≥50 yr	Data currently under analysis	Schantz et al. 1996

[a] PCBs, polychlorinated biphenyls.
[b] NS, not stated.
[c] PCDFs, polychlorinated dibenzofurans.
[d] DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene.
[e] HCB, hydrochlorobenzene.
[f] PCDDs, polychlorinated dibenzo-*p*-dioxins.

effects of accidental exposures, there was little opportunity for consistent assessment of PCBs in cord blood or in maternal and infant serum. Thus, lack of good exposure measures makes assessment of a dose-response relationship difficult (Schantz 1996).

Lake Michigan

A series of studies on the cognitive development in offspring of women exposed to low concentrations of PCBs in their diet was conducted by Jacobson and Jacobson (1996) and Jacobson et al. (1985). A sample group of white, urban, predominantly middle-class women was recruited between 1980 and 1981 from four western Michigan hospitals for a longitudinal study (Michigan/Maternal Infant Cohort Study) of intrauterine and postnatal PCB exposure. The initial sample included 313 subjects: 242 women who reported moderate consumption of Lake Michigan sport fish (at least 11.8 kg during a 6-yr period) and 71 women (chosen as a 4.6% random sample) who reported no consumption of fish. Umbilical cord blood, maternal blood samples taken shortly after delivery, and breast milk samples taken 0.2-4.5 mo (median 1 mo) after delivery were analyzed for PCBs. PCBs were not detected in 70% of cord serum samples or in 22% of maternal serum samples. The mean concentrations of PCBs were 6 ng/mL in maternal serum, 3 ng/mL in cord serum, and 841 ng/g in breast milk.

The children of the exposed women were tested at 7 mo old with Fagan's test of visual recognition. The investigators controlled for infants whose gestational age was less than 38 wk by excluding them from the analysis, and they controlled for confounding variables. Preference for novelty decreased in a dose-dependent fashion with increasing prenatal PCB exposure, although postnatal exposure was not related. The effect of PCB exposure on recognition memory in newborns was not mediated by smaller body size or by behavioral deficits associated with PCB exposure; infants performing poorly at 7 mo had not necessarily exhibited deficits at birth (Jacobson et al. 1985).

When the same cohort of infants was tested at 4 yr (n = 236), prenatal exposure to PCBs was a good predictor of poorer short-term memory function on verbal and quantitative tests in a dose-dependent fashion; those effects persisted after controlling for a broad range of confounding variables (Jacobson et al. 1992). When the same children were tested on tasks designed to evaluate cognitive processing, prenatal exposure to PCBs was associated with less-efficient visual discrimination and with more errors in short-term memory, but not with a lack of sustained attention. Although much greater quantities of PCBs are transferred postnatally through breast-feeding, postnatal exposure was unrelated to cognitive and visual performance, suggesting that the critical period of exposure is during prenatal development (Jacobson et al. 1992).

Jacobson and Jacobson (1996) published a follow-up of this cohort assessed at 11 yr old. They examined 212 children, 68% of the 313 newborns studied in

1980-1981. Blood samples were analyzed for seven organochlorine pesticides and for lead. Hair samples also were taken for mercury analysis. Of the 212 children, 176 had been classified as fish-eaters. Children were tested at home at a mean age of 11 ± 0.2 yr for reading comprehension and with the WISC IQ test by testers blind to the exposure history of the child. Prenatal exposure to PCBs was associated with significantly lower full-scale and verbal IQ scores ($p = .02$). The IQ score of the most highly exposed group (1.25 μg/g breast milk, 4.7 ng in cord serum, or 9.7 ng in maternal serum) averaged 6.2 points lower than the other four groups after adjusting for potential confounders, including socioeconomic status, maternal age, age at testing, delivery complications, maternal drinking and smoking, parity, and number of children in household ($p = .007$). The deficit in IQ in the highly exposed children is similar to the effect of low-concentration exposure to lead (Bellinger et al. 1992). This highly exposed group was also three times more likely to have low average IQ scores ($p < 0.001$) and to have a twofold increased risk of being 2 yr behind in reading comprehension ($p = 0.03$). Intellectual impairments occurred only in relation to transplacental exposure, consistent with other studies. The investigators concluded that "in utero exposure to PCBs in concentrations slightly higher than those in the general public can have a long-term impact on intellectual function" (Jacobson and Jacobson 1996). Although further corroboration from other studies is needed, this finding is consistent with the Jacobsons' earlier work (Jacobson et al. 1985, 1990, 1992) and with that of Lonky et al. (1996), reported below.

Several criticisms of the Jacobson studies must be considered (Paneth 1991; Schantz 1996; Seegal 1996). It has been charged that the work suffers from sample selection bias, differential loss to follow-up of the study cohort, and incomplete or inconsistent exposure assessment. Paneth (1991) questions the initial sample selection and comparability between exposed and control groups. The control group was slightly less than one-third the size of the exposed group (n = 71) (Schantz 1996).

Taken together, those criticisms suggest that the studies should be interpreted cautiously (Schantz 1996; Seegal 1996). However, Seegal (1996) points out that the Jacobson studies are the most intensive and detailed work to date on cognitive and developmental effects of human in utero exposure to complex mixtures.

Recently, the Agency for Toxic Substances and Disease Registry (ATSDR) evaluated the public-health implications of persistent toxic substances in the Great Lakes and St. Lawrence basins (Johnson et al. 1998). With regard to neurologic effects, ATSDR noted that "The findings of elevated PCB levels in persons who consume large amounts of Great Lakes-St. Lawrence fish, together with findings of developmental deficits and neurologic problems in children of fish-consuming mothers, are compelling." It was concluded that "neurobehavioral and developmental deficits occur in newborns and continue through school-age children from in utero exposure to persistent toxic substances, e.g., PCBs."

North Carolina

Rogan et al. (1986a) conducted the North Carolina Breast Milk and Formula Project, a prospective birth-cohort study of 930 children born between 1978 and 1982. These children were followed to study the possible neurologic consequences of prenatal and postnatal exposure to environmental concentrations of PCBs and DDE. Women were recruited at or near term from hospitals, childbirth classes, and private and public prenatal clinics. No attempt was made to assemble a random sample. Of the women studied, 92% were white and well-educated, and most worked outside the home. A questionnaire was administered during hospitalization, and samples of placenta, maternal and cord blood, and breast milk and colostrum were assayed for PCB and DDE concentrations. Subsequently, additional serum and breast milk samples were obtained. The results from all available milk samples were combined, after scaling to account for differences between matrices and over time, to estimate the concentration of each chemical in milk fat at birth. No associations were seen for PCBs or DDE and either birth weight, head circumference, or hyperbilirubinemia, end points that had been associated with exposure to PCBs in the Taiwan and Yusho episodes. Neonatal testing was conducted during the first month of life using the Brazelton Neonatal Behavioral Assessment Scales (BNBAS). Because of attrition, results for this testing were obtained for 867 infants (93%) (Rogan et al. 1986a). The BNBAS analysis controlled for mother's age, education, occupation, smoking, drinking, and consumption of sport fish, as well as infant's sex, race, birth weight, and presence of jaundice. Tonicity and reflex cluster scores were significantly related to exposure to PCB or DDE. Exposure to high concentrations of PCBs in milk fat (≥3.5 ppm) was associated with hypotonicity and hyporeflexia; and DDE exposure through milk fat (≥4.0 ppm) was related to hyporeflexia. There was no evidence of deficit in memory function (Rogan et al. 1986b).

At the ages of 6 and 12 mo, 802 of the children were retested, using two instruments, the Bayley Mental Development Index (MDI) and the Bayley Psychomotor Development Index (PDI). Neither postnatal PCB exposure nor postnatal DDE exposure was related to either score at 6 or 12 mo, nor was the PDI related to prenatal DDE concentrations. However, the Bayley PDI decreased significantly with increasing prenatal PCB exposure at both points; 0.96/ppm (2.1 SE) and 1.34/ppm (2.2 SE) at 6 and 12 mo, respectively. A significant association between estimated prenatal DDE concentrations and Bayley MDI score was seen at 6 mo, but not at 12 mo (Gladen et al. 1988).

The children were again examined using the Bayley scales at 18 and 24 mo. At both ages, adjusted scores on the psychomotor scales were 4 to 9 points lower among children in the top quintile of exposure to transplacental PCB, and scores differed significantly at 24 mo. As with earlier test results from this cohort, no significant effects were seen in association with exposure through breast milk (Rogan and Gladen 1991). When tested at older ages (5.5-10.5 yr), using the

McCarthy Scales of Children's Abilities, the developmental changes seen at younger ages in relation to transplacental exposure to PCBs were no longer detectable (Gladen and Rogan 1991). This series of studies of neurologic function in children exposed to concentrations of PCBs and DDE that were ambient to the southeastern United States in 1978-1982 suggests small, but significant, neuromotor deficits that are no longer detected after early childhood.

Oswego, New York

An ongoing prospective longitudinal study has been designed to examine the behavioral effects of maternal consumption of Lake Ontario fish that contained a range of persistent chemicals, including PCBs, hexachlorobenzene (HCB), polychlorinated dibenzo-*p*-dioxin (PCDD), dieldrin, lindane, chlordane, cadmium, mercury, and mirex (Daly et al. 1996; Lonky et al. 1996). The study involves pregnant women recruited at 20 wk gestation and their offspring born between 1991 and 1994. Women were classified into exposure groups of high (consumed >40 equivalent pounds of fish; n = 152), low (consumed <40 equivalent pounds of fish; n = 243) and no exposure (n = 164). The parameter "equivalent pounds of fish" is a score based on fish species, number of years eating fish, number of meals per year, and serving size. Behavioral development was tested by the Neonatal Behavioral Assessment Scale. Trained observers, who were blind to the exposure status of the children, conducted the tests. Children were assessed 12-24 hr after birth and again at 25-48 hr. Principal component analyses controlled for three sets of variables: demographics, substances consumed during pregnancy, and labor-delivery-birth characteristics. The study sample was similar to the hospital population with respect to demographics and characteristics of labor and delivery, as were the three study groups. The study controlled for more than 100 confounders.

No significant associations were found between exposure and either weight or head circumference. Newborns in the high-exposure group scored more poorly than did the low or unexposed groups on three of the seven clusters: reflex, autonomic maturity, and habituation. The findings of poorer reflex functioning and greater autonomic immaturity in highly exposed infants are similar to the findings of Jacobson et al. (1985) and Rogan et al. (1986b) and thus strengthen the findings from those studies, which have been criticized on methodologic grounds. Further follow-up will be done to determine the persistence of effects as the children mature.

Efforts are under way to study the effect of recency of maternal fish consumption on neonatal coping behavior and infant temperament in this cohort at 24 mo (Darvill et al. 1997, as cited in Johnson et al. 1998).

Netherlands

An ongoing study of perinatal exposure to PCBs is being carried out in the Netherlands (Huisman et al. 1995a). The protocol involves 418 children of normal birth (37-42 wk gestation), half of whom were breast fed. PCB exposure was determined with umbilical cord blood, maternal serum, and breast-milk samples. Neurologic optimality of the newborns was evaluated with the Prechtl neurologic examination between postnatal d 10 and 21. Children were classified as normal, suspect, or abnormal, and two clusters were scored: postural tone and reflex. PCB concentrations in cord and maternal sera were not related to neurologic dysfunction. However, higher concentrations of PCB, PCDD, and PCDF congeners in breast milk were associated with reduced neonatal neurologic optimality. Higher concentrations of planar PCBs in breast milk were related to an increased incidence of hypotonia (Huisman et al. 1995a).

A subset of these children (105 breast-fed and 102 formula-fed infants) were tested at 3, 7, and 18 mo with the Bayley Scales of Infant Development (Koopman-Esseboom et al. 1996). Prenatal exposure to PCBs had a small negative effect on psychomotor scores at 3 mo, but had no effect on mental scores. PCB and dioxin exposure through breast feeding had adverse effects on psychomotor outcome at 7 mo, while mental outcome was positively influenced in children exposed pre- and postnatally compared with children who were exposed prenatally only. At 18 mo, no adverse effects on psychomotor or mental development were found.

The entire cohort of 418 children were also tested with the Neurologic Examination for Toddler-Age at 18 mo. After adjustment for covariates (father's education, smoking of father, and parity), it was determined that transplacental PCB exposure is significantly and negatively related to motor function (grasping, sitting, crawling, standing, and walking) at 18 mo (β coefficient –0.149; p = .003). Father's smoking modified this association, which was seen predominantly in children whose fathers did not smoke. On the other hand, little association was seen with additive lactational exposure, consistent with other studies (Huisman et al. 1995b).

Recently, 395 children from the original 418 mother-infant pairs in this study were tested with the Dutch version of the Kaufman Assessment Battery for Children (sequential and simultaneous processing) at 3.5 yr. Prenatal exposure to PCBs was measured using maternal and umbilical cord plasma samples, postnatal exposure to PCBs and dioxins was measured using breast-milk samples, and current exposure to PCBs was determined in the child's plasma. After adjustments for covariates, prenatal exposure to PCBs was significantly associated with lower cognitive scores, while postnatal exposure to PCBs and dioxin and current exposure to PCBs were not (Patandin et al. 1999). The children were also evaluated for motor function using the Touwen/Hempel method (motor functions prehension, sitting, crawling, standing, and walking) at 3.5 yr, and no effect on neurologic condition was found (Lanting et al. 1998).

In collaboration with the studies in the Netherlands, Winneke et al. (1998) investigated the neurodevelopmental effects of perinatal exposure to PCBs in 171 newborns in Düsseldorf, Germany. Children were evaluated using the Bayley scales of infant development (BSID), psychomotor development tests, and the Fagan visual recognition memory test at 7 mo. Mean concentrations of PCBs were 0.55 ng/mL in cord blood and 427 ng/g in the fat of breast milk. After adjusting for confounders, the only significant association was a negative association between PCB concentrations in breast milk and mental development index.

Great Lakes

A neurologic assessment of an aging population of Great Lakes fisheaters is currently being conducted by Schantz et al. (1996). In all, 104 fisheaters and 84 nonfisheaters, age 50 or older, were enrolled in the study. Blood samples from 180 of the 188 study participants were analyzed for PCBs and ten other contaminants. The blood concentrations of PCBs and DDE in the fish-eaters were found to be elevated in comparison to age- and sex-matched nonfisheaters. A battery of neuropsychologic tests, including tests of motor function, memory and learning, executive functions, visual-spatial function, and abstract reasoning, have been conducted, but the data are still under analysis.

A study has also been conducted that compares nervous system function in adult consumers of fish from the upper St. Lawrence River lakes to nonfisheaters (Mergler et al. 1998). No significant differences were found between the groups in tests of sensory function, visual memory and recognition, fine motor performance, and some motor tests. However, the fisheaters performed more poorly on tests requiring cognitive flexibility, word naming, auditory recall, and more complex motor task compared with individuals who do not eat fish. It should be noted that the observations were made within a larger study of early neurotoxic effects of environmental exposure to manganese, and was not designed to examine the effects of fish eating. Furthermore, paired analyses showed that consumers of fish had higher levels of organic mercury and lead in their blood.

New Bedford, Massachusetts

Mother-infant pairs residing near the New Bedford Superfund site, which is known to be significantly contaminated with PCBs, are being evaluated for adverse neurologic effects (Korrick 1998 as cited in ATSDR 1999). Preliminary data suggest that cognitive development, as measured by the Fagan Test of Infant Intelligence, is impaired by in utero exposure to PCBs.

SUMMARY AND CONCLUSIONS

Most of the human data on neurologic effects of HAAs involve studies of newborns and children because the developing nervous system is thought to be the most sensitive to toxic agents. Long-term epidemiologic studies of cognitive and neurobehavioral development have been conducted in Michigan, New York, North Carolina, and the Netherlands on children exposed pre- and postnatally to PCBs from maternal consumption of contaminated fish or other food products. Studies of cognitive development (i.e., short-term memory, visual discrimination, and IQ scores) in Michigan show consistent correlations between prenatal exposure to PCBs and deficits at up to 11 yr (Jacobson and Jacobson 1996). Similarly, in the Netherlands, lower cognitive scores were associated with prenatal exposure when tested in 3.5-yr-old children (Patandin et al. 1999). In contrast, studies in North Carolina (Gladen et al. 1988; Gladen and Rogan 1991; Rogan and Gladen 1991) and Germany (Winneke et al. 1998) found no association between prenatal exposure and cognitive development.

In studies of neurobehavioral development (i.e., sitting, crawling, and walking), deficits were observed in the North Carolina cohort of children until 2 yr but not thereafter. Neurobehavioral deficits have also been reported in the Michigan and New York cohorts. Reports regarding neurobehavioral development from the Netherlands are inconsistent. For example, one study measuring psychomotor development at 18 mo with the Neurologic Examination for Toddler-Age found a significant reduction in neurologic function in prenatally exposed children (Huisman et al. 1995b), while another study of psychomotor development using the Bayley Scales of Infant Development found slight effects at 3 mo but none at 7 or 18 mo (Koopman-Esseboom et al. 1996). A study conducted with children at 3.5 yr also found no effects (Lanting et al. 1998). Some adverse neurobehavioral effects have been reported from postnatal exposure in studies of 10-21-d-old children (Huisman et al. 1995a) and 7-mo-old children (Koopman-Esseboom et al. 1996) in the Netherlands. However, no significant effect on neurobehavioral development was found in perinatally exposed children in Germany (Winneke et al. 1998).

Thus, data from the United States and some of the data from the Netherlands suggest a correlation between prenatal exposure to PCBs (due to maternal consumption of contaminated fish or other food products) and effects on cognitive and behavioral development in children. For the most part, postnatal exposure via breast milk does not appear to contribute significantly to these outcomes. Studies of PCBs in laboratory animals support these findings. In particular, studies of monkeys exposed prenatally to PCBs have shown impaired learning ability and prental exposure of rats and mice to PCBs have also shown impaired locomotor ability and learning. Taken together, the results of animal and human studies indicate that prenatal exposure to PCBs can affect neurologic development. The mechanisms underlying these effects have yet to be determined.

RECOMMENDATIONS

The majority of the data reviewed by the committee on the neurologic effects of HAAs focused on only a few chemicals, namely PCBs, PCDF, dioxin, and DDT. Based on the committee's evaluation of those data, the following are recommended:

—Human populations suspected of being affected by HAAs should continue to be monitored for adverse neurologic effects. Longitudinal tests should be conducted on developmental landmarks or milestones from conception through adulthood. A standardized set of criteria should be established to measure functional and social development and physical and clinical parameters. Burdens of HAAs should be measured in relation to the effects.

—Further attempts should be made to identify the specific agents and the mechanisms of action underlying the human neurologic effects that have been associated with exposure to PCBs, PCDFs, dioxins, and DDT.

7

Immunologic Effects

THIS CHAPTER DISCUSSES the immunologic effects attributed to persistent organochlorines and other hormonally active agents (HAAs). Effects of specific HAAs (such as the halogenated aromatic hydrocarbons (HAHs), dichlorodiphenyltrichloroethane (DDT), chlordecone (Kepone), endrin, aldrin, dieldrin, lindane, chlordane, toxaphene, endosulfan, and hexachlorobenzene) observed in laboratory studies and to a lesser extent in field and human studies are compared and correlated with information on exposure. Because these agents are postulated to act by means of hormonally mediated mechanisms, a brief discussion of how hormones affect the immune system is presented below. As more information becomes available, this will provide a context for evaluating the immunologic effects of HAAs.

It has been well documented that immunity can be modulated by hormones (Grossman 1984, 1985). The presence of steroid hormone receptors in a strikingly wide variety of immunologic tissues is a strong indication that cells and tissues of the immune system must be targets for steroid hormones, and that steroid hormones elicit regulatory effects in these cells and tissues. The heterogeneity of responses caused by interactions of various steroid hormones (such as corticoids, estrogens, androgens, and progestins) between the immune and endocrine systems has been documented (Grossman 1984, 1985, 1989, 1994; Grossman et al. 1991; Berczi 1994; Chapman and Michael 1994; Dardeene and Savino 1994; Fabris 1994; McCruden and Stimson 1994; Rivier 1994; Wira et al. 1994). Thus, a variety of stimuli (including exposure to environmental toxicants) could mediate nonspecific, stress like effects.

In the case of steroid hormones, it is clear that the actions of estrogens and androgens are important in the reported differences in immune response between male and female laboratory animals (Batchelor and Chapman 1965; Terres et al.

1968; Grossman 1984, 1985, 1989, 1994; Grossman et al. 1991). Termed "immunologic sexual dimorphism," the results of the actions are a general increase in humoral immunity in females compared with males (Batchelor and Chapman 1965; Terres et al. 1968) and differences in some cell-mediated immune responses (Graff et al. 1966, 1969; Kittas and Henry 1979, 1980) in females and males. For example, females tend to be far more susceptible than males to such autoimmune diseases as Hashimoto's disease (Tunbridge et al. 1977; Grossman et al. 1991), Grave's disease (Grossman et al. 1991), rheumatoid arthritis (Vandenbroucke 1982; Vandenbroucke et al. 1982; Lotz and Vaughan 1988; Grossman et al. 1991), systemic lupus erythematosus (Roubinian et al. 1979a,b; Lahita 1985; Grossman et al. 1991), thyroid disease (Tunbridge et al. 1977), and demyelinating disease (Arnason and Richman 1969).

Immunologic differences are also observed between pregnant and nonpregnant females. Immune responses in pregnant women are depressed compared with nonpregnant women. This depression of the immune system might be necessary during pregnancy to prevent fetal rejection and abortion before term (Grossman and Roselle 1987), and might be due, in part, to the presence of sex steroids that are elevated during pregnancy. However, it also could be partly responsible for the reported increases in the susceptibility of pregnant women to such infectious diseases as smallpox, polio, viral hepatitis, varicella-zoster, influenza, cytomegalovirus, and pulmonary and systemic mycoses (Grossman and Roselle 1987).

Diethylstilbestrol (DES) has been studied extensively in humans, and this estrogen has been shown to alter immunity (Dodds et al. 1938; Ablin et al. 1974; Korach et al. 1978; Dean et al. 1980; Kalland and Forsberg 1981; Haukaas et al. 1982; Fugmann et al. 1983; Luster et al. 1984; Morahan et al. 1984; Pung et al. 1984, 1985; Noller et al. 1988). These studies provide examples of how hormones can affect the immune system, but whether all HAAs act in this manner remains to be determined.

HAAs AND STEROID HORMONES

HAAs and Lymphatic Tissue Structure

Some reports of architectural changes in primary and secondary lymphatic tissues exposed to HAAs are available. Of particular interest are studies that describe thymic atrophy, since the thymus is a major site of early T-lymphocyte development, as well as a source of immunologic regulatory hormones (Dardeene and Savino 1994) in the adult. Thus, it follows that disorganization of thymic structure in the embryo could also result in immunologic abnormalities. In mammalian species, thymic atrophy and disruption of the secondary lymphatic organs has been generally observed as a result of PCB exposure (McKinney et al. 1976; Safe 1985; Thomas and Faith 1985), and these compounds can alter lymphoid

development of thymus and bursa (Andersson et al. 1991). Notably, TCDD promotes thymic involution in fish (Spitsbergen et al. 1986) and mice (Luster and Rosenthal 1986; Kerkvliet et al. 1990) and cellular depletion in thymus, spleen, and lymph nodes (Clark et al. 1981).

The action of some HAAs, such as TCDD and some PCBs, are mediated through the aryl hydrocarbon (Ah) receptor mechanism (Kerkvliet et al. 1990; Andersson et al. 1991; Kerkvliet and Burleson 1994; K. White et al. 1994), and may not be construed as direct acting HAAs. It is important to keep in mind that the regulatory pathways between the endocrine and immune systems are complex. Alterations in thymic structure and function can affect sex- and adrenal-hormone regulation of immunity, as mediated by the various thymic-hypothalamic-pituitary axes (Grossman 1984, 1985, 1989; Grossman et al. 1991).

HALOGENATED AROMATIC HYDROCARBON COMPOUNDS

It has been well documented that HAHs such as TCDD, polychlorinated dibenzofurans (PCDFs), and PCBs, affect immune response, and they appear to affect all functional arms of the immune system (innate immunity and host resistance, cell-mediated immunity, and humoral immunity) (Table 7-1).

Laboratory Studies

Specific examples of the immunologic effects of PCBs and TCDD on laboratory animals are detailed in Table 7-2. These HAHs cause atrophy of the thymus, the primary lymphoid organ in which stem cells are selected and differentiated into T-cells. Thymic atrophy has been induced in adrenalectomized animals (Vos and Luster 1989; Lundberg 1991). HAHs cause thymic involution and decrease the number of colony-forming stem cells in animals—and these effects are more dramatic when exposures occur either perinatally or postnatally (Lundberg et al. 1990; Holladay et al. 1991; Lundberg 1991; De Waal et al. 1992; De Heer et al. 1994). This suggests that HAHs target the developing immune system (Fine et al. 1990). Because TCDD directly affects the thymic cortical epithelium, it has been hypothesized that the hormonal factors necessary for lymphocyte maturation are not produced and that thymocytes are pushed into premature terminal differentiation (Greenlee et al. 1985; Lundberg et al. 1990).

Exposure to HAHs decreases cell-mediated immune (CMI) responses against bacteria and viruses. This is shown in the decreased host resistance reported in Table 7-1. Treatment with HAHs before immunization with sheep red blood cells—a T-dependent antigen—results in dose- and structure-dependent suppression of immune response in mice (Silkworth et al. 1986; Davis and Safe 1988, 1990; Dickerson et al. 1990; Kerkvliet et al. 1990; Tomar and Kerkvliet 1991). TCDD also has been shown to suppress delayed-type hypersensitivity (Vos and Luster 1989) and to suppress generation and lytic activity of cytotoxic T-cells in

a dose- and strain-dependent manner (Clark et al. 1981, 1983; Nagarkatti et al. 1984). The suppression of cytotoxic T-cells could be related to a concurrent increased number of T-suppressor cells and increased suppressor activity (Clark et al. 1981, 1983; Holsapple et al. 1986); however, that hypothesis is the subject of controversy.

Although the mechanism by which HAHs alter CMI responses is unknown, studies have shown that HAHs affect these responses without decreasing T-cell proliferation, IL-2 production, or the number of IL-2 receptors (Dooley et al. 1990). There is evidence that TCDD targets activated lymphocytes rather than resting cells and that TCDD specifically inhibits the activation of antigen-specific T-cells (Dooley et al. 1990; Lundberg et al. 1992).

HAHs have been shown to affect humoral immune response. This response is characterized by B-cell antibody production, and it requires B-cell interaction with T-cells and interleukins, which are necessary for B-cell activation and differentiation. B-cells produce antibodies to specific antigens presented by T-cells (T-dependent antigen) or to antigens that cross-link surface immunoglobulins on the B-cell membrane (T-independent antigen). Exposure to HAHs followed by immunization with either T-dependent or T-independent antigens results in a dose- and structure-dependent decrease in antibody production without affecting B-cell proliferation (Davis and Safe 1988, 1990; Kerkvliet et al. 1990; Holsapple et al. 1991; Harper et al. 1995). Although the mechanism by which HAHs suppress the humoral immune response is unknown, it appears that HAHs act by means of the Ah receptor (Silkworth and Grabstein 1982; Lubet et al. 1984; Silkworth et al. 1984, 1986; Kerkvliet et al. 1985, 1990; Davis and Safe 1988, 1990; Howie et al. 1990; Tomar and Kerkvliet 1991; Howie 1992) and that immunotoxic potency correlates with binding affinity. However, it has been reported that components of immunosuppression induced by some HAHs act independently of the Ah receptor (Howie et al. 1990; Kerkvliet et al. 1990; Howie 1992).

Field Studies

An observational study was conducted between 1992 and 1994 to determine whether contaminant-associated immunosuppression occurs in prefledgling Caspian terns and herring gulls of the Great Lakes (Grasman et al. 1996). The phytohemagglutinin skin test for T-cell mediated immunity was conducted on 3-wk-old chicks at colonies distributed across a broad gradient of organochlorine contamination (primarily PCBs). In both species, there was a strong exposure-response relationship between organochlorines and suppressed T-cell-mediated immunity. Suppression was most severe (30-45%) in colonies in Lake Ontario and Saginaw Bay for Caspian terns and herring gulls, and in western Lake Erie for herring gulls. Although there were significant differences in total antibody and IgG titers among sites, there was no consistent exposure-response relationship with organochlo-

TABLE 7-1 Effects of Halogenated Aromatic Hydrocarbons (HAHs) on Immunity

HAH	Effect	Reference
Innate Immunity and Host Resistance		
TCDD[a]	Reduced survival rates of mice infected with influenza virus, herpes simplex type II virus, and *Salmonella bern* or *S. typhimurium*	Thigpen et al. 1975; Clark et al. 1983; House et al. 1990
TCDD	Decreased serum complement C3 and decreased resistance to *Streptococcus pneumoniae* infection in mice	White et al. 1986
TCDD	In utero exposure to mice significantly increased progeny mortality after infection with *Listeria monocytogenes*	Luster et al. 1980
TCDD	Longer duration of parasitic infection in mice exposed to *Plasmodium yoelii*	Tucker et al. 1986
TCDD	Persistent infection in mice treated with *Trichinella spiralis*	Luebke et al. 1994
Cell-Mediated Immunity		
TCDD	Thymic involution and decrease in the number of colony-forming stem cells in rats and mice; effects were more dramatic when exposures occurred perinatally or postnatally	Lundberg et al. 1990; Holladay et al. 1991; Lundberg 1991; De Waal et al. 1992; De Heer et al. 1994
TCDD PeCDF[b] TCDF[c] PCB[d] HpCDF[e]	Dose- and structure-dependent suppression of immune response in mice after immunization with sheep red blood cells	Silkworth et al. 1986; Davis and Safe 1988, 1990; Dickerson et al. 1990; Kerkvliet et al. 1990; Tomar and Kerkvliet 1991

TCDD	Enhanced immune response in rats to sheep red blood cells	Smialowicz et al. 1994
TCDD	Suppressed delayed-type hypersensitivity reactions to tuberculin and oxazalone in guinea pigs and mice, as well as graft vs. host reactivity in mice and rabbits	Vos and Luster 1989
TCDD	Suppressed generation and lytic activity of cytotoxic T-cells in dose- and strain-dependent manner	Clark et al. 1981, 1983; Nagarkatti et al. 1984
TCDD	Altered lymphocyte populations in marmosets	Neubert et al. 1992
TCDD	Humans exposed to TCDD showed an increased frequency of anergy for delayed-type hypersensitivity responses to the following recall antigens: tetanus, diphtheria, *Streptococcus*, tuberculin, *Candida, Proteus,* and *Trichophyton*.	Hoffman et al. 1986
PCB	PCB-exposed children had nonsignificant decrease in CD4+:CD8+ T-cell ratio compared with nonexposed children	Lan et al. 1990
Humoral Immunity		
TCDD PeCDF TCDF PCB	Exposure to HAHs followed by immunization with either T-dependent antigen (SRBC) or T-independent antigen trinitrophenyl-lipopolysaccharide caused dose- and structure-dependent decrease in antibody production without affecting B-cell proliferation	Davis and Safe 1988, 1990; Kerkvliet et al. 1990; Holsapple et al. 1991

[a] TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.
[b] PeCDF, 1,2,3,7,9-pentachlorodibenzofuran.
[c] TCDF, 2,3,7,8- or 1,3,6,8-tetrachlorodibenzofuran.
[d] PCBs, polychlorinated biphenyls.
[e] HpCDFs, heptachlorodibenzofurans.

SOURCE: Adapted from Harper 1995.

TABLE 7-2 Laboratory Studies on Halogenated Aromatic Hydrocarbons

Concentration	Species	Effect	Reference
Polychlorinated Biphenyls (PCBs)			
104-464 mg/kg Aroclor 1260, 1254, 1248, 1242, 1016, 1232	Mouse	Murine splenic PFC[a] response to SRBC[b] was functionally inhibited in a dose-dependent fashion; higher chlorinated PCB preparations were more potent	Davis and Safe 1989
25 mg/kg/d[c] Aroclor 1242	Mouse	Mice fed PCBs for 6 wk had increased mortality, possibly due to the immunosuppressive effects of PCBs on humoral immunity; PFC response to SRBC antigen by spleen cells was significantly reduced; there was a reduction in serum IgG_1, IgA, and IgM; significant reduction in secondary immune response	Loose et al. 1977
5 µg/kg PCB	Chick	Thymic and spleen involution; marked depletion of lymphocytic cell types in both organs	McKinney et al. 1976
4 µg/kg egg PeCB[d] 50 µg/kg egg TCB[e] 300 µg/kg egg HCB[f]	Chick	Inhibition of lymphoid cell development in the thymus and bursa	Andersson et al. 1991
25-100 ppm Aroclor 1254	Duck	Increased mortality following challenge with duck hepatitis virus	Friend and Trainer 1970
5-80 µg/kg/d Aroclor 1254	Rhesus monkey	Monkeys fed Aroclor 1254 for 23 or 55 mo showed dose-dependent suppression of anti-SRBC antibody production, no effect on antipneumococcus antibody response; at 80 g, decrease in T-helper cell population and increase in T-suppressor cell population with no change in the mitogen-activated lymphocyte stimulation; at 55 mo, increase in natural killer cell activity and in thymosin a-1, but not B-4 or tumor necrosis factor	Tryphonas et al. 1989, 1991a,b

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)

5-50 μg/kg	Mouse	Dose-dependent alteration in T-lymphocyte development	Lundberg et al. 1990
100 μg/kg	Mouse	Depletion of cells from thymus and spleen	Pung et al. 1984
40 μg/kg	Mouse	Depletion of thymic cells only; impaired antibody response to antigens SRBC and trinitrophenyl-Brucella abortus	Pung et al. 1984
4 μg/kg	Mouse	Thymic cell depletion; delayed hypersensitivity response to oxazolone depressed	Pung et al. 1984
0.00125 μg/kg/d[c]	Rhesus monkey	Monkeys fed TCDD for 4 yr showed selective increase in CD8+ T-cells and decrease in CD4+ T-cells, no apparent significant effect demonstrated on T-cell function; natural killer cell activity appeared normal; offspring had significant increases in generation of antitetanus toxoid antibody production postimmunization correlated with TCDD tissue concentrations	Hong et al. 1989
10 ng/kg	Marmoset	Marmosets injected with TCDD showed decreased circulating helper-inducer T-cells and increased suppressor-inducer T-cells, no change in total number of T-cells; no-observed-effect level was 3 ng/kg	Neubert et al. 1990
1.5 ng/kg/wk	Marmoset	Marmosets treated for 3 wk had depressed helper-inducer T-cells	Neubert et al. 1992

[a] PFC, plaque-forming cell.
[b] SRBC, sheep red blood cell.
[c] Estimated dose based on general assumptions of weight and feed intake.
[d] PeCB, 3,3′,4,4′,5-pentachlorobiphenyl.
[e] TCB, tetrachlorobiphenyl.
[f] HCB, hexachlorbenzene.

rines. In 1992, altered white blood cell numbers were associated with elevated organochlorine concentrations in Caspian terns but not herring gulls. Although the identity of the specific organochlorine(s) responsible for the suppression of T-cell-mediated immunity could not be determined, the researchers noted that PCBs were the most closely associated with immunosuppression.

Field studies of the immunologic effects of HAHs also have been conducted with seals, whales, and dolphins. de Swart et al. (1994, 1996) reported that innate and acquired immune responses were functionally impaired in harbor seals fed herring from PCB-contaminated waters (Baltic Sea) for 126 wk. The estimated intake of PCBs was 1,460 μg/d. Ross et al. (1995) reported similar findings in captive harbor seals fed fish from the PCB-contaminated Baltic Sea. These seals demonstrated impaired ability to mount a delayed hypersensitivity response when challenged with ovalbumin, and they generated 37% less antibody to ovalbumin after antigen challenge than did seals fed fish from the relatively uncontaminated Atlantic Ocean. In exposed seals, the combined concentration of mono-PCB (International Union of Pure and Applied Chemistry (IUPAC) numbers 118, 156, and 189) and diortho-PCB (IUPAC number 180), measured in nanograms of toxic equivalent per kilogram of lipid, was 140.0; it was 35.5 in unexposed seals.

De Guise et al. (1994, 1995) studied beluga whales living in the highly contaminated St. Lawrence estuary of Quebec, Canada, and compared them with belugas living in the much less contaminated arctic. They observed that belugas from the St. Lawrence had numerous severe and disseminated infections caused by mildly pathogenic bacteria. They suggested that the generalized immunosuppression was caused by organochlorine contamination. In addition, 75 tumors have been reported in whales worldwide, 28 (37%) of which were found in 18 St. Lawrence beluga whales. The researchers tentatively concluded that this could result from depression in immunosurveillance caused by exposure to environmental contaminants or carcinogens, or both. However, because the reported results were obtained through highly selective (nonrandom) sampling, selection bias might also skew these conclusions.

In a study of 15 bottlenose dolphins along the west coast of Florida, peripheral blood lymphocyte responses to Concanavalin A (ConA) and phytohemagglutinin were determined in vitro and compared by regression analysis with contaminant concentrations in whole blood from five of the dolphins (Lahvis et al. 1995). Reduction in ConA-induced lymphocyte responses was correlated with increasing whole blood concentrations of tetrachloro-PCBs (1-18 ng/g), pentachloro-PCBs (4-44 ng/g), hexachloro-PCBs (13-322 ng/g), heptachloro-PCBs (7-293 ng/g), and octachloro-PCBs (2-81 ng/g), and similar correlations were also found with DDT (see below). Immunosuppression caused by exposure to environmental contaminants also could account for the severity and extent of morbillivirus epizootics observed among seals and dolphins (de Swart et al. 1995). However, a direct cause-and-effect relationship has not been proven.

Human Studies

Alterations in immune responses caused by exposure to HAHs have been documented in a few human studies. Lu and Wu (1985) describe the acnegenic and hepatotoxic effects in residents of Yu-Cheng, Taiwan, who ingested high concentrations of PCBs that were accidentally leaked into rice oil. The resulting effects were related primarily to increased respiratory infection; decreased serum concentrations of IgA and IgM; decreased CD4+ T-cells and increased CD8+ T-cells; suppressed dermal delayed hypersensitivity responses to a combination of streptokinase and streptodormase and to tuberculosis antigens; and augmentation of the in vitro lymphocyte mitogen stimulation to phytohemagglutinin (PHA) and pokeweed mitogen (PWM), but not to ConA. Average blood concentrations of PCBs in the affected individuals were 89 ± 6.9 ppb.

In a study of Wisconsin infants whose mothers ate PCB-contaminated fish, maternal serum PCB levels were positively associated with the number and type of infectious illnesses, such as colds, earache, and flu symptoms, that occurred in infants during the first 4 mo of life (Smith 1984). The authors concluded that prenatal exposure to PCBs was the cause of the increased infections. However, as noted by Swain (1991) in a critique of the study, these results should be interpreted carefully because blood concentrations of PCBs were only measured after birth and not during pregnancy.

In a study of Dutch infants, 105 breast-fed and 102 formula-fed infants were evaluated from birth until 18 mo to determine whether prenatal and postnatal exposure to background concentrations of PCBs and dioxins had an effect on the incidences of rhinitis, bronchitis, tonsillitis, and otitis (Weisglas-Kuperus et al. 1995). Humoral immunity was also measured by detecting antibody levels to mumps, measles, and rubella as a result of vaccinations. Prenatal exposure was estimated by PCBs in maternal blood and the total toxic equivalent (TEQ) level in breast milk (measured as pg TEQ/g milk fat), and postnatal exposure was calculated as a product of the total TEQ level in breast milk multiplied by the weeks of breast feeding. Umbilical cord and venous blood was taken from a subgroup of 55 infants at 3 and 18 mo for white blood cell counts and immunologic marker analysis. No relationship was found between pre- and postnatal PCB/dioxin exposure and upper or lower respiratory symptoms or humoral antibody production. However, higher prenatal and postnatal exposures to PCBs/dioxins were associated with lower monocyte and granulocyte counts at 3 mo, and increases in the total number of T-cells and in the number of cytotoxic T-cells were observed at 18 mo.

Recent studies of Inuit people exposed to organochlorines in their diet via sea-mammal fat have reported serum lipid concentrations of 4.1 mg/kg lipids PCBs and 184.2 ng/kg lipids 2,3,7,8-TCDD (Ayotte et al. 1997). Health risk assessments for newborns in these populations indicate a correlation between PCB/dioxin exposures in breast milk and suppressed levels of white blood cells

in infants (Ayotte et al. 1996). The breast milk of Inuit women contained 7 times more PCBs (sum of the PCB congeners = 1,052 ng/g, lipid) than the milk from women from urban, industrialized areas south of Quebec (Dewailly et al. 1993b). Researchers are investigating the possible connection between unusually high rates of infectious disease, particularly acute ear infections, among Inuit children and exposure to PCBs. Such studies must be interpreted carefully because comparisons of organochlorine concentrations over time are unreliable.

Webb et al. (1989) found that humans exposed to TCDD had increased CD8+ T-lymphocyte populations; no change in CD4+ T-cells; no change in lymphocyte response to the mitogens ConA, PHA, and PWM; no change in cytotoxic T-cells; and increased serum IgA. Of the 41 individuals studied, 16 had TCDD concentrations below 20 ppt in their adipose tissue, 13 had concentrations of 20-60 ppt, and 12 had concentrations above 60 ppt (the maximum was 750 ppt).

DICHLORODIPHENYLTRICHLOROETHANE

DDT has been reported to possess estrogenic and antiandrogenic properties (Kupfer and Bulger 1980), supporting the hypothesis that it acts by binding to steroid receptors in immunologic target tissues. Laboratory studies have demonstrated that DDT can alter both the primary and the secondary humoral immune response, immunoglobulin production, splenic plaque-forming cell (PFC) response, histamine concentrations, and mast cell numbers. Specific examples of DDT's immunologic effects in laboratory animals are detailed in Table 7-3. DDT has been demonstrated to trigger some immunologic effector mechanisms in animal and bird models (Barnett and Rodgers 1994).

In studies of harbor seals, a diet of DDT-contaminated fish from the Baltic Sea was shown to impair immune response, as measured by delayed hypersensitivity in the skin to ovalbumin and in vitro lymphocyte assays (de Swart et al. 1994; Ross et al. 1995). In a study of bottlenose dolphins, Lahvis et al. (1995) reported that ConA mitogen assays of peripheral lymphocytes demonstrated a correlation between reduced immune response and increasing concentrations of *p,p′*-DDT (0-24 ng/g) and *p,p′*-DDE (13-536 ng/g). 1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p′*-DDE) is a metabolite of DDT. It has been suggested that, with elevated concentrations of these contaminants, a reduction in immune response can be correlated with an increased incidence of infection (Svensson et al. 1994).

Overall, the data are suggestive of DDT-mediated immunosuppression in animals and birds being detected at about 10 mg per kilogram of body weight (BW) per day. Additional laboratory studies are needed to identify the functional parameters and immunologic effector cells involved in DDT-mediated immunotoxicity. Until human data are available for comparison, no conclusions can be made about the effects of DDT on the human immune system.

TABLE 7-3 Laboratory Studies on Dichlorodiphenyltricholroethane (DDT)

Concentration	Species	Effect	Reference
10 mg/kg/d[a]	Rat	Increased spleen weight, reduced antiovalbumin Ab production, reduced globin fraction of serum proteins, increased albumin observed in rats fed DDT for 35 d	Wassermann et al. 1969
2.2 mg/kg/d	Rat	Slight but significant decrease in mast cells in rats fed DDT for 31 d	Gabliks et al. 1975
3, 7.5, 15 mg/kg/d[a]	Mouse	Mice fed 15 mg/kg DDT for 12 wk had significant reduction in primary and secondary anti-SRBC[b] (IgM) and (IgG) titers, significant reduction in direct splenic PFC[c] response; effects appear to be dose and time dependent: less or no immunosuppression was observed at the lower doses or with shorter treatments	Banerjee et al. 1986
25, 50 mg/kg/d[a]	Chick	Chicks fed 50 mg/kg (but not 25 mg/kg) for 5 wk showed significantly depressed production of IgG and IgM; no effect on antibody production when stimulated with bovine serum albumin antigen; no effect on PFC response to SRBC	Glick 1974
125 mg/kg/d[a]	Chick	DDT was lethal to 75% of the chicks by week 2	Glick 1974
12.5 mg/kg/d[a]	Chick	Chicks fed DDT for 40 d had decreased anti-SRBC (IgG) titers and increased anti-SRBC (IgM) titers; decreased metabolic activity in tissue from bursa, spleen, thymus	Subba Rao and Glick 1977
6.5 mg/kg/d	Rabbit	No apparent effect on the titers of anti-SRBC hemagglutinin in rats fed DDT for 57 d	Street and Sharma 1975
25 mg/kg/d	Guinea pig	Guinea pigs injected with DDT for 3 d had reduced histamines and mast cells; no statistical analyses were performed.	Askari and Gabliks 1973

[a] Estimated dose based on general assumptions of weight and feed intake (FDA 1975).
[b] SRBC, sheep red blood cell.
[c] PFC, plaque-forming cell.

CHLORDECONE

Chlordecone binds to estrogen receptors (Hammond et al. 1979) and could thus modulate immunity via binding to them. The only hard evidence in support of an immunomodulatory effect of chlordecone is from a study of the immunologic effects of malnutrition and administration of chlordecone in rats. Chetty et al. (1993) reported that 95-100% of malnourished rats fed 5 mg/kg/d died; that 0.5 mg/kg/d decreased body weight and increased spleen weight; that both malnutrition and chlordecone increased PFC; and that 0.5 and 5 mg/kg/d plus a calcium diet increased PFC response, but that 5 mg/kg/d plus a protein diet decreased PFC response.

ENDRIN, ALDRIN, AND DIELDRIN

Data from laboratory studies with animals show that organochlorine pesticides, such as endrin, aldrin, and dieldrin, can be immunotoxic at very low doses (0.065-36 ppm) (depending on the model system and the route and duration of exposure). Organochlorine pesticides affect immune system functions either because they directly interact with immune effector cells or because their metabolic products do so. Specific examples of the immunologic effects of endrin and dieldrin in laboratory animals are shown in Table 7-4. The primary action appears to be in the macrophage processing of antigen (Loose et al. 1981; Loose 1982).

Limited information is available about the immunologic consequences of human exposure to organochlorine pesticides. In one case study (Muirhead et al. 1959), a pesticide sprayer developed immunohemolytic anemia after multiple exposures to dieldrin, heptachlor, and toxaphene. Specifically, the individual had circulating antibodies against dieldrin-coated erythrocytes and heptachlor-coated erythrocytes. However, because the patient also was exposed to other pesticides, including DDT, the usefulness of the information is limited. Immunohemolytic anemias also have been found after multiple dieldrin exposures in workers (Hamilton et al. 1978), or after consumption of dieldrin-contaminated fish (Hamilton et al. 1978). Loose et al. (1981) suggest that the human threshold for dieldrin immunotoxicity is 7×10^{-5} mg/kg/d. Given the limited amount of human data, definitive conclusions cannot be drawn about the effects of this family of pesticides on human health.

LINDANE

Lindane (primarily the Y-isomer of hexachloro-cyclohexane or benzene hexachloride) has been reported to perturb immune function after prenatal and postnatal exposure. Specific examples of the immunologic consequences of in vitro and laboratory exposures to animals are presented in Table 7-5. These effects appear to encompass both the nonspecific and the specific arms of the

TABLE 7-4 Laboratory Studies on Endrin and Dieldrin

Concentration	Species	Effect	Reference
Endrin			
3, 4.5, 6 mg/kg	Rat	Enhanced nitric oxide production by peritoneal macrophages at 24 and 48 hr	Akubue and Stohs 1992
4 mg/kg	Mouse	3-fold increase in hepatic mitochondrial lipid peroxidation; increase in reactive oxygen species by peritoneal macrophages	Bagchi et al. 1993a,b
Dieldrin			
10 M	Rat cells	Significant stimulation in vitro of polymorphonuclear neutrophils to release superoxide; dependent on the presence of extracellular calcium	Hewett and Roth 1988
0.65 mg/kg/d	Mouse	After infection with *Leishmania*, Kupffer cells stimulated to generate soluble factor that stimulated T-suppressor cell activity in mice treated for 10 wk	Loose 1982
18-36 mg/kg	Mouse	Intraperitoneal injection prolonged recovery from hepatitis infection; depressed production of antiviral IgG Ab	Krzystyniak et al. 1985, 1986
6 mg/kg	Mouse	Dysfunction of cellular cooperation during the induction phase of the immune response with suppressed production of anti-sheep red blood cells IgM and IgG, and anti-lipopolysaccharide IgM	Bernier et al. 1987
0.065 mg/kg/d	Mouse	Decreased antigen processing by alveolar macrophages observed in mice fed dieldrin for 2 wk	Loose et al. 1981
36 mg/kg	Mouse	Lymphoid cells had strong but transient inhibition of the mixed-lymphocyte reaction; mitogen response unaffected in mice administered intraperitoneal dieldrin	Hugo et al. 1988
16.6 mg/kg	Mouse	Inhibition of mixed-lymphocyte reaction	Fournier et al. 1988

TABLE 7-5 In Vitro and Laboratory Studies on Lindane

Concentration	Species	Effect	Reference
In Vitro Studies			
0.2-20 µg/mL	Human progenitor cells	Partial elimination of cell growth at 0.2 µg/mL; complete elimination of all cell growth at 2-µ20 g/mL	Parent-Massin et al. 1994
0.2-200 µg/mL	Rat GM-CFU[a] cells	0.2 and 2 g/mL significantly stimulated cell growth; 20 and 200 g/mL significantly reduced, but did not completely eliminate, growth	Parent-Massin et al. 1994
60, 80, 100 ppm	Amoeba	Phagocytic activity of labeled *Escherichia coli* inhibited, possibly because of alterations in receptor-mediated mechanisms	Gayatri and Chatterjee 1993
Laboratory Studies			
10, 100 mg/kg	Mouse	Alteration in cellular and humoral immunity in pups; pups from dams exposed prenatally to 10 mg/kg displayed significantly increased delayed hypersensitivity to SRBC[b]; 100 mg/kg significantly impaired delayed hypersensitivity response to SRBC. At 10 mg/kg, mitogen response of spleen cells was 2-fold greater for ConA[c] and 8-fold greater for LPS[d]; PFC[e] response was 2-fold greater. At 100 mg/kg, no effect on mitogen or PFC response	Das et al. 1990
0.012-1.2 mg/kg	Mouse	Dose-dependent initial stimulation in cell-mediated and humoral immunity, followed by suppression, in mice fed lindane for 24 wk; no apparent effect on macrophage function	Meera et al. 1992
0.012-1.2 mg/kg	Mouse	Biphasic effect of lindane corroborated; enhanced calcium uptake during initial stimulation, return to normal, and suppression in mice fed lindane for 24 wk	Meera et al. 1993
15 mg/kg/d	Mouse	After oral immunization with SRBC, a 2-fold increase in the titers of anti-SRBC IgG 2b antibody in mice fed lindane for 1 mo; duration of *Giardia muris* infection significantly increased; mice developed systemic anti-*Giardia* antibodies more frequently	Andre et al. 1983
100 mg/kg	Mouse	No significant change after 30 d treatment	Cornacoff et al. 1988

300 mg/kg/d	Mouse	44% reduction in natural killer cell activity, significant depression in lympho-proliferation as measured by phytohemagglutinin, ConA, and LPS response in mice fed lindane for 30 d	Cornacoff et al. 1988
1.3 mg/L	Fish	Reduced leukocyte counts; no statistical analyses performed	Saxena et al 1992
10, 50, 100 mg/kg/d	Fish	Fish treated with intraperitoneal injections for 45 d; after injection with *Yersinisa ruckeri* vaccine, slight suppression of antibody secretion observed with 10 mg/kg, effect magnified at higher doses	Dunier and Siwicki 1994
100 mg/kg	Fish	Stimulation of *Y. ruckeri*-antibody-secreting cells and lymphocyte proliferation by nitrogranulogen more efficient after immunosuppression induced by lindane	Siwicki and Dunier 1994
1 mg/kg	Fish	Depression of nonspecific immune system observed in fish treated for 30 d; decreased phorbol 12-myristate 13-acetate-induced phagocytosis by pronephric cells, depression lasted 2 wk, returned to normal after 1.5 mo	Dunier et al. 1994
10, 50, 100 mg/kg/d	Fish	Fish treated with intraperitoneal lindane for 45 d had depressed B-lymphocyte, but not T-lymphocyte, proliferation; B-lymphocytes reduced in the head kidney	Dunier et al. 1994
10, 50 mg/kg	Fish	30 d after single intraperitoneal injection of 10 mg/kg significantly reduced Ig+ lymphocytes, decreased proliferation of B-lymphocytes; effects not observed with 50 mg/kg. Significant modification in sera lysozyme and ceruloplasmin observed at each dose	Dunier et al. 1995

[a] GM-CFU, granulocyte-macrophage colony-forming unit.
[b] SRBC, sheep red blood cell.
[c] ConA, concanavalin A.
[d] LPS, lipopolysaccharide.
[e] PFC, plaque-forming cell.

immune system, including effects on humoral and cell-mediated immunity. Notably, lindane at concentrations of 0.012-10 mg/kg stimulated antibody production; production was inhibited at 100 mg/kg. Mitogen response and delayed hypersensitivity displayed similar biphasic effects (stimulation at 10 mg/kg; inhibition at 100 mg/kg). Although lindane at 1 mg/kg inhibited phagocytosis, higher concentrations were needed to inhibit other nonspecific elements of immunity (10 mg/kg for lysozyme concentrations; 300 mg/kg for natural killer-cell activity). Given the various concentrations of lindane tested, diverse routes of administration, differences in length of treatment, and variety of animal models, the exact sites of action of this pesticide and its mechanisms of action on the immune system remain clouded in speculation. Furthermore, with just one limited in vitro laboratory study on the effects of lindane on human immunity, it is premature to extrapolate broad conclusions about its effects in humans.

CHLORDANE

Some studies that use laboratory animal models suggest that exposure to chlordane (also under the trade names Octochlor and Velsicol 1068) leads to moderate immunotoxicity. Specific examples of the effects of this pesticide in laboratory animals are presented in Table 7-6. In inhalation studies, chlordane administered at 1 or 10 mg/m^3 for 90 d increased lymphocyte numbers in female rats; in vitro tests that used 10 M chlordane showed mitogenic activity. In addition, prenatal exposure to chlordane was reported to alter immune responses—such as delayed hypersensitivity, macrophage activation, and colony-forming unit activity—in offspring of exposed rats. The majority of studies—regardless of the concentration, route of administration, or time of application—found no histologic or functional changes that could be attributed to chlordane treatment.

Very limited information is available on the possible immunotoxic effects of chlordane exposure for humans. McConnachie and Zahalsky (1992) report significant changes in cell-mediated immunity and humoral immunity in humans exposed to chlordane aerosols for 3-15 mo. They report impairment in lymphoproliferation to mitogens ConA and phytohemagglutinin A (PHA) and increased titers of autoantibodies in 11 of the 12 subjects tested. These tests were performed from 4 mo to 10 yr after exposure, implying long-term immunotoxicity for this agent. However, the studies are too limited to support any conclusions about the effects of chlordane on human health.

TOXAPHENE

There are few data to describe the immunologic effects of toxaphene in laboratory animal models. In one study (Trottmann and Desaiah 1980), thymus weight was reduced in mice after oral administration of toxaphene at 22.5 and 30 mg/kg/d for 14 d. Koller et al. (1983) observed depressed IgG antibody produc-

tion in rats treated with 0.5, 1.5, and 10 mg/kg/d for 6 wk. Allen et al. (1983) treated mice with toxaphene at 15 and 30 mg/kg/d for 8 wk and report depressed IgG antibody production. No effect on delayed hypersensitivity was reported.

In a prenatal exposure study (Chernoff et al. 1990), pregnant rats were administered 32 mg/kg/d toxaphene by gavage from the onset of pregnancy until gestation d 8, 12, or 16. Rats were killed at these time points or on d 20 of gestation. Spleen weight was significantly reduced in fetuses from rats sacrificed on d 8, 16, and 20; thymus weight was reduced in fetuses from rats sacrificed on d 8 and 20.

The studies above are limited, and additional studies are required before a reliable assessment of the possible immunologic effect of toxaphene on animals or humans can be made.

ENDOSULFAN

Exposure to endosulfan has been reported to produce immunotoxic changes in nonspecific immunity and in humoral and cell-mediated responses in laboratory animals. Immunologic studies with endosulfan are summarized in Table 7-7. Oral exposure to endosulfan induced immunologic effects at 1-5 mg/kg/d (from 6-22 wk), but inhalation and dermal routes did not. The endosulfan studies are limited, and there is little information on the immunologic effects of this compound in humans.

HEXACHLOROBENZENE

Exposure to hexachlorobenzene has been reported to produce histologic changes in lymphoid tissue architecture in laboratory animals. Vos et al. (1983) report that prenatal and postnatal exposure to hexachlorobenzene at 4 mg/kg/d in feed enhanced humoral and cellular immune responses in rats. Hexachlorobenzene also promoted accumulation of macrophages in the rats' lungs. The high endothelial venules present in the lymph nodes underwent abnormal proliferation, accompanied by lymphoid hyperplasia in the splenic white pulp, in rats fed this compound at 25-100 mg/kg/d for 3 wk (Vos et al. 1979). Hyperplasia of lymphoid tissue in the stomach has been induced in dogs fed hexachlorobenzene at 6.5-10 mg/kg (1 mg/d) for 12 mo (Gralla et al. 1977).

No information is available about the possible immunotoxicity of hexachlorobenzene in humans, and the data from laboratory studies are too limited to support any conclusions about how this compound affects human immune response.

SUMMARY AND CONCLUSIONS

There are very few studies of the immunologic effects of human exposure to HAAs, but for some chemicals there are adequate data for laboratory animals.

TABLE 7-6 Laboratory Studies on Chlordane

Concentration	Species	Effect	Reference
Inhalation Studies			
5.8, 28.2 mg/m^3	Rat	In rats treated 8 hr/d, 5 d/wk for 28 d, reduction in thymic weight of females exposed to 28.2 mg/m^3 chlordane	Khasawinah et al. 1989
1, 10 mg/m^3	Rat	In rats treated 8 hr/d, 5 d/wk for 90 d, increased lymphocyte numbers in female rats; no significant change in thymic weight	Khasawinah et al. 1989
1, 10 mg/m^3	Monkey	No significant changes in lymphocyte numbers and no histologic changes in either lymph nodes or spleen in monkeys treated 8 hr/d, 5 d/wk for 90 d.	Khasawinah et al. 1989
Oral Studies			
200 mg/kg	Rat	No histologic changes in the spleen	Truhaut et al. 1974, 1975
16 mg/kg/d	Rat	No effects on spleen weight or spleen histology in rats fed chlordane for 407 d	Ambrose et al. 1953
1.25 mg/kg/d	Rat	No effects on spleen weight or spleen histology in rats fed chlordane for 2-9 mo	Ortega et al. 1957
200 mg/kg	Mouse	No histologic changes in the spleen	Truhaut et al. 1974, 1975
8 mg/kg/d	Mouse	No evidence of immune dysfunction, although leukocytosis and lymphocytosis were present in mice administered chlordane by gavage for 14 d	Johnson et al. 1986
1200 mg/kg	Hamster	No histologic changes in the spleen	Truhaut et al. 1974, 1975

4, 8 mg/kg/d	Mouse	Offspring from dams fed chlordane for 18 d during gestation had depressed in vitro in GM-CFU[a] and SCFU[b]; viability of bone marrow cells unaffected; no effect on GM-CFU or SCFU activity in dams	Barnett et al. 1990a,b
0.16, 8.0 mg/kg/d	Mouse	Offspring of dams fed 8.0 mg/kg/d through d 19 of gestation had depression in delayed hypersensitivity to oxazolone; no difference in humoral immunity as measured by plaque-forming cell assay	Spyker-Cranmer et al. 1982
4.0, 8.0, 16.0 mg/kg/d	Mouse	Offspring from pregnant mice fed 8 and 16 mg/kg/d through d 19 of gestation had significant depression in delayed hypersensitivity responses to oxazolone; delayed hypersensitivity response to influenza type A depressed; antiviral antibody titers significantly increased in female offspring only	Barnett et al. 1985a,b Menna et al. 1985
8 mg/kg/d	Mouse	Offspring from mice fed chlordane for 18 d during gestation had significant decrease in 5′-nucleotidase response; macrophages expressed advanced stage inflammatory responses	Theus et al. 1992
Other Studies			
67 mg/kg/d	Guinea pig	Attempts to generate dermal hypersensitivity with chlordane over 90 d unsuccessful; these animals were not presensitized to chlordane before dermal application	Datta et al. 1977
10, 80 μM	Monkey cells	In vitro T-cell mitogenic response modified in peripheral blood mononuclear cells; at 10 μM, chlordane acted as a T-cell mitogen in the absence of conventional mitogens; 80 μM completely impaired T-cell function	Chuang et al. 1992

[a] GM-CFU, granulocyte-macrophage colony forming unit.
[b] SCFU, spleen colony forming unit.

TABLE 7-7 Studies on Endosulfan

Concentration	Species	Effect	Reference
Inhalation Studies			
2 mg/m^3/d	Rat	Rats exposed for 6 hr/d, 5 d/wk for 21 or 29 d showed no histologic change in lymph nodes, thymus, spleen	Hoechst 1984a
Oral Studies			
1, 2, 3, 5 mg/kg/d	Rat	Significantly depressed humoral response, specifically in serum IgG and IgM circulating gammaglobulin fraction, in rats fed endosulfan for 6-22 wk	Banerjee and Hussain 1986, 1987
1, 2 mg/kg/d	Rat	Spleen weight reduced in rats fed 1.0 mg/kg for 22 wk; macrophage migration inhibition and leukocyte migration inhibition response significantly reduced dependent on dose	Banerjee and Hussain 1986, 1987
7.3 mg/kg/d	Mouse	Male mice fed endosulfan for 13 d had significantly reduced spleen weight	Hoechst 1984b
Dermal Studies			
597 mg/kg/d	Guinea pig	No measurable sensitization observed in guinea pigs treated for 6 hr/d, 3 d/wk for 3 wk	Hoechst 1983
81 mg/kg/d	Rat	No effect on thymus weight in male rats treated for 6 hr/d, 5 d/wk for 30 d	Hoechst 1985
27 mg/kg/d	Rat	No effect on thymus weight in female rats treated for 6 hr/d, 5 d/wk for 30 d	Hoechst 1985
1,000 mg/kg/d	Rat	Spleen weight reduced in 1 male rat after exposure to chlordane for 6 hr, 5 d/wk	Hoechst 1989
1% solution	Human	No skin sensitivity to endosulfan in 48 hr closed patch test of 14 farm workers	Schuman and Dobson 1985

Animal studies have identified immunotoxic and immunomodulatory effects. The most extensively studied compounds are the HAHs. Laboratory studies have shown that HAHs affect the functional arms of the immune system. Field studies of birds show a strong exposure-response relationship between organochlorines and immune suppression. Experimental studies have also shown that innate and acquired immune responses were impaired in seals fed fish from the contaminated Baltic Sea. This immunosuppression is believed to be the reason for increased incidences of bacterial and viral infections in seal populations found in contaminated waters. There have only been a few studies of the effects of HAAs in humans, but the results of laboratory and wildlife studies suggest that HAAs have the potential to affect human immune functions. Certainly, additional clinical immunologic end points must be studied. As noted by Kerkvliet and Burleson (1994) "massive retrospective studies on poorly defined exposure groups cannot be justified to try to 'prove' that immune modulation has occurred in these people." The authors state that "research must focus on the definition of sensitive end points (i.e., biomarkers) of immune dysfunction in humans. . . . In particular it is important to determine in animal models how well changes in immune function in the lymphoid organs (e.g., spleen and lymph nodes) correlate with changes in the expression of lymphocyte subset/activation markers in peripheral blood. Until such correlations are established, the interpretation of changes observed in subset/activation markers in human peripheral blood lymphocytes in terms of health risk will be limited to speculation." HAHs are thought to act through Ah receptor binding, but some components of HAH-mediated immune suppression could function through other, independent mechanisms.

The data available on DDT suggest that DDT mediates immunosuppression in laboratory animals and in birds. Certainly, DDT possesses estrogenic properties, and it could act by binding to steroid receptors to modulate immunity. Chlordecone also could modulate immunity through steroid-receptor-binding pathways. Studies of endrin, aldrin, and dieldrin are limited, but the immunologic effects reported for these chemicals in laboratory studies appear to involve macrophage processing of antigen. Reported effects from lindane encompass both the specific and the nonspecific arms of the immune system, and in laboratory animals such responses have included effects on humoral and cell-mediated immunity. Exposure to endosulfan in laboratory studies also has produced immunotoxic effects on nonspecific immunity and altered humoral and cell-mediated responses. In studies of chlordane, moderate immunotoxicity has been observed in laboratory animals, but most studies have not identified histologic or functional changes. Histologic changes in lymphoid tissue architecture were found in laboratory animals after exposure to hexachlorobenzene. There is little information available on the immunologic effects of toxaphene.

Generally, the available data neither support nor refute the premise that the

actions of HAAs is mediated either directly or indirectly through endocrine pathways. It can be stated with some degree of certainty that some HAAs affect one or more aspects of immune function, at least in animal models. Field studies, especially in marine mammals, generally support this view, although there is intrinsic uncertainty for such studies because the conditions of exposure to the environmental contaminants responsible are not known and mixtures of the compounds are not always clearly defined.

In human studies, the cause-and-effect relationship between HAAs and immunotoxicity is not clear cut. Thomas (1995) considers all the available data on Great Lakes residents exposed to potentially immunotoxic agents through the food chain and concludes that, based on "uncertainties with regard to exposure levels, predictability of tests, suitability of the animal models, and immune reserve . . . there is no definite evidence as yet that environmental [exposure] to these xenobiotics poses a significant threat to the human immune system." In the few human studies available, exposure to mixtures, extended delays between time of exposure and performance of immunologic tests, and the effects of other confounding variables—age, sex, lifestyle, underlying disease—all tend to limit support for any definitive conclusion. In addition, immunosuppressive effects of background exposures have not been determined. Thus, although animal studies suggest that HAAs can cause immunologic effects, underlying mechanisms are not clear. It is also unclear whether they have similar effects on the human immune system.

RECOMMENDATIONS

Based on the committee's review of the extensive laboratory animal data on immunologic effects of HAAs, as well as the limited information from wildlife and human studies, the following are recommended:

—Comprehensive epidemiologic studies that evaluate a variety of health effects, including immunologic effects, of human populations suspected of being affected by HAAs should be initiated. Especially needed are studies of cohorts established either through registries or directed effort to assess the prevalence of autoimmune problems in offspring whose mothers were exposed during pregnancy. To address the potential problem of measuring exposure in a case-control or cohort design, it is suggested that populations known to have been heavily exposed to HAAs (such as the Seveso population) be used for cohort studies. Ideally, such cohorts should be followed throughout their lifetime.

Epidemiologic studies should use clinically relevant immunologic assays, such as those for monitoring concentrations of circulating antibodies to thymus-dependent antigens; antigen test banks to monitor delayed-hypersensitivity skin reactions; quantitative lymphocyte subclass identifications; in vitro measurements

of lymphocyte cytokine production and possibly mitogenic responsiveness; and the lytic action attributed to cytotoxic lymphocytes and natural killer cells in exposed populations to clarify the relationship between HAA exposure and human health.

—Because much of the available immunologic laboratory data on HAAs is on chemicals that have been regulated and, in most cases, are no longer used in the United States, future studies should focus on chemicals that are being used, such as endosulfan and lindane.

8

HAAs and Carcinogenesis in Animals

IT HAS BEEN HYPOTHESIZED that environmental exposure to hormonally active agents (HAAs) results in an elevated risk of hormone-related cancers in humans. Human and experimental animal data link these cancers to exposure to endogenous hormones and diethylstilbestrol (DES) (see Appendix) (Herbst et al. 1971; Sonnenschein et al. 1974; Wiklund and Gorski 1982; Key and Pike 1988; Greenman et al. 1990; Brinton and Hoover 1993; Mittendorf 1995; Nandi et al. 1995). Endogenous estrogens have been associated with the development of tumors in the breast and endometrium in humans (Key and Pike 1988; Brinton and Hoover 1993; Nandi et al. 1995), and in the mammary, pituitary, and thyroid glands in animals (Sonnenschein et al. 1974; Wiklund and Gorski 1982; Greenman et al. 1990; Nandi et al. 1995). Endogenous estrogens could act as "initiators" by inducing DNA mutations (Liehr et al. 1986), as "promoters" by inducing cell proliferation (Russo and Russo 1978), or by allowing the persistence of tissues that normally regress or differentiate during development (Takasugi 1976; Bern 1992a). For a detailed discussion of the molecular mechanisms of estrogen-induced carcinogenesis, see Yager and Liehr (1996). The introduction of HAAs into the environment in the past 50-60 yr has preceded and overlapped the increasing incidence rates of some kinds of cancer. Because there is a lag period between exposure to a carcinogen and the induction of clinically apparent neoplasias, it is reasonable to investigate the association between HAAs and cancer.

This chapter reviews and evaluates data from animal studies relating environmental HAAs to cancers of the female and male reproductive systems and endocrine organs. The committee limited its review to cancer sites that are known from ancillary data to have some hormonal dependence and where activity should be most evident. However, the committee recognizes that some of the compounds discussed have been shown to cause cancer in other organ systems,

and in a variety of species. Compounds discussed in this chapter, with the exception of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), have been shown to possess estrogenic activity in at least one in vitro or in vivo bioassay (see Chapter 2). Finally, with the exception of DDT, it must be emphasized that this chapter focuses on postnatal exposures because no data are available on the carcinogenic effects of perinatal exposure to environmental HAAs to the F_1 or succeeding generations.

BIOASSAYS

Bioassays of the following compounds were evaluated with regard to carcinogenic effects in selected reproductive organs (i.e., endometrium/uterus, ovaries, testicles, and prostate gland) and endocrine organs (e.g., mammary, pituitary, thyroid, and adrenal glands): aldrin and dieldrin, 4,4′isopropylidenediphenol (bisphenol A), butyl benzyl phthalate (BBP), chlordecone, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD), 1,1- dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE), dichlorodiphenyltrichloroethane (DDT), endosulfan, endrin, lindane, methoxychlor, polychlorinated biphenyls (PCBs), TCDD, and toxaphene. Table 8-1 presents the experimental details and the specific results of the bioassays. A summary of those findings is presented below, and is followed by a discussion of their strengths and limitations. Negative results should be interpreted to mean that the HAA did not cause tumor formation under the conditions tested. Different test conditions may yield different results in the incidence of tumors.

Aldrin and Dieldrin

Aldrin was tested in bioassays in $B6C3F_1$ and C_3HeB/Fe mice, Osborne-Mendel rats, and mongrel dogs (Davis and Fitzhugh 1962; Fitzhugh et al. 1964; NCI 1978e). Dieldrin was tested in C_3HeB/Fe, CF1, or $B6C3F_1$ mice (Davis and Fitzhugh 1962; Thorpe and Walker 1973; Walker et al. 1973; NCI 1978e); Fischer 344, Osborne-Mendel, and Carworth Farm "E" strain (CF "E") rats (Fitzhugh et al. 1964; Walker et al. 1969; NCI 1978e,f); Syrian Golden hamsters (Cabral et al. 1979); and beagle and mongrel dogs (Fitzhugh et al. 1964; Walker et al. 1969). None of the bioassays involved perinatal exposure. Overall, there was no evidence that either aldrin or dieldrin induced tumors of the endometrium/uterus, ovaries, testicles, prostate gland, mammary gland, thyroid gland, pituitary gland, or adrenal glands.

Bisphenol A

Bisphenol A was tested for carcinogenicity in Fischer 344 rats and $B6C3F_1$ mice (NTP 1982b). These tests involved exposure to adult animals only. An increase in testicular tumors (interstitial-cell tumors) was observed in 96% of the

rats of the low-dose group ($p = 0.001$) and in 94% of the high-dose group ($p = 0.003$), compared with 71% in the control group. However, the committee noted that aging Fischer 344 rats have a high incidence (more than 90%) of this type of tumor. Incidence data from the treated groups and the historical controls were not significantly different.

No increases were found in the incidence of tumors of the endometrium/uterus, ovaries, prostate gland, mammary gland, thyroid gland, pituitary gland, or adrenal glands.

Butyl Benzyl Phthalate

Bioassays of BBP were conducted using Fischer 344/N rats (NTP 1997) and B6C3F$_1$ mice (NTP 1982c). None of the studies involved prenatal exposure to BBP. There was no evidence that BBP increased the incidence of tumors of the endometrium/uterus, ovaries, testicles, prostate gland, mammary gland, thyroid gland, pituitary gland, or adrenal glands compared with controls.

Chlordecone

Chlordecone was tested in bioassays using Osborne-Mendel rats and B6C3F$_1$ mice (NCI 1976). Tests were conducted only on adult animals. There was no evidence that chlordecone increased the incidence of tumors of the endometrium/uterus, ovaries, testicles, prostate gland, mammary gland, thyroid gland, pituitary gland, or adrenal glands compared with controls. However, there is some evidence that chlordecone might induce cancers in the liver, which led the International Agency for Research on Cancer (IARC) to classify it as possibly carcinogenic to humans (IARC 1987).

DDD

DDD was evaluated for carcinogenicity in Osborne-Mendel rats and B6C3F$_1$ mice (NCI 1978c). The tests involved exposure to adult animals only. DDD increased the incidence of thyroid tumors in male rats. The incidence of follicular-cell adenoma, follicular-cell carcinoma, c-cell adenoma, c-cell carcinoma, and adenoma was 50% in the low-dose group, 32% in the high-dose group, and 10% in control groups. The difference between the DDD-treated groups and the control groups was significant in the case of male rats with follicular-cell carcinoma and follicular-cell adenoma. There was no significant increase in the incidence of thyroid tumors in female rats. However, the authors indicate that the Cochran-Armitage test revealed a significant positive association between dose and combined incidence of follicular-cell adenomas and carcinomas in females. It is not known whether DDD induced the thyroid tumors through a hormonally mediated mechanism, although there is evidence that natural estrogens and DES

TABLE 8-1 Environmental HAAs and Cancer

Species (Reference)	Dose	Results[a] and Limitations
ALDRIN AND DIELDRIN		
Rat		
Osborne-Mendel (NCI 1978e)	**Aldrin.** Males and females (50 per group) received 30 or 60 ppm (1.5 or 3.0 mg/kg/d) in diet for 74 (males) or 80 (females) wk.	**Aldrin.** No statistically significant increased incidence of tumors in reproductive or other endocrine organs. There was a significant positive linear trend in the incidence of follicular-cell adenoma or follicular-cell carcinoma of the thyroid in the male and female low-dose groups when compared with the pooled control group, but not when compared with the matched control group. Both male and female high-dose groups failed to confirm the significance seen in the low-dose groups. Additionally, cortical adenomas of the adrenal gland were observed in females in significant proportions (p = .001) in the low-dose group, but not in the high-dose group, when compared to the pooled control group. These increased incidences were not consistently significant when compared to matched rather than pooled control groups. **Males.** Mammary fibrosarcoma: 2%, 0%, 0%; testicular: NTR; prostate: NTR; pituitary chromophobe adenoma and chromophobe carcinoma: 35%, 33%, 33%; thyroid follicular cell adenoma, follicular cell carcinoma, c-cell adenoma, and c-cell carcinoma: 48%, 29%, 71%; adrenal cortical adenoma, cortical carcinoma, pheochromocytoma: 6%, 5%, 20%. **Females.** Uterine leiomyosarcoma and endometrial stomal polyp: 13%, 21%, 0%; mammary papillary adenocarcinoma: 20%, 14%, 30%; ovarian granulosa cell tumor: 2%, 9%, 0%; pituitary chromophobe adenoma: 35%, 23%, 44%; thyroid follicular cell adenoma, follicular cell carcinoma, c-cell adenoma, and c-cell carcinoma: 41%, 37%, 22%; adrenal cortical adenoma: 18%, 2%, 0%.
Osborne-Mendel (NCI 1978e)	**Dieldrin.** Males and females (50 per group) received 29 or 65 ppm (1.45 or 3.25 kg/mg/d) in diet for 80 (low-dose group) or 59 (high-dose group) wk.	**Dieldrin.** No statistically significant increased incidence of tumors in reproductive or other endocrine organs. Females showed a significant (p = .007) difference between combined incidence of adrenal cortical adenoma or carcinoma in the low-dose group and that in the pooled control group. However, the incidence in the high-dose group was not significant, and the incidences were not significant when matched controls were used for comparison. Males did not show a statistically significant difference between treated and control groups.

(table continues)

TABLE 8-1 Continued

Species (Reference)	Dose	Results[a] and Limitations
		Males. Mammary fibroma: 2%, 0%, 10%; testicular interstitial-cell tumor and mesothelioma NOS: 4%, 0%, 0%; prostate: NTR; pituitary chromophobe adenoma, chromophobe carcinoma, acidophil adenoma: 34%, 32%, 30%; thyroid follicular cell adenoma, follicular cell carcinoma, c-cell adenoma, and c-cell carcinoma: 26%, 28%, 0%; adrenal cortical adenoma, pheochromocytoma, sarcoma NOS, ganglioneuroma: 2%, 11%, 10%. **Females.** Uterine leiomyosarcoma and endometrial stromal polyp: 11%, 3%, 10%; mammary adenoma NOS, adenocarcinoma NOS, fibroadenoma: 29%, 12%, 10%; ovarian granulosa cell tumor: 4%, 2%, 0%; pituitary chromophobe adenoma, chromophobe carcinoma: 27%, 27%, 50%; thyroid follicular cell adenoma, follicular cell carcinoma, c-cell adenoma, and c-cell carcinoma: 37%, 34%, 25%; adrenal cortical adenoma and carcinoma and pheochromocytoma: 13%, 8%, 0%.
Fischer 344 (NCI 1978f)	**Dieldrin.** Males and females (24 per group) received 2, 10, or 50 ppm (0.1, 0.5, or 2.5 mg/kg/d) in diet for 104-105 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. This study is limited because too few animals were used and also, the thyroids were not routinely examined microscopically. **Males.** Mammary: NTR; testicular interstitial cell tumor: 96%, 100%, 83%, 100%; prostate: NTR; pituitary adenoma: 13%, 4%, 4%, 0%; thyroid small cell carcinoma and adenoma NOS: 0%, 0%, 100%, 7%; adrenal gland: NTR. **Females.** Uterine adenocarcinoma NOS, leiomyoma, endometrial stromal polyp, endometrial stromal sarcoma: 80%, 63%, 46%, 59%; mammary adenoma, adenocarcinoma, cystadenoma, fibroma, fibroadenoma: 12%, 4%, 0%, 16%; ovary: NTR; pituitary adenoma NOS: 30%, 17%, 9%, 8%; thyroid: NTR; adrenal gland: NTR.
Osborne-Mendel (Fitzhugh et al. 1964)	**Aldrin and Dieldrin.** Males and females (12 per group) received 0.5, 2, 10, 50, 100, or 150 ppm (0.025, 0.1, 0.5, 2.5, 5, 7.5 mg/kg/d) in diet for 2 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs. This study is flawed because too few animals were used, the number of animals examined microscopically was limited, and there were high levels of early mortality with insufficient numbers of animals surviving until termination of the study.

Carworth Farm "E" strain (Walker et al. 1969)	**Dieldrin.** Males and females (25 per group) received 0.1, 1, or 10 ppm (0.005, 0.05, or 0.5 mg/kg/d) in diet for 2 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs. This study is flawed because the number of animals examined microscopically was limited.
Mouse B6C3F$_1$ (NCI 1978e)	**Aldrin.** Males (50 per group) received 4 or 8 ppm (0.6 or 1.2 kg/mg/d) in diet for 80 wk. Females (50 per group) received 3 or 6 ppm (0.45 or 0.9 kg/mg/d) in diet for 80 wk.	**Aldrin.** No statistically significant increased incidence of tumors in reproductive or other endocrine organs. **Males.** Mammary: NTR; testicular interstitial cell tumor: 0%, 0%, 0%, 10%; prostate: NTR; pituitary: NTR; thyroid follicular cell adenoma: 13%, 2%, 0%,0%; adrenal gland: NTR.
	Dieldrin. Males and females (50 per group) received 2.5 or 5 ppm (0.37 or 0.75 mg/kg/d) in diet for 80 wk.	**Females.** Uterine endometrial stromal polyp: 2%, 0%, 0%; mammary leiomysarcoma: 0%, 0%, 10%; ovarian leiomysarcoma: 0%, 0%, 10%; pituitary chromophobe adenoma: 2%, 0%, 0%; thyroid adenoma: 2%, 0%, 0%; adrenal leiomysarcoma: 0%, 0%, 10%. **Dieldrin.** No statistically significant increased incidence of tumors in reproductive or other endocrine organs. **Males.** Mammary: NTR; testicular: NTR; prostate: NTR; pituitary: NTR; thyroid : NTR; adrenal gland: NTR. **Females.** Uterine endometrial stromal polyp: 0%, 2%, 0%, 0%; mammary: NTR; ovary: NTR; pituitary chromophobe adenoma: 0%, 3%, 3%, 0%; thyroid follicular cell adenoma: 0%, 0%, 0%, 10%; adrenal gland: NTR.
C_3HeB/Fe (Davis and Fitzhugh 1962)	**Aldrin and Dieldrin.** Males and females (approximately 36 per group) received 10 ppm (1.5 mg/kg/d) in diet for 2 yr.	No significantly increased incidence of tumors in reproductive or other endocrine organs.
Carworth Farm No. 1 (Walker et al. 1973)	**Dieldrin.** Males and females (250-400 per group) received 0.1, 1, or 10 ppm (0.015, 0.15, 1.5 mg/kg/d) in diet for 2 yr.	No significantly increased incidence of tumors in reproductive or other endocrine organs.

(table continues)

TABLE 8-1 Continued

Species (Reference)	Dose	Results[a] and Limitations
Carworth Farm No. 1 (Thorpe and Walker 1973)	**Dieldrin.** Males and females (30 per group) received 10 ppm (1.5 mg/kg/d) in diet for 2 yr.	No significantly increased incidence of tumors in reproductive or other endocrine organs.
Hamster Syrian Golden (Cabral et al. 1979)	**Dieldrin.** Males and females (36 per group) received 20, 60, or 180 ppm (0.8, 2.4, or 7.2 mg/kg/d) in diet for life span.	No significantly increased incidence of tumors in reproductive or other endocrine organs.
Dog Mongrel (Fitzhugh et al. 1964)	**Aldrin and dieldrin.** Animals (sex not specified, 26 total) received 8-400 ppm (0.2-10 mg/kg/d) in diet for up to 25 mo.	No significant increased incidence of tumors in reproductive or other endocrine organs. This study is flawed because too few animals were used and the length of administration was not adequate for a carcinogenicity bioassay.
Beagle (Walker et al. 1969)	**Dieldrin.** Males and females (5 per group) received 0.1 or 1 ppm (0.005 or 0.05 mg/kg/d) in diet for 2 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs. This study is flawed because too few animals were used and the length of administration was not adequate for a carcinogenicity bioassay.
BISPHENOL A		
Rat Fischer 344 (NTP 1982b)	Males and females (50 per group) received 1,000 or 2,000 ppm (50 mg/kg/d or 100 mg/kg/d) in diet for 103 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. However, the incidence of mammary fibroadenomas in males was increased in the high-dose group compared with the control group (8% in the high-dose group; 0% in the control group). That is a statistically significant dose-response trend based on the Cochran-Armitage test (p = .015). **Males.** Mammary fibroadenoma: 0%, 8%, 0%; testicular interstitial-cell tumors: 96%, 94%, 71%; pituitary carcinoma and adenoma: 27%, 31%, 28%; thyroid C-cell adenoma and C-cell carcinoma: 12%, 13%, 19%; adrenal-gland cortical adenoma, cortical carcinoma, and pheochromocytoma: 14%, 15%, 31%.

		Females. Mammary adenoma: 2%, 0%, 0%; ovarian granulosa-cell tumor and fibrosarcoma: 2%, 0%, 4%; pituitary adenoma: 41%, 50%, 52%; thyroid C-cell adenoma and C-cell carcinoma: 13%, 11%, 8%; adrenal-gland neoplasm, cortical adenoma, pheochromocytoma, ganglioneuroma: 18%, 16%, 30%.
Mouse $B6C3F_1$ (NTP 1982b)	Males (50 per group) received 1,000 or 5,000 ppm (150 mg/kg/d or 750 mg/kg/d) and females (50 per group) received 5,000 or 10,000 ppm (750 mg/kg/d or 1,500 mg/kg/d) in diet for 103 wk.	No statistically significantly increased incidence of tumors in reproductive or other endocrine organs. However, the incidence of pituitary chromophobe carcinomas in males was increased in the high-dose group compared with the control group (7% in the high-dose group; 0% in the control group). That is a statistically significant dose-response trend based on the Cochran-Armitage test (p = .016). **Males.** Reproductive organs: NTR; pituitary chromophobe carcinoma: 0%, 7%, 0%; adrenal cortical adenoma and sarcoma: 2%, 6%, 2%. **Females.** Endometrial stromal polyp and leiomyosarcoma: 2%, 4%, 0%; mammary adenocarcinoma and adenoma–squamous metaplasia: 2%, 2%, 0%; ovarian papillary adenoma and granulosa-cell tumor: 0%, 4%, 0%; pituitary chromophobe adenoma and chromophobe carcinoma: 0%, 3%, 5%; thyroid follicular-cell adenoma: 0%, 0%, 3%.
BUTYL BENZYL PHTHALATE		
Rat Fischer 344/N (NTP 1997)	Males (60 per group) received 3,000, 6,000, or 12,000 ppm (120, 240, or 500 mg/kg/d) in diet for 2 yr. Females (60 per group) received 6,000, 12,000, or 24,000 ppm (300, 600, 1200 mg/kg/d) in diet for 2 yr.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. Females exposed to the highest dose had an incidence of fibroadenomas of the mammary gland that was statistically significantly decreased compared to control animals (p = .001). This decreased incidence was attributed to lower mean body weights in the dosed group. **Males.** Mammary carcinoma and fibroadenomas: 12%, 4%, 0%, 4%; testicular adenocarcinoma (metastatic), interstitial cell adenoma: 92%, 98%, 90%, 88%; prostate adenocarcinoma (metastatic): 0%, 2%, 0%, 0%; pituitary adenoma and carcinoma: 24%, 24%, 20%, 20%; thyroid c-cell adenoma, follicular cell adenoma, follicular cell carcinoma: 8%, 6%, 8%, 10%; adrenal pheochromocytoma: 20%, 22%, 20%, 22%.

(table continues)

TABLE 8-1 Continued

Species (Reference)	Dose	Results[a] and Limitations
		Females. Uterine deciduoma, leiomyoma, leiomyosarcoma, polyp stromal, sarcoma stromal: 20%, 14%, 16%, 16%; mammary adenoma, carcinoma, and fibroadenomas: 62%, 66%, 22%, 65%; ovarian arrhenoblastoma NOS and granulosa cell tumor: 0%, 2%, 0%, 4%; pituitary adenoma and carcinoma: 52%, 52%, 26%, 45%; thyroid c-cell adenoma, c-cell carcinoma, follicular cell adenoma, follicular cell carcinoma: 16%, 6%, 6%, 10%; adrenal ademona, carcinoma, ganglioneuroma, and pheochromocytoma: 16%, 4%, 2%, 4%.
Mouse B6C3F$_1$ (NTP 1982c)	Males and females (50 per group) received 6,000 or 12,000 ppm (900 or 1,800 mg/kg/d) in diet for up to 103 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. **Males.** Mammary: NTR; testicles: NTR; prostate: NTR; pituitary: NTR; thyroid follicular cell adenomas and follicular cell carcinoma: 2%, 0%, 4%. **Females.** Uterine leiomyoma, leiomyosarcoma, endometrial stromal polyp, and endometrial stromal sarcoma: 0%, 6%, 6%; mammary: NTR, ovarian: cystadenocarcinoma NOS: 0%, 2%, 0%; pituitary adenoma NOS: 0%, 0%, 2%; thyroid: NTR; adrenal cortical adenoma: 2%, 0%, 0%.
CHLORDECONE		
Rat Osborne-Mendel (NCI 1976)	Males (50 per group) received 8 or 24 ppm (0.4 or 1.2 mg/kg/d) in diet for 80 wk. Females (50 per group) received 18 or 26 ppm (0.9 or 1.3 mg/kg/d) in diet for 80 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs.[b] **Males.** Mammary fibroadenoma, adenoma, fibroma, adenocarcinoma, fibrolipoma: 2%, 5%, 20%; testicular: NTR; prostate: NTR; pituitary chromophobe adenoma and adenocarcinoma: 24%, 14%, 40%; thyroid follicular cell carcinoma, follicular cell adenoma, c-cell adenoma, c-cell carcinoma: 18%, 0%, 0%; adrenal cortical adenoma: 2%, 0%, 0%. **Females.** Uterine endometrial/stromal polyp, malignant lymphoma, squamous-cell carcinoma: 8%, 4%, 0%; mammary fibroadenoma, adenoma, adenocarcinoma, fibrolipoma: 14%, 4%, 50%; ovarian arrhenoblastoma and granulosa-cell tumor: 2%,

		2%, 0%; pituitary chromophobe adenoma: 27%, 9%, 30%; thyroid follicular cell carcinoma, follicular cell adenoma, c-cell adenoma, c-cell carcinoma: 6%, 7%, 0%; adrenal cortical adenoma: 0%, 0%, 4%.
Mouse B6C3F$_1$ (NCI 1976)	Males (50 per group) received 20 or 23 ppm (3 or 3.45 mg/kg/d) in diet for 80 wk. Females (50 per group) received 20 or 40 ppm (3 or 6 mg/kg/d) in diet for 80 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. **Males.** Mammary: NTR; testicular: NTR; prostate: NTR; pituitary: NTR; thyroid: NTR; adrenal gland: NTR. **Females.** Uterine/endometrial: NTR; mammary: NTR; ovarian cystadenoma: 0%, 2%, 0%; pituitary: NTR; thyroid: NTR; adrenal gland: NTR.
DDD		
Rat Osborne-Mendel (NCI 1978c)	Males (50 per group) fed 1,647 ppm or 3,294 ppm (82.3 mg/kg/d or 164.7 mg/kg/d) in diet for 78 wk. Females (50 per group) fed 850 ppm or 1,700 ppm (42.5 mg/kg/d or 85.0 mg/kg/d) in diet for 78 wk.	Increased incidence of thyroid tumors in males; positive association between dose and combined incidence of thyroid tumors in females. The high incidence of tumors in control animals (37% mammary tumors, 21% pituitary tumors, 21% thyroid tumors in females) raises concern about the interpretation of this study. **Males.** Mammary fibroadenoma: 0%, 2%, 0%; testicle: NTR; prostate: NTR; pituitary chromophobe adenoma and glioma: 27%, 24%, 5%; thyroid follicular-cell adenoma, follicular-cell carcinoma, C-cell adenoma, C-cell carcinoma, and adenoma: 50%, 32%, 10%; adrenal-gland pheochromocytoma: 0%, 5%, 0%. **Females.** Endometrial–uterine squamous-cell carcinoma: 3%, 3%, 0%; mammary fibroadenoma and adenocarcinoma: 29%, 22%, 37%; ovary: NTR; pituitary chromophobe adenoma: 47%, 36%, 21%; thyroid follicular-cell adenoma, follicular-cell carcinoma, C-cell adenoma, and C-cell carcinoma: 31%, 22%, 21%; adrenal-gland cortical adenoma, cortical carcinoma, and pheochromocytoma: 4%, 6%, 0%.
Mouse B6C3F$_1$ (NCI 1978c)	Males and females (50 per group) fed 411 ppm or 822 ppm (61.6 mg/kg/d or 123.3 mg/kg/d) in diet for 78 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. **Males.** Mammary: NTR; testicle: NTR; prostate: NTR; pituitary: NTR; thyroid: NTR; adrenal gland: NTR.

(table continues)

TABLE 8-1 Continued

Species (Reference)	Dose	Results[a] and Limitations
		Females. Endometrial stromal polyp: 3%, 0%, 0%; mammary: NTR; ovary: NTR; pituitary: NTR; thyroid: NTR; adrenal gland: NTR.
DDE		
Rat Osborne-Mendel (NCI 1978c)	Males (50 per group) fed 437 ppm or 839 ppm (21.8 mg/kg/d or 42.0 mg/kg/d) in diet for 78 wk. Females (50 per group) fed 242 ppm or 462 ppm (12.1 mg/kg/d or 23.1 mg/kg/d) in diet for 78 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. The high incidence of tumors in control animals (30% mammary tumors, 50% pituitary tumors, 15% thyroid tumors in females; 30% thyroid tumors in males) raises concern about the interpretation of this study. **Males.** Mammary adenoma and fibroadenoma: 0%, 2%, 5%; testicular interstitial-cell tumors: 0%, 6%, 0%; prostate sarcoma: 0%, 6%, 0%; pituitary carcinoma and chromophobe adenoma: 22%, 5%, 0%; thyroid follicular-cell adenoma, follicular-cell carcinoma, C-cell adenoma, and C-cell carcinoma: 30%, 23%, 30%; adrenal gland: NTR. **Females.** Endometrial–uterine sarcoma, leiomysarcoma, and stromal polyp: 9%, 8%, 0%; mammary adenoma, adenocarcinoma, and fibroadenoma: 24%, 16%, 30%; ovarian cystadenoma: 3%, 0%, 0%; pituitary carcinoma and chromophobe adenoma: 30%, 52%, 50%; thyroid follicular-cell adenoma, follicular-cell carcinoma, C-cell adenoma, and C-cell carcinoma: 35%, 29%, 15%; adrenal-gland cortical adenoma: 3%, 4%, 0%.
Mouse $B6C3F_1$ (NCI 1978c)	Males and females (50 per group) fed 148 ppm or 261 ppm (22.2 mg/kg/d or 39.1 mg/kg/d) in diet for 78 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. **Males.** Mammary: NTR; testicular interstitial-cell tumors: 2%, 0%, 0%; prostate: NTR; pituitary: NTR; thyroid: NTR; adrenal gland: NTR. **Females.** Endometrial–uterine adenocarcinoma, endometrial stromal polyp, and hemangioma: 0%, 4%, 6%; mammary adenocarcinoma and fibroadenocarcinoma: 4%, 0%, 5%; ovary: NTR; pituitary: NTR; thyroid follicular-cell carcinoma: 0%, 3%, 0%; adrenal gland: NTR.

DDT

Rat Osborne-Mendel (NCI 1978c)	Males (50 per group) fed 321 ppm or 642 ppm (16.0 mg/kg/d or 32.1 mg/kg/d) in diet for 78 wk. Females (50 per group) fed 210 ppm or 420 ppm (10.5 mg/kg/d or 21.0 mg/kg/d) in diet for 78 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. However, a positive correlation between dose and incidence of pheochromocytoma in females was reported in this study. The high incidence of tumors in control animals (40% mammary tumors, 68% pituitary adenoma, 26% thyroid tumors in females; 16% pituitary tumors, 52% thyroid tumors in males) raises concern about the interpretation of this study. **Males.** Mammary adenocarcinoma and fibroadenoma: 2%, 0%, 5%; testicle: NTR; prostate: NTR; pituitary chromophobe adenoma: 18%, 14%, 16%; thyroid follicular-cell adenoma, follicular-cell carcinoma, C-cell adenoma, and C-cell carcinoma: 55%, 51%, 52%; adrenal-gland pheochromocytoma: 4%, 0%, 0%. **Females.** Endometrial stromal polyp: 5%, 13%, 0%; mammary adenoma, adenocarcinoma, and fibroadenoma: 26%, 14%, 40%; ovary: NTR; pituitary chromophobe adenoma: 41%, 48%, 68%; thyroid follicular-cell adenoma, follicular-cell carcinoma, C-cell adenoma, and C-cell carcinoma: 37%, 26%, 26%; adrenal-gland cortical adenoma and pheochromocytoma: 3%, 13%, 0%.
Osborne-Mendel (Fitzhugh and Nelson 1947)	Males and females (24 per group) fed 200–800 ppm (5.0–40.0 mg/kg/d) in diet for 2 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs.
Carworth (Treon and Cleveland 1955)	Males and females (40 per group) fed 2.5 ppm, 12.5 ppm, or 25 ppm (0.12 mg/kg/d, 0.25 mg/kg/d, or 0.5 mg/kg/d) in diet for 2 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs.
Osborne-Mendel (Deichmann et al. 1967)	Males and females (30 per group) fed 80 ppm or 200 ppm (4.0 mg/kg/d or 10.0 mg/kg/d) in diet for 2 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs.
Osborne-Mendel (Radomski et al. 1965)	Males and females (30 per group) fed 80 ppm (4.0 mg/kg/d) in diet for 2 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs.

(table continues)

TABLE 8-1 Continued

Species (Reference)	Dose	Results[a] and Limitations
Mouse $B6C3F_1$ (NCI 1978c)	Males (50 per group) fed 22 ppm or 44 ppm (3.3 mg/kg/d or 6.6 mg/kg/d) in diet for 78 wk. Females (50 per group) fed 87 ppm or 175 ppm (13.5 mg/kg/d or 26.2 mg/kg/d) in diet for 78 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. **Males.** Mammary: NTR; testicle: NTR; prostate: NTR; pituitary: NTR; thyroid follicular cell adenoma: 0%, 2%, 0%; adrenal gland: NTR. **Females.** Endometrial–uterine: NTR; mammary: NTR; ovary: NTR; pituitary chromophobe adenoma: 7%, 0%, 5%; thyroid follicular-cell adenoma, follicular-cell carcinoma, and C-cell adenoma: 5%, 8%, 0%; adrenal gland: NTR.
BALB/c (Tarján and Kemény 1969)	Males and females (28-30 per group) fed p,p′-DDT at 3 ppm (0.45 mg/kg/d) in diet for 6 mo; 5-generation study.	No significant increased incidence of tumors in reproductive or other endocrine organs.
CF1 (Turusov et al. 1973)	Males and females (60 per group) fed 2 ppm, 10 ppm, 50 ppm, or 250 ppm (0.3 mg/kg/d, 1.5 mg/kg/d, 7.5 mg/kg/d, or 37.5 mg/kg/d) in diet for life span; 6-generation study.	No significant increased incidence of tumors in reproductive or other endocrine organs.
BALB/c (Terracini et al. 1973)	Males and females (60 per group) fed 2 ppm, 20 ppm, or 250 ppm (0.3 mg/kg/d, 3.0 mg/kg/d, or 37.5 mg/kg/d) in diet for life span; 2-generation study.	No significant increased incidence of tumors in reproductive or other endocrine organs.
A strain (Shabad et al. 1973)	Males and females (total of 264 animals) administered 50 ppm (7.5 mg/kg/d) to F_0 generation and 10 ppm (1.5 mg/kg/d) to F_{1-5} generations by gavage for life span; 6-generation study.	No significant increased incidence of tumors in reproductive or other endocrine organs. Early deaths occurred in the F_0, F_1, and F_2 generations.

CF1 (Walker et al. 1973)	Males and females (64 per group) fed p,p′-DDT at 50 ppm or 100 ppm (7.5 mg/kg/d or 15.0 mg/kg/d) in diet for 2 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs.
CF1 (Thorpe and Walker 1973)	Males and females (30 per group) fed p,p′-DDT at 100 ppm (15.0 mg/kg/d) in diet for 110 wk.	No significant increased incidence of tumors in reproductive or other endocrine organs.
Hamster (Rossi et al. 1983)	Males and females (at least 40 per group) fed 1,000 ppm (40 mg/kg/d) in diet for 30 mo.	Statistically significant increased incidence of adrenal-gland tumors in females.
(Cabral et al. 1982)	Males and females (29-40 per group) fed 1,000 ppm (40.0 mg/kg/d) in diet for 28 mo.	Increased incidence (not statistically significant) of adrenal-gland tumors in males.
(Agthe et al. 1970)	Males and females (30 per group) fed 500 ppm or 1,000 ppm (20.0 mg/kg/d or 40.0 mg/kg/d) in diet for 48 wk.	No significant increased incidence of tumors in reproductive or other endocrine organs. Study is of limited value because of early deaths.
(Graillot et al. 1975)	Males and females (15 per group) fed 250 mg/kg, 500 mg/kg, or 1,000 mg/kg in diet for 18 mo.	No significant increased incidence of tumors in reproductive or other endocrine organs. Study is limited because length of administration was less than the life span.
Monkey (Adamson and Sieber 1983)	Animals (24) fed 400 ppm (20.0 mg/kg/d) in diet for up to 10.8 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs.
(Durham et al. 1963)	Animals (22) fed 5-5,000 ppm in diet for up to 7 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs.

(table continues)

TABLE 8-1 Continued

Species (Reference)	Dose	Results[a] and Limitations
Dog (Lehman 1965)	Males and females (22 total) fed 400–3,200 ppm (30.0–240.0 mg/kg/d) in diet for up to 4 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs.
ENDOSULFAN		
Rat Osborne-Mendel (NCI 1978b)	Males (50 per group) fed 408 ppm or 952 ppm (20.4 mg/kg/d or 47.6 mg/kg/d) in diet for 78 wk. Females (50 per group) fed 223 ppm or 445 ppm (11.1 mg/kg/d or 22.2 mg/kg/d) in diet for 78 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs in females.[c] The study is limited in that the dose was toxic to males. The high incidence of tumors in control animals (35% in mammary gland, 58% pituitary adenoma in females; 16% pituitary adenoma in males) raises concern about the interpretation of this study. **Males.** Mammary fibroadenoma: 2%, 0%, 0%; testicle: NTR; prostate lipoma: 3%, 0%, 0%; pituitary chromophobe adenoma: 2%, 0%, 16%; thyroid follicular-cell adenoma: 0%, 0%, 5%; adrenal gland: NTR. **Females.** Endometrial–uterine lipoma, leiomyosarcoma, stromal polyp: 8%, 2%, 10%; mammary fibroadenoma and adenocarcinoma: 32%, 24%, 35%; ovarian granulosa-cell tumors, lipoma, and leiomysarcoma (metastatic): 4%, 0%, 5%; pituitary chromophobe adenoma: 33%, 19%, 58%; thyroid follicular-cell adenoma, follicular-cell carcinoma, and C-cell adenoma: 2%, 4%, 11%; adrenal-gland cortical adenoma: 4%, 0%, 0%.
Mouse $B6C3F_1$ (NCI 1978b)	Males (50 per group) fed 3.5 ppm or 6.9 ppm (0.52 mg/kg/d or 1.0 mg/kg/d) in diet for 78 wk. Females (50 per group) fed 2.0 ppm or 3.9 ppm (0.3 mg/kg/d or 0.58 mg/kg/d) in diet for 78 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs in females. The study is of limited value because the dose was toxic to males. **Males.** Mammary adenocarcinoma: 0%, 2%, 0%; testicle: NTR; prostate: NTR; pituitary: NTR; thyroid: NTR; adrenal gland: NTR.

		Females. Endometrial–uterine hemangiosarcoma: 0%, 2%, 5% (adenocarcinoma); mammary adenocarcinoma: 2%, 2%, 0%; ovarian granulosa-cell tumors: 0%, 2%, 0%; pituitary chromophobe adenoma: 0%, 2%, 0%; thyroid: NTR, adrenal gland: NTR.
ENDRIN		
Rat		
Osborne-Mendel (NCI 1979b)	Males (50 per group) received 2.5 or 5 ppm (0.12 or 0.24 mg/kg/d) in diet for 80 wk. Females (50 per group) received 3 or 6 ppm (0.15 or 0.3 mg/kg/d) in diet for 80 wk.	The Cochran-Armitage test for the incidence of adenomas of the pituitary in females showed significance (p = .015) using the pooled controls, and the results of the Fisher exact test showed that the incidence in the high dose group was statistically significantly higher (p = .016) than that in pooled controls. There was a statistically significantly increased incidence of combined adenomas and carcinomas of the adrenal gland in the low and high dose group males and in the low dose group females. The incidence of these tumors in the matched controls of either sex were higher than those of the corresponding pooled controls, and the incidences in the matched controls equaled or exceeded those in any of the respective dosed groups. NCI concluded that these tumors cannot be clearly related to administration of the test chemcial. **Males.** Mammary: NTR; testicles: NTR; prostate: NTR; pituitary adenoma NOS and chromophobe adenoma: 31%, 31%, 40%; thyroid follicular cell adenoma, follicular cell carcinoma, and c-cell adenoma: 9%, 7%, 11%; adrenal carcinoma NOS and adenoma NOS: 8%, 18%, 22%. **Females.** Uterine adenocarcinoma NOS, papillary adenoma, leiomyosarcoma, endometrial stromal polyp: 14%, 4%, 26%; mammary adenoma NOS, adenocarcinoma NOS, fibroma, fibroadenoma: 16%, 8%, 10%; ovarian fibroma 0%, 2%, 0%; pituitary carcinoma NOS, adenoma NOS, adenocarcinoma NOS, chromophobe adenoma: 48%, 45%, 29%; thyroid follicular cell adenoma: 11%, 8%, 0%; adrenal carcinoma NOS, adenoma NOS, pheochromocytoma: 31%, 17%, 33%.
Osborne-Mendel (Deichmann et al. 1970)	Males and females (50 per group) received 2, 6, or 12 ppm (0.1, 0.3, or 0.6 mg/kg/d) in diet for 2.4 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs. The study is limited in that not all tissues were examined microscopically.

(table continues)

TABLE 8-1 Continued

Species (Reference)	Dose	Results[a] and Limitations
Osborne-Mendel (Reuber 1978a)	Males and females (24 per group) received 0.1-25 ppm (0.005-1.25 mg/kg/d) in diet for 104 wk.	Carcinomas and sarcomas were present in the mammary gland and thyroid of both males and females. It is not stated whether the incidences of tumors were significant.
Mouse $B6C3F_1$ (NCI 1979b)	Males (50 per group) received 1.6 or 3.2 ppm (0.24 or 0.48 mg/kg/d) in diet for 80 wk. Females (50 per group) received 2.5 or 5 ppm (0.37 or 0.75 mg/kg/d) in diet for 80 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. This study is limited in that survival of the low dose males could not be evaluated, due to the accidental administration of excessive quantities of endrin to this group during wk 66. **Males.** Mammary: NTR; testicular: NTR; prostate: NTR; pituitary: NTR; thyroid : NTR; adrenal gland: NTR. **Females.** Uterine sarcoma NOS: 0%, 2%, 0%; mammary: NTR; ovarian cystadenoma: 0%, 2%, 0%; pituitary: NTR; thyroid : NTR; adrenal gland: NTR.
LINDANE		
Rat Osborne-Mendel (NCI 1977)	Males (50 per group) fed 236 ppm or 472 ppm (11.8 mg/kg/d or 23.6 mg/kg/d) in diet for 80 wk. Females (50 per group) fed 135 ppm or 270 ppm (6.75 mg/kg/d or 13.5 mg/kg/d) in diet for 80 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs.[d] The high incidence of tumors in control animals (50% in mammary gland, 43% pituitary tumors in females; 34% follicular-cell carcinoma of thyroid in males) raises concern about the interpretation of this study. **Males.** Mammary carcinoma and adenoma: 2%, 4%, 0%; testicle: NTR; prostate: NTR; pituitary carcinoma, adenoma, and chromophobe adenoma: 12%, 9%, 0%; thyroid follicular-cell adenoma, follicular-cell carcinoma, and C-cell adenoma: 25%, 14%, 34%; adrenal-gland cortical adenoma: 0%, 3%, 0%. **Females.** Endometrial–uterine adenocarcinoma, leiomyosarcoma, stromal polyp, and carcinoma: 15%, 20%, 11%; mammary carcinoma, adenoma, adenocarcinoma, fibroma, and fibroadenoma: 36%, 22%, 50%; ovarian sertoli-cell tumors: 0%, 2%,

		0%; pituitary carcinoma, adenoma, and chromophobe adenoma: 33%, 25%, 43%; thyroid follicular-cell adenoma, follicular-cell carcinoma, and C-cell adenoma: 13%, 9%, 0%; adrenal-gland cortical adenoma: 7%, 5%, 0%.
Wistar (Ito et al. 1975)	Males (18-24 per group) fed 500 ppm (25.0 mg/kg/d) in diet for 24–48 wk.	No significant increased incidence of tumors in reproductive or other endocrine organs.
Wistar (Fitzhugh et al. 1950)	Males and females (10 per group) fed 5–1600 ppm (0.25–80.0 mg/kg/d) in diet for up to 107 wk.	No significant increased incidence of tumors in reproductive or other endocrine organs.
Mouse $B6C3F_1$ (NCI 1977)	Males and females (50 per group) fed 80 ppm or 160 ppm (12.0 mg/kg/d or 24.0 mg/kg/d) in diet for 80 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. **Males.** Mammary: NTR; testicle: NTR; prostate: NTR; pituitary: NTR; thyroid: NTR; adrenal gland: NTR. **Females.** Endometrial–uterine sarcoma: 0%, 2%, 0%; mammary adenoma: 0%, 0%, 10%; ovary: NTR; pituitary: NTR; thyroid follicular-cell adenoma: 0%, 5%, 0%; adrenal-gland cortical carcinoma: 2%, 0%, 0%.
dd (Ito et al. 1973)	Males (20-40 per group) fed 455 ppm (68.2 mg/kg/d) in diet for 24 wk.	No significant increased incidence of tumors in reproductive or other endocrine organs.
dd (Hanada et al. 1973)	Males and females (10-11 per group) fed 546 ppm (81.9 mg/kg/d) in diet for 32 wk.	No significant increased incidence of tumors in reproductive or other endocrine organs. Dose might have exceeded maximum tolerated dose.
CF1 (Thorpe and Walker 1973)	Males and females (30 per group) fed 400 ppm (60.0 mg/kg/d) in diet for 104 wk.	No significant increased incidence of tumors in reproductive or other endocrine organs.
IRC-JCL (Goto et al.1972)	Males fed 600 ppm (90.0 mg/kg/d) in diet for 26 wk.	No significant increased incidence of tumors in reproductive or other endocrine organs.

(table continues)

TABLE 8-1 Continued

Species (Reference)	Dose	Results[a] and Limitations
NMRI (Herbst et al.1975)	Males and females (50 per group) fed 12.5 ppm, 25 ppm, or 50 ppm (1.8 mg/kg/d, 3.7 mg/kg/d, or 7.5 mg/kg/d) in diet for 80 wk.	No significant increased incidence of tumors in reproductive or other endocrine organs.
METHOXYCHLOR		
Rat Osborne-Mendel (NCI 1978a)	Males (50 per group) fed 448 ppm or 845 ppm (22.4 mg/kg/d or 42.2 mg/kg/d) in diet for 78 wk. Females (50 per group) fed 750 ppm or 1,385 ppm (37.5 mg/kg/d or 69.2 mg/kg/d) in diet for 78 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs.[e] The high incidence of tumors in control animals (45% in mammary gland, 40% pituitary adenoma in females; 17% in males) raises concern about the interpretation of this study. **Males.** Mammary adenoma: 2%, 2%, 0%; testicular interstitial-cell tumors: 0%, 5%, 0%; prostate: NTR[f]; pituitary chromophobe adenoma: 38%, 18%, 17%; thyroid follicular-cell adenoma, follicular-cell carcinoma, C-cell adenoma, and C-cell carcinoma: 23%, 24%, 16%; adrenal-gland cortical adenoma and pheochromocytoma: 6%, 5%, 5%. **Females.** Endometrial stromal polyp: 3%, 11%, 0%; mammary adenoma, adenocarcinoma, and fibroadenoma: 34%, 28%, 45%; ovarian cystadenoma and fibrosarcoma: 3%, 3%, 0%; pituitary chromophobe adenoma: 22%, 28%, 40%; thyroid follicular-cell adenoma, C-cell adenoma, and C-cell carcinoma: 11%, 10%, 0%; adrenal gland: NTR.
Strain not specified (Hodge et al. 1952)	Males and females (25 per group) fed 25–1,600 ppm (1.25–80 mg/kg/d) in diet for 2 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs.
Osborne-Mendel (Radomski et al. 1965)	Males and females (30 per group) fed 80 ppm (4 mg/kg/d) in diet for 2 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs

Osborne-Mendel (Deichmann et al. 1967)	Males and females (30 per group) fed 1,000 ppm (50 mg/kg/d) in diet for 27 mo.	No significant increased incidence of tumors in reproductive or other endocrine organs.
Mouse $B6C3F_1$ (NCI 1978a)	Males (50 per group) fed 1,746 ppm or 3,491 ppm (262 mg/kg/d or 523 mg/kg/d) in diet for 78 wk. Females (50 per group) fed 997 ppm or 1,994 ppm (149.5 mg/kg/d or 299 mg/kg/d) in diet for 78 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. **Males.** Mammary: NTR; testicle: NTR; prostate: NTR; pituitary: NTR; thyroid: NTR; adrenal gland: NTR. **Females.** Endometrial stromal polyp: 0%, 8%, 5%; mammary: NTR; ovary cystadenoma: 0%, 7%, 0%; pituitary: NTR; thyroid: NTR; adrenal-gland cortical adenoma: 0%, 0%, 5%.
PCB		
Rat Fischer 344 (NCI 1978d)	Males and females (24 per group) fed Aroclor 1254 at 25 ppm, 50 ppm, or 100 ppm (1.25 mg/kg/d, 2.5 mg/kg/d, or 5.0 mg/kg/d) in diet for 104–105 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. The high incidence of tumors in control animals (17% pituitary adenoma in females, 100% interstitial-cell tumors in males), as well as the low number of animals in some groups, raises concern about the interpretation of this study. **Males.** Mammary: NTR; testicular interstitial-cell tumors: 100%, 83%, 83%, 100%; prostate: NTR; pituitary adenoma (NOS): 8%, 0%, 0%, 0%; thyroid: NTR; adrenal-gland pheochromocytoma: NTR. **Females.** Endometrial–uterine adenocarcinoma, adenoma in adenomatous polyp, leiomyoma, and stromal polyp: 58%, 33%, 84%, 14%; mammary adenoma and adenocarcinoma: 17%, 0%, 0%, 4%; ovarian granulosa-cell tumors: 100% (1 animal), 0%, 0%, 0%; pituitary adenoma: 5%, 5%, 4%, 17%; thyroid: NTR; adrenal gland: NTR.
Sherman (Kimbrough et al. 1975)	Females (200 per group) fed Aroclor 1260 at 100 ppm (5.0 mg/kg/d) in diet for approximately 21 mo.	No significant increased incidence of tumors in reproductive or other endocrine organs.

(table continues)

TABLE 8-1 Continued

Species (Reference)	Dose	Results[a] and Limitations
Sprague-Dawley (Norback and Weltman 1985)	Males and females (70 per group) fed approximately 70 ppm (3.5 mg/kg/d) Aroclor 1260 in diet for 24 mo.	No significant increased incidence of tumors in reproductive or other endocrine organs.
Wistar (Schaeffer et al. 1984)	Males (141-152 per group) fed Clophen A–60 or Clophen A–30 at 100 ppm (5.0 mg/kg/d) in diet for up to 28 mo.	No significant increased incidence of tumors in reproductive or other endocrine organs.
TCDD		
Rat Sprague-Dawley (Kociba et al. 1978)	Males and females (50 per group) fed 0.02–2.0 ppb (0.001-0.1 µg/kg/d) in diet for 2 yr.	No significant increased incidence of tumors in reproductive or other endocrine organs. Significantly reduced incidence of tumors of the uterus, pituitary, mammary, and adrenal gland.
Osborne-Mendel (NTP 1982a)	Males and females (50 per group) received 0.2, 1.0, or 10.0 ppb (0.01, 0.05, or 0.5 µg/kg/wk) twice per wk for 104 wk by gavage.	In males, the incidence of follicular-cell adenoma in the thyroid was dose related and significantly (p = .001) higher at the high dose than in controls. Increased incidence (not statistically significant) of thyroid tumors in females. **Males.** Mammary adenoma, adenocarinoma, papillary adenoma, and fibroadenoma: 0%, 10%, 2%, 4%; testicular interstitial-cell tumors and lipoma: 0%, 4%, 0%, 0%; pituitary adenoma (NOS) and chromophobe adenoma: 2%, 7%, 8%, 0%; thyroid follicular-cell adenoma, follicular-cell carcinoma, C-cell adenoma, and C-cell carcinoma: 14%, 26%, 30%, 0%; adrenal-gland pheochromocytoma and cortical adenoma: 18%, 26%, 20%, 21%. **Females.** Uterine squamous-cell carcinoma, adenocarcinoma, leiomyoma, and leiomyosarcoma: 4%, 0%, 2%, 8%; mammary fibrosarcoma, lipoma, fibroadenoma, and adenocarcinoma: 50%, 46%, 41%, 24%; ovarian thecoma, luteoma, and

		granulosa-cell tumors: 2%, 0%, 6%, 0%; pituitary adenoma and chromophobe adenoma: 11%, 5%, 9%, 14%; thyroid follicular-cell adenoma, C-cell adenoma, and C-cell carcinoma: 10%, 18%, 26%, 17%; adrenal-gland adenoma, cortical adenoma, cortical carcinoma, and pheochromocytoma: 18%, 10%, 30%, 21%.
Sprague-Dawley (Brown et al. 1998)	Pregnant females (8 per group) received 1 µg/kg on d 15 post-conception by gavage. Female offspring received 30 mg DMBA/kg at 50 d of age.	**Females.** Mammary adenocarcinomas in offspring after DMBA treatment: 90% and 79%.
Mouse $B6C3F_1$ (NTP 1982a)	Males (50 per group) received 0.0015, 0.0075, or 0.075 ppb (0.01, 0.05, or 0.5 µg/kg/wk) twice per wk for 104 wk by gavage. Females (50 per group) received 0.006, 0.03, or 0.3 ppb (0.01, 0.05, or 0.5 µg/kg/wk) twice per wk for 104 wk by gavage.	In females, the incidence of follicular-cell adenoma in the thyroid was dose related and was significantly higher ($p = .009$) at the high dose than in controls. **Males.** Reproductive organs: NTR; pituitary meningioma (invasive): 0%, 0%, 3%, 0%; adrenal heptocellular carcinoma (metastatic) and pheochromocytoma: 2%, 0%, 0%, 4%; thyroid adenoma and follicular-cell adenoma: 6%, 0%, 0%, 5%. **Females.** Mammary fibrosarcoma and fibroadenoma: 2%, 2%, 0%, 0%; uterine fibroma, leiomyoma, carcinoma (NOS): 0%, 2%, 0%, 12%; ovarian papillary adenoma, granulosa-cell tumor, and teratoma (benign): 4%, 2%, 2%, 0%; pituitary adenoma (NOS) and chromophobe adenoma: 5%, 0%, 6%, 5%; adrenal cortical adenoma and pheochromocytoma: 0%, 0%, 2%, 8%; thyroid adenoma (NOS) and follicular-cell adenoma: 6%, 2%, 11%, 4%.
TOXAPHENE		
Rat Osborne-Mendel (NCI 1979a)	Males (50 per group) fed 556 ppm or 1,112 ppm (27.8 mg/kg/d or 55.6 mg/kg/d) in diet for 80 wk. Females (50 per group) fed 540 ppm or 1,080 ppm (27.0 or 54.0 mg/kg/d) in diet for 80 wk.	In males the incidence of thyroid tumors was dose related ($p = .007$) using pooled controls. In females the incidence of thyroid tumors was dose related using either matched ($p = .022$) or pooled ($p = .008$) controls. The high incidence of tumors in control animals (38% pituitary adenoma, 17% thyroid tumors in females; 43% pituitary tumors, 14% thyroid tumors, 55% adrenal-gland tumors in males) raises concern about the interpretation of study.

(table continues)

TABLE 8-1 Continued

Species (Reference)	Dose	Results[a] and Limitations
		Males. Mammary carcinoma: 2%, 0%, 0%; testicle: NTR; prostate sarcoma: 0%, 3%, 0%; pituitary carcinoma, adenoma, and chromophobe adenoma: 31%, 16%, 43%; thyroid follicular-cell adenoma, follicular-cell carcinoma, C-cell adenoma, and C-cell carcinoma: 21%, 26%, 14%; adrenal-gland adenoma, cortical adenoma, cortical carcinoma, and pheochromocytoma: 12%, 11%, 55%. **Females.** Endometrial–uterine carcinoma, papillary adenoma, and stromal polyp: 24%, 13%, 0%; mammary adenoma, adenocarcinoma, papillary adenocarcinoma, fibroma, fibroadenoma, and teratoma (malignant): 28%, 32%, 10%; ovarian carinoma and granulosa-cell tumors: 3%, 3%, 0%; pituitary adenoma, chromophobe adenoma, and chromophobe carcinoma: 37%, 59%, 38%; thyroid follicular-cell adenoma and C-cell carcinoma: 2%, 17%, 17%; adrenal-gland cortical adenoma and cortical carcinoma: 7%, 14%, 0%.
Mouse $B6C3F_1$ (NCI 1979a)	Males and females (50 per group) fed 99 ppm or 198 ppm (14.8 mg/kg/d or 29.7 mg/kg/d) in diet for 80 wk.	No statistically significant increased incidence of tumors in reproductive or other endocrine organs. **Males.** Mammary: NTR; testicle: NTR; prostate: NTR; pituitary: NTR; thyroid: NTR; adrenal gland: NTR. **Females.** Endometrial–uterine leiomyoma, stromal polyp: 4%, 0%, 0%; mammary adenoma: 2%, 0%, 0%; ovary: NTR; pituitary: NTR; thyroid hyperplasia (follicular cell): 0%, 3%, 0%; adrenal gland: NTR.

[a] Result values are shown in order of low-dose, mid-dose (if applicable), high-dose, and control groups.

[b] Reuber (1978b) reanalyzed the data and reported an increased incidence of pituitary tumors in low dose rats (27% for males; 27% for females) compared to controls (0% for males; 4% for females).

[c] Reuber (1981) reanalyzed the data and concluded that the pooled incidence of all tumors of the female genital tract was increased (62%) compared to controls (30%).

[d] Reuber (1979) reanalyzed the data and reported a significant increase in the incidence of ovarian tumors in the low-dose (45%) and high-dose (57%) groups compared to controls (0%); a significant increase in pituitary tumors in the low-dose (37% in males; 59% in females) and high-dose groups (40% in males; 64 in females) compared to pooled controls (16% in males; 21% in females); a significant increase in the incidence of thyroid tumors in males of the low-dose group (42%) and in females of the low-dose (24%) and high-dose (28%) groups compared with pooled controls (18% for males; 7% for females); a significant increase in the incidence of adrenal gland tumors (malignant and benign) in males and females of the low-dose (35% in males; 45% in females) and high-dose (60% in males; 57 in females) groups compared to pooled controls (16% for males; 7% for females).

[e] Reuber (1980) reanalyzed the data and reported an increased incidence of mammary gland tumors in female rats in the low-dose (33%) and high-dose (30%) groups compared to the control group (15%); a significant increase in the incidence of ovarian carcinomas in the low-dose (11%) and high-dose (23%) groups compared to controls (0%); twice as many adenomas and carcinomas of the pituitary in female rats of the high-dose group (43%) compared to controls (20%); an increased incidence of adrenal gland tumors in females of the low-dose (30%) and high-dose (38%) groups compared to controls (15%); an increase in thyroid tumors in treated rats (no data were provided).

[f] NTR, no tumors reported.

induce follicular-cell adenoma in mice (Greenman et al. 1990). However, male and female mice fed DDD did not develop thyroid tumors.

There was no evidence that DDD induced tumors of the endometrium/uterus, ovaries, testicles, prostate gland, mammary gland, pituitary gland, or adrenal glands.

DDE

DDE was tested for carcinogenicity in Osborne-Mendel rats and $B6C3F_1$ mice (NCI 1978c). The tests involved exposure to adult animals only. There was no evidence in either species that DDE caused an increase in the incidence in tumors of the endometrium/uterus, ovaries, testicles, prostate gland, mammary gland, thyroid gland, or adrenal glands. An increased incidence of pituitary tumors was observed in rats of the low- and high-dose groups compared with control rats, but the effect was not dose related. It should be noted that 50% of the female controls had pituitary tumors, which is a higher incidence than normally observed in the historical controls. No pituitary tumors were observed in the study with mice.

DDT

DDT was tested in many bioassays in two strains of rat (Osborne-Mendel and Carworth) (Fitzhugh and Nelson 1947; Treon and Cleveland 1955; Radomski et al. 1965; Deichmann et al. 1967; NCI 1978c), four strains of mouse ($B6C3F_1$, BALB/c, CF1, and A strain) (Tarján and Kemény 1969; Shabad et al. 1973; Terracini et al. 1973; Thorpe and Walker 1973; Turusov et al. 1973; Walker et al. 1973; NCI 1978c), hamsters (Agthe et al. 1970; Graillot et al. 1975; Cabral et al. 1982; Rossi et al. 1983), monkeys (Durham et al. 1963; Adamson and Sieber 1983), and dogs (Lehman 1965). Four of the studies with mice were multigeneration studies that involved exposure to DDT during fetal life, lactation, and after weaning for either 6 mo (Tarján and Kemény 1969) or for their life span (Shabad et al. 1973; Terracini et al. 1973; Turusov et al. 1973).

Two studies (Cabral et al. 1982; Rossi et al. 1983) reported an increased incidence of adrenal gland tumors (adrenocortical adenomas) in male and female hamsters. The increase was significant for the females. In two other studies with hamsters, DDT did not induce adrenal gland tumors, but early deaths occurred in one study (Agthe et al. 1970), and the length of administration was less than lifetime in the other (Graillot et al. 1975). In the bioassay conducted by NCI (1978c), DDT administration did not result in an increase in the incidence of adrenal gland tumors in rats or mice, but the Cochran-Armitage tests found a positive association between DDT and the incidence of pheochromocytoma in female rats. No increased incidence of adrenal gland tumors was observed in other bioassays with adult mice, rats, monkeys, or dogs or in multigeneration bioassays with mice exposed perinatally.

There was no evidence that DDT increased the incidence of tumors of the endometrium/uterus, ovaries, testicles, prostate gland, mammary gland, or pituitary gland in animals treated prenatally or in adulthood. However, there is some evidence that DDT might induce cancers in other organs, such as the liver, which led IARC to classify it as possibly carcinogenic to humans (IARC 1991).

A minor component of DDT— *o,p′*-DDT—is estrogenic at a dose of more than 25 mg/kg of body weight per day. That compound promoted the growth of MT2 mammary adenocarcinoma cells injected into ovariectomized Wistar-Furth rats (Robison et al. 1985b), which demonstrates that DDT can support the growth of an estrogen-responsive tumor.

Endosulfan

Endosulfan was tested in bioassays using Osborne-Mendel rats and $B6C3F_1$ mice (NCI 1978b). The tests involved exposure to adult animals only. There was no evidence that endosulfan induced tumors of the endometrium/uterus, ovaries, testicles, prostate gland, mammary gland, thyroid gland, pituitary gland, or adrenal glands, but endosulfan was toxic to male rats and mice.

Endrin

Bioassays of endrin were conducted with Osborne-Mendel rats (Deichmann et al. 1970; Reuber 1978a; NCI 1979b) and $B6C3F_1$ mice (NCI 1979b). All of these studies involved exposure to adult animals. One study (Reuber 1978a) reported an increased incidence of mammary gland and thyroid tumors in Osborne-Mendel rats, but two other bioassays in rats did not find an increase in those types of tumors (Deichmann et al. 1970; NCI 1979b). A bioassay with $B6C3F_1$ mice (NCI 1979b) was also negative. Reuber's criteria for classifying tissues as tumorigenic were not consistent with those of other investigators (EPA 1979b).

There are data showing that endrin administered in the diet for up to 2 yr induced pituitary tumors in female but not in male Osborne-Mendel rats (NCI 1979b). The result for the Cochran-Armitage test for the incidence of adenomas of the pituitary in females was significant ($p = .015$) using the pooled controls, and the results of the Fisher exact test showed that the incidence in the high dose group was higher ($p = .016$) than that in the pooled controls. However, when the combined incidence of adenomas, carcinomas, adenocarcinomas, and chromophobe adenomas of the pituitary in female rats was compared with pooled controls, the results were not significant. In addition, two other studies did not report an increased incidence of pituitary tumors in Osborne-Mendel rats fed endrin for up to the 116 wk (Deichmann et al. 1970; Reuber 1978a). No pituitary tumors were reported in male or female $B6C3F_1$ mice fed endrin for up to 2 yr (NCI 1979b).

There was no evidence that endrin increased the incidence of tumors of the endometrium/uterus, ovaries, testicles, or prostate gland compared with controls.

Lindane

Bioassays of lindane have been conducted in two strains of rat (Osborne-Mendel and Wistar) (Fitzhugh et al. 1950; Ito et al. 1975; NCI 1977) and five strains of mouse ($B6C3F_1$, dd, CF1, IRC-JCL, and NMRI) (Goto et al. 1972; Hanada et al. 1973; Thorpe and Walker 1973; Herbst et al. 1975; NCI 1977). None of the studies involved prenatal exposure to lindane. Overall, there was no evidence that lindane increased the incidence of tumors of the endometrium/uterus, ovaries, testicles, prostate gland, mammary gland, thyroid gland, pituitary gland, or adrenal glands compared with controls. However, there is some evidence that lindane might induce tumors of the liver, which led IARC (1987) to classify it as possibly carcinogenic to humans.

Methoxychlor

Bioassays of methoxychlor were conducted in Osborne-Mendel rats (Radomski et al. 1965; Deichmann et al. 1967; NCI 1978a), an unspecified species of rat (Hodge et al. 1952), and $B6C3F_1$ mice (NCI 1978a). None of the studies involved prenatal exposure to methoxychlor. Overall, there was no evidence that methoxychlor increased the incidence of tumors of the endometrium/uterus, ovaries, testicles, prostate gland, mammary gland, thyroid gland, pituitary gland, or adrenal glands compared with controls.

PCBs

Bioassays of PCBs have been conducted in four strains of rat (Fischer 344, Sherman, Sprague-Dawley, and Wistar) (Kimbrough et al. 1975; NCI 1978d; Schaeffer et al. 1984; Norback and Weltman 1985). None of these studies involved perinatal exposure to PCBs. There was no evidence in any of the studies that PCBs induced tumors of the endometrium/uterus, ovaries, testicles, prostate gland, mammary gland, thyroid gland, pituitary gland, or adrenal glands. However, there is some evidence that PCBs induce cancers of the liver, which led IARC to classify it as probably carcinogenic to humans (IARC 1987).

TCDD

TCDD has been evaluated for carcinogenicity in Sprague-Dawley rats (Kociba et al. 1978; Brown et al. 1998), Osborne-Mendel rats (NTP 1982a), and $B6C3F_1$ mice (NTP 1982a). Of the reproductive and endocrine organs considered by the

committee, an increased incidence of tumors from adult exposure to TCDD was observed only in the thyroid gland. There was an increase in the incidence of thyroid tumors in male and female Osborne-Mendel rats and in female $B6C3F_1$ mice. The incidence of thyroid tumors was significantly increased ($p = 0.001$) in male rats and slightly increased in female rats administered 10 ppb TCDD by gavage twice a week for 2 yr (NTP 1982a). A significant increase ($p = 0.009$) in thyroid tumors was found in female, but not male, mice administered 0.3 ppb TCDD by gavage twice a week for 2 yr. However, there was no evidence that TCDD induced thyroid tumors in Sprague-Dawley rats fed up to 2 ppb TCDD in the diet for 2 yr (Kociba et al. 1978).

One study was conducted to investigate the effects of prenatal exposure to TCDD on chemically induced carcinogenesis (Brown et al. 1998). Eight pregnant rats were gavaged with 1 μg/kg TCDD on d 15 post-conception. Female offspring were subsequently treated with dimethylbenzanthracene (DMBA) at 50 d of age. Ninety percent of the animals developed mammary adenocarcinomas compared with 79% of control animals that were exposed to TCDD during gestation.

There was no evidence that adult exposure to TCDD caused tumors of the endometrium/uterus, ovaries, testicles, prostate gland, mammary gland, pituitary gland, or adrenal glands. In fact, a significant decrease in pituitary and adrenal gland tumors was observed in Sprague-Dawley rats (Kociba et al. 1978).

The committee restricted its evaluation of carcinogenicity to selected reproductive and endocrine organs, but TCDD has been shown to induce tumors in laboratory animals in other organs, including the liver, lungs, thymus, hard palate, nasal turbinates, and skin. IARC (1997) concluded there is sufficient evidence in experimental animals that TCDD is carcinogenic, and classified TCDD as a human carcinogen. Experimental evidence suggests that the carcinogenic effects of TCDD are mediated through the Ah receptor which is a nongenotoxic, epigenetic mechanism (Ahlborg et al. 1995).

Toxaphene

Toxaphene was tested in bioassays using Osborne-Mendel rats and $B6C3F_1$ mice (NCI 1979a). The tests involved exposure to adult animals only. There was some evidence that toxaphene induced thyroid tumors in rats. In male rats, the incidence of thyroid follicular-cell adenoma or carcinoma was 17% in the low-dose group, 25% in the high-dose group, and 4.5% in the control group. This increase was dose related ($p = 0.007$) when compared with pooled control data. In female rats, the incidence of thyroid follicular-cell carcinoma was 2% in the low-dose group, 17% in the high-dose group, and 2% in the control group. This increase was dose related using either matched ($p = 0.022$) or pooled ($p = 0.008$) control data. It is not known whether toxaphene induced the thyroid tumors through a hormonally mediated mechanism, although there is evidence that natu-

ral estrogens and DES induce follicular-cell adenoma in mice (Greenman et al. 1990). In the bioassay of toxaphene with mice, there was no evidence of an increased incidence of thyroid tumors.

There was no evidence that toxaphene induced tumors of the endometrium/uterus, ovaries, testicles, prostate gland, mammary gland, pituitary gland, or adrenal glands. However, there is some evidence that toxaphene might induce cancers of the liver, as well as the thyroid gland, which led IARC to classify it as possibly carcinogenic to humans (IARC 1987).

Strengths and Limitations of the Cancer Bioassays

Several factors must be taken into consideration when interpreting the results of cancer bioassays. The factors include the genetic background of animals, the appropriateness of the animal model, the dose and route of administration, and the timing and duration of exposure during an animal's life span. In addition, the data themselves can be subject to different interpretations. The factors will influence the certainty expressed in conclusions drawn from the underlying data base.

Genetics

The genetic background of an animal strain can influence the results of a study. For example, when Fisher, Wistar-Furth, and Sprague-Dawley rats were administered the chemical carcinogens 7,12-dimethylbenzanthracene (DMBA) or nitroso-methylurea, more than 80% of the rats developed mammary tumors (Daniel and Joyce 1984; el Abed et al. 1987; Kort et al. 1987; Thompson et al. 1995; Russo and Russo 1996). Other rats, such as Copenhagen, had a significantly lower incidence of tumor development.

Different strains of rat and mice can also have varying incidences of spontaneous tumor formation that must be considered in the evaluation of carcinogenic agents. F344 rats are typically used in NCI bioassays because they have a low spontaneous incidence of mammary tumors, and $B6C3F_1$ mice are used because they have a median incidence of liver tumors compared with other strains of mice (Eugene McConnell, Raleigh, N.C., personal commun., 1997). Many of the NCI bioassays reviewed used Osborne-Mendel rats, and no explanation was given for choosing this strain over F344 rats.

In cases where the spontaneous incidence of tumors in the control animals is high, detecting an increase in the frequency of tumor formation in treated animals would require larger sample sizes than typically found in animal bioassays, which normally employ 20-25 animals per group. For example, a high incidence of pituitary tumors was found in the control animals in studies of lindane (NCI 1977), methoxychlor (NCI 1978a), endosulfan (NCI 1978b), DDT (NCI 1978c), and DDE (NCI 1978c). Although the incidence of those tumors in experimental animals was not increased in comparison with the controls, the high incidence of

spontaneous pituitary tumors in the controls raises concern about the testing conditions.

In addition, it should be noted that tumor incidence can be altered by dietary modulation. For example, it has been shown that reducing calorie intake increases survival, decreases the incidence of spontaneous tumors, and might alter susceptibility to chemical carcinogens in laboratory animals (NRC 1996a). Thus, the sensitivity of rodent bioassays might be altered by the administered diet.

Animal Model

In the NCI bioassays, both rats and mice were typically tested because of the belief that a given event occurring in both species could be expected to occur in a third species (for example, humans) (Weisburger 1983). With the exception of PCBs, all the HAAs reviewed by the committee were tested in rats and mice; PCBs were tested in rats only. However, the bioassays described in this chapter may or may not have used the most susceptible species or strains of animals. For example, the spontaneous development of prostate cancer is a rare event in most nonhuman species. Only a few rat strains, such as Noble and Lobund-Wistar, have been used as models of prostate cancer. In those rat strains, androgens and estrogens increase the incidence of prostate cancer. The Noble rat appears to be uniquely sensitive to sex hormones for prostate cancer induction (Ofner et al. 1992). In this model, dysplasia in the dorsolateral lobe of rats treated with testosterone and estradiol is almost identical to the premalignant lesions described in the human gland (McNeal and Bostwick 1986). None of the studies on environmental HAAs described in this chapter were conducted using the Noble or Lorbund-Wistar strains. Also, there are no adequate animal models to test for endometrial or germinal-cell testicular cancers (which are the type of testicular tumor that is increasing in certain human populations).

Dose and Route of Administration

In NCI studies, animals are exposed via the most likely route of exposure in humans. Test animals are typically administered the maximum tolerated dose (MTD) and one-half of the MTD in cancer bioassays. The MTD is defined as the highest dose that does not alter the test animal's longevity or well-being because of noncancer effects (NRC 1993). Thus, the doses are not designed to be representative of environmental exposures. In the NCI (1978b) study on endosulfan, the sublethal doses administered to the male animals caused severe secondary effects and early death; therefore, carcinogenicity could not be assessed adequately. Because the highest dose used in a chronic toxicity study is based on the results from a prechronic toxicity study, the MTD is exceeded in some cases, as in the NCI endosulfan study. The other NCI and NTP studies discussed in this chapter showed no indication that the MTD was exceeded. However, there are

concerns about the design of the typical long-term rodent bioassay, including the use of the MTD (see NRC 1993).

Timing and Duration of Exposure

The duration of exposure and period of observation must be considered when interpreting the results of a cancer bioassay. Experimental animals should be exposed to a range of doses of an agent for up to the life span of the animal to detect the effects of carcinogens with long latency periods and with multiple modes of action (for example, initiation or promotion). It is also important to take into account the stage in an animal's life cycle that exposure occurs. DES has been shown to cause cancer in humans and animals (Newbold 1995) when exposure occurs during critical periods of development but not when exposure occurs after the critical period. The timing of exposure during postnatal life also could affect carcinogenicity. In DMBA-induced mammary cancer in rats, a "window of vulnerability" was identified between d 45 and 55 of life (Russo and Russo 1978); the administration of the carcinogen during that period significantly increased the incidence of carcinoma and decreased the latency period. The effect is explained by the induction of intense proliferative activity of structures called terminal end buds, from which new gland ducts originate during the period (Russo and Russo 1987).

Perinatal exposure was not addressed in the bioassays described in this chapter with the exception of several bioassays in which mice were exposed to DDT during fetal life, lactation, and after weaning by feeding or gavage for up to 6 mo or their life span. Those studies produced negative findings.

In a recent report, the U.S. Environmental Protection Agency's (EPA) FIFRA/Scientific Advisory Panel (SAP) reviewed an EPA analysis of combined perinatal and adult exposure, perinatal only exposure, and adult only exposure carcinogenesis bioassays to determine if the age of initial exposure to a chemical influences the carcinogenic response (EPA 1997). In the analysis, 69 carcinogenesis bioassays were reviewed. The studies analyzed included six NTP bioassays (Chhabra et al. 1992, 1993a,b), 13 unpublished FDA bioassays, and other studies previously reviewed by McConnell (1992). Chemicals tested in the bioassays reviewed by McConnell (1992) include three HAAs (dieldrin, DDT, and TCDD). The SAP agreed with the EPA's conclusion that perinatal exposure rarely identifies carcinogens that are not found in standard (adult only) carcinogenesis bioassays, and combined perinatal and adult exposure slightly increases the incidence of a given type of tumor. With respect to the latter, it is not known if this reflects the effect of an increased length of exposure, a heightened sensitivity of the young animal to the carcinogenic effects of the chemical, or variability in the experimental design or results.

EPA noted the available data for drawing conclusions are not very robust and has developed criteria for the inclusion of perinatal exposure into the standard

carcinogenesis bioassay. The criteria include factors such as the likelihood of widespread exposure to women of childbearing age, infants and children, and specific toxicity to the developing fetus. A weight-of-the-evidence approach is applied to these factors to determine if a chemical is a candidate for perinatal carcinogenesis testing. The Food and Drug Administration (FDA) (1982) has also developed a set of criteria for determining candidates for perinatal carcinogenesis bioassays that are based on use, exposure, and toxicity seen in developmental toxicity and reproductive studies.

Other Interpretations

One investigator has published different interpretations of some of the NCI and FDA studies described in this chapter (see Table 8-1, footnotes b, c, d, and e). Reuber (1978b, 1979, 1980, 1981) reported positive results in reanalyses of NCI (1976, 1977, 1978a,b) studies and unpublished FDA studies, all of which reported negative results. Reuber concluded that methoxychlor increased the incidence of mammary, ovarian, testicular, pituitary, thyroid, and adrenal gland tumors, that endosulfan increased the incidence of all tumors of the female genital tract, and lindane increased the incidence of ovarian, pituitary, thyroid and adrenal gland tumors, and that chlordecone increased the incidence of pituitary tumors. Because Reuber (1978b, 1979, 1980, 1981) did not indicate why he considered the original NCI (1976, 1977, 1978a,b) interpretation of the tissues questionable or how the tissues were reexamined, his reanalysis cannot be independently verified.

SUMMARY AND CONCLUSIONS

Given that there are data from animal and human studies that indicate endogenous estrogens play some role in increasing the incidence of tumors in various endocrine glands in humans (Key and Pike 1988; Brinton and Hoover 1993; Nandi et al. 1995), and animals (Sonnenschein et al. 1974; Wiklund and Gorski 1982; Greenman et al. 1990; Nandi et al. 1995) and that the introduction of HAAs into the environment has preceded and overlapped the increasing incidence rates of some types of cancer, an association between HAAs and cancer is a reasonable hypothesis. Animal bioassays have been conducted to assess the carcinogenicity of aldrin and dieldrin, bisphenol A, BBP, chlordecone, DDD, DDE, DDT, endosulfan, endrin, lindane, methoxychlor, PCBs, TCDD, and toxaphene. Overall, the available animal data do not support an association between environmental HAAs and cancers of the female and male reproductive systems and endocrine organs. However, some of the HAAs evaluated have been shown to induce tumors in other organs. IARC has classified lindane, toxaphene, *p,p′*-DDT, and chlordecone as possibly carcinogenic, PCBs as probably carcinogenic, and TCDD as carcinogenic in humans.

The few human studies that are available are in general agreement with the animal studies described in this chapter. Human studies conducted to date do not find an association between breast, endometrial, or testicular cancers and DDT, DDE, PCBs, or TCDD (see Chapter 9). However, several of these studies are limited by factors such as small case numbers and lack of exposure measurements in body fluids and tissues. Based on the available data, animal studies have also not found an association between DDT, DDE, PCBs, or TCDD and breast, endometrial, or testicular tumors.

Liver cancer in wild fish populations has been conclusively linked to the presence of known carcinogens, especially polynuclear aromatic hydrocarbons (PAHs), in the environment (Harshbarger and Clark 1990; Baumann and Harshbarger 1995). The liver is an estrogen-responsive organ in fish, and laboratory experiments in two-stage models of carcinogenesis in two species have indicated that estradiol is a tumor promoter in some fish (Nunez et al. 1989; Cooke and Hinton 1999). It is possible that environmental estrogens act in this way in wild fish, but it has not been confirmed. Tumors in endocrine organs of fish occur in regions contaminated with pesticides; but it is not known whether those chemicals are the causative agents (Harshbarger and Clark 1990).

Body burdens of PCBs, DDT, and DDE have been decreasing over the past 30 yr largely due to regulatory intervention. However, the body burden and exposure to the majority of environmental HAAs, including other estrogenic pesticides, antioxidants, and plasticizers is not known, and the potential cumulative effects of environmental HAAs is yet to be fully explored. In addition, not all HAAs have yet been tested for carcinogenicity, including some phthalates and alkyl phenols. The large number of studies reviewed by the committee that examined the carcinogenicity of some of the most studied HAAs in many species under a great variety of experimental circumstances failed to adduce compelling evidence that HAAs induce cancers of the female and male reproductive systems and other endocrine organs. However, most of the available studies involved high-dose, postnatal testing. Little or no data are available on whether prenatal exposure to HAAs can cause cancers of the endocrine system.

RECOMMENDATIONS

Because perinatal exposure for the most part has not been addressed with respect to carcinogenesis, research in laboratory animals is needed on the role of prenatal exposure to suspected chemicals in inducing cancers later in life or in subsequent generations. Initial studies should focus on HAAs that have been shown to induce cancer of the thyroid, pituitary, and adrenal glands in some laboratory animals.

9

HAAs and Carcinogenesis in Humans

LABORATORY ASSAY SYSTEMS have been designed to test chemical compounds for their potential estrogenicity, androgenicity, antiestrogenicity, and antiandrogenicity. The systems, the chemical compounds, and the results obtained from the assays are fully elaborated elsewhere in this report. Although the evidence from various in vitro and in vivo studies contributes substantially to our understanding of the action of hormonally active agents (HAAs), that information is not a substitute for actual data obtained from human beings. This chapter reviews the data (primarily epidemiologic) that bear on putative associations between HAAs identified in the environment and risk of various cancers in humans.

This review concentrates on data reported in the published literature. This constraint limits both the environmental agents and the specific cancer sites that are covered. Although a broad range of environmental agents that have been identified in laboratory systems to mimic sex steroid hormone activity, the data that exist for evaluating the relationship between HAAs and human cancers are essentially limited to organochlorines, mostly 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (DDT) (and its metabolite 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE)), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and polychlorinated biphenyls (PCBs). There are some 209 PCB congeners, but only a few recent epidemiologic studies have distinguished among them.

The mechanism of action of DDT, PCBs, and TCDD and their metabolites are diverse because, as discussed in Chapter 2, those compounds exhibit varying hormonal properties in in vitro systems. The DDT isomer *o,p′*-DDT has estrogenic properties (Johnson et al. 1988; Soto et al. 1994), while the DDT metabolite *p,p′*-DDE acts as an antiandrogen (Kelce et al. 1995). There is evidence that PCB mixtures and congeners have both estrogenic and antiestrogenic (Gellert 1978b;

Moore et al. 1997) properties. TCDD is generally considered to be an antiestrogen (Gallo et al. 1986; Romkes et al. 1987; Astroff and Safe 1990).

The committee focused on cancer sites that are known from ancillary data to have some hormonal dependence. If HAAs operate in humans, the activity should be most evident in tissues that are known targets for endogenous and exogenous sex steroid hormones. Thus, breast and endometrial cancers in women and prostate and testicular cancers in men were selected. Each of the cancers reviewed in this chapter does not receive equal attention. Breast cancer predominates merely because the majority of studies have focused on the breast. Analysis of the incidence of endometrial cancer should be the most informative with respect to HAA effects, because the lining of the uterine fundus (endometrium) is an exquisitely sensitive tissue both to estrogenic and to antiestrogenic effects in women.

Where associations are demonstrated, either positive or negative, between HAAs and a particular cancer site, this does not in itself prove causation. Additional considerations are involved in such a determination, many of which are highlighted in epidemiologic theory and methods. For an overview of epidemiologic research and its strengths and weaknesses, the reader is referred to evaluations presented in other National Research Council reports (NRC 1999). Most important for this chapter is the concept of synthesis; that is, the integration of data from the multiple disciplines represented in this report, as well as the relative consistency (or lack of it) in the epidemiologic studies reviewed in this chapter.

One further caution is noted concerning the possible mechanisms responsible for any associations demonstrated. Many of the compounds under study are toxic agents in laboratory systems. Thus, one cannot be confident that a hormonally mediated mechanism accounts for an observed association between an HAA and a hormonally sensitive target tissue. Multiple mechanisms of action could be responsible for the toxic effects of these compounds.

The data in this chapter are introduced in a review of the known risk factors for each cancer site, emphasizing those factors that could indicate hormonal alteration. The chapter focuses on studies that specifically address the question of a relationship between environmental HAAs and cancer in humans. The studies are characterized as to type (case control, occupational cohort, unplanned experiment, or ecologic). Methodologic features that enhance or detract from the validity of various study designs are noted when relevant. The final section of the chapter lists the conclusions that can be drawn from the data presented.

BREAST CANCER

Breast-Cancer Risk Profile

Breast cancer is the second most common cancer among women in the world, and in developed countries it is the most common (Parkin et al. 1992).

Worldwide, cancer of the uterine cervix is the most common. For the other major common cancers—lung, colon, and prostate—age-specific rates increase exponentially with increasing age in developed countries. This is not true for gynecologic cancers, however. By the year 2000, it is estimated that breast cancer will account for 500,000 deaths annually (Pisani et al. 1993). Age-specific incidence rates rise rapidly until about age 50, the approximate age of natural menopause. Thereafter, the rate of increase declines, a phenomenon not seen with other common cancers. This inflection in the age incidence curve is attributed to the precipitous drop in ovarian steroid hormone production at the menopause.

Country of birth has a marked correlation with risk. Rates in developed countries are significantly higher than are those in less-developed countries (Pisani et al. 1993). Japan is an exception; there, breast cancer rates are half as high as are those in North America and northern Europe (Coleman et al. 1993), although they are on the rise. When women migrate from one country to another, their breast cancer rates assume a pattern more similar to the host country's, although that change can require two or three generations. This observation has been well documented in Asian-born migrants and among Asian-American women born in the United States (Standford et al. 1995).

Hormonally mediated endogenous factors are known to affect risk of breast cancer (Hulka et al. 1994). Lifetime risk reduction is evident in women who undergo bilateral oophorectomy at an early age. Risk is increased by early age at menarche, by late age at menopause, by nulliparity, or by late age at first pregnancy (Hulka and Stark 1995). Obesity in postmenopausal women increases risk, whereas prolonged lactation in premenopausal women decreases risk (Hulka and Stark 1995). Increasing cumulative lifetime exposure to endogenous estrogen, supplemented by cyclic progesterone, provides a unifying theory to explain many of these observations (Pike et al. 1993).

It is estimated that in 1997 there would be 180,200 new cases of breast cancer and 43,900 deaths from breast cancer in the United States (Ries et al. 1997). Breast-cancer mortality rates stayed fairly constant during the 1970s and 1980s (Miller et al. 1993), and there was a small decline in the 1990s (Parker et al. 1997). Breast cancer incidence rates have been increasing since the 1940s, with a 23% increase between 1973 and 1994 (Ries et al. 1997). Superimposed on this secular trend in incidence rates was an abrupt increase of more than 2% each year in women older than 50 during the 1980s. No increase was evident in women under 40, and there were only small increases in the 40- to 49-yr-old group. The increased rates have been attributed to the large rise in the use of screening mammography during the early and middle part of the decade (Sondik et al. 1989). This attribution is consistent with increased rates of certain tumors (localized invasive tumors and carcinomas in situ) and with the targeted screening of women age 50 and above. Since 1987, breast-cancer rates have reached a plateau (Hankey et al. 1994), although high use of screening mammography continues. It is likely that the reservoir of prevalent breast-cancer cases has been

depleted through prior screening and that cases being detected in the 1990s are true incident disease.

Many factors could account for the long-term increasing incidence of breast cancer. Changes in reproductive patterns over the past 50 yr, including fewer pregnancies and later age at first pregnancy, could have had some effect (White 1987). Some researchers suggest that increasing cumulative lifetime exposure to estrogens could be responsible (Pike et al. 1993). In addition to endogenous sources of estrogen, hormone-replacement therapy (HRT) and hormonal contraceptives are commonly prescribed. Estimates of oral-contraceptive use vary by age group of respondent, but by age 40, 80% of U.S. women have used them (IOM 1991). For HRT, use patterns vary by geographic region, age, and social class (Matthews et al. 1989). For women ages 50-59 in 1985, use prevalence in the western United States was reported at 43%; it was only 15% on the East Coast (Hemminki et al. 1988). Use of HRT by postmenopausal women is encouraged because of its potential for protection against cardiovascular disease, osteoporosis, and fractures.

Breast Cancer and HAAs

The data relating HAAs in the environment to human cancer are limited to PCBs, DDE, and TCDD. The amount and quality of those data is greatest for breast cancer—a high-incidence, high-profile disease among women. The studies have been varied: They include case-control studies, in which the chemical dose is measured in body adipose tissue or serum; occupational studies of highly exposed individuals, and ecologic analyses. Case-control studies are most likely to provide valid data because of the greater accuracy of the exposure measurement. Data are reviewed below.

Case-Control Studies

Before 1995, at least seven studies were published that contained data on organochlorine concentrations in tissue or sera as shown in Table 9-1 (Adami et al. 1995). The earliest was a 1976 report by Wasserman et al. (1976), in which formalin-fixed breast tissue from nine women with breast cancer and five women in a control group was analyzed for several metabolites of DDT and PCB congeners. They found higher concentrations of several PCB congeners in extracted lipids of malignant breast tissue than in adjacent normal tissue or control tissues. However, the concentrations of *p,p′*-DDE, the major persistent metabolite of DDT, were significantly higher in control than in case tissues.

Unger et al. (1984) used gas chromatography to analyze PCB and DDE in breast tissue from two sets of cases and controls: a postmortem series (N = 53) and a biopsy series (N = 35). Although there was no significant difference in analyte concentrations between cases and controls in either series, the postmor-

TABLE 9-1 Summary of Case-Control Studies of Breast Cancer with Ratio of Mean Concentrations as the Effect Measure

Exposure	Cases	Controls	Case Mean ± SD[a]	Control Mean ± SD	Ratio of Means (CI[b])	Reference
p,p′-DDE adipose tissue	9	5	1.53 ± 1.39 ppm	4.32 ± 1.66 ppm	0.35 (0.18-0.70)	Wasserman et al. 1976
DDE biopsy cases and controls	14	21	1.23 ± 0.63 ppm	1.25 ± 0.76 ppm	0.98 (0.68-1.43)	Unger et al. 1984
PCB biopsy cases and controls	14	21	3.89 ± 0.97	3.93 ± 1.33	0.99 (0.81-1.20)	
PCB postmortem cases and controls	18	35	6.47 ± 2.35	5.12 ± 2.38	1.26 (1.01-1.59)	
p,p′-DDE	41	33	0.96 ± 0.63 ppm	0.98 ± 0.89 ppm	0.98 (0.68-1.42)	Mussalo-Rauhamaa et al. 1990
PCB	41	33	1.05 ± 0.63	1.30 ± 0.75	0.81 (0.62-1.06)	
p,p′-DDE (wet weight basis)	20	20	1877 ± 1283 ng/g	1174 ± 630 ng/g	1.60 (1.09-2.34)	Falck et al. 1992
PCB (wet weight basis)	20	20	1669 ± 894	1105 ± 424	1.51 (1.13-2.02)	
p,p′-DDE serum	58	171	11.0 ± 9.1 ng/mL	7.7 ± 6.8 ng/mL	1.43 (1.11-1.84)	Wolff et al. 1993
PCB serum	58	171	8.0 ± 4.1	6.7 ± 2.9	1.19 (1.03-1.38)	
DDE	18	17	1370.6 ± 2077.7 µg/kg	765.3 ± 526.9 µg/kg	1.79 (0.83-3.88)	Dewailly et al. 1994[c]
PCB	18	17	368.1 ± 150.5	397.0 ± 161.5	0.93 (0.71-1.22)	
p,p′-DDE serum	150	150	43.3 ± 25.9 ppb	43.1 ± 23.7 ppb	1.01 (0.88-1.14)	Krieger et al. 1994
PCB serum	150	150	4.4 ± 1.8	4.8 ± 2.5	0.92 (0.82-1.02)	
DDE	—	—	—	—	1.08 (0.98-1.19)	Summary analysis
PCB	—	—	—	—	1.03 (0.96-1.10)	

[a] SD, standard deviation.
[b] CI, 95% confidence interval.
[c] ER+ and ER− combined.

SOURCE: Adapted from Adami et al. 1995.

tem series is difficult to interpret because of the potential for fat or organochlorine loss in the course of progressive, fatal breast cancer.

Mussalo-Rauhamaa et al. (1990) analyzed adipose tissue from the breast tissue of 44 living cases and 33 postmortem controls for several organochlorines. No difference was found between cases and controls in either PCBs or DDT metabolites, but the concentrations of β-hexachlorocyclohexane (β-HCH) were higher in the tissues of cases than in controls. The use of postmortem controls is not an optimal study-design feature, nor is the practice of excluding individuals with nondetectable concentrations of the analyte from the analysis, which for β-HCH was approximately half of each group.

Falck et al. (1992) analyzed seven organochlorine compounds in the tissues of women presenting for diagnosis of a breast mass. Among 20 cases and 20 controls, they found higher DDE and PCB concentrations in cases than in controls, although there was no difference in DDE concentrations after adjusting for age and smoking.

Dewailly et al. (1994) attempted to differentiate estrogen-receptor positive (ER+) versus estrogen-receptor negative (ER–) breast-tumor tissues with respect to several organochlorines. In an analysis of 16 compounds in tissues from 17 control subjects and 18 cases, evenly divided between ER+ and ER–, they found mirex (an ant insecticide) and PCB congener 118 to be decreased in ER– cases relative to controls, and DDE and PCB congener 99 elevated in ER+ cases relative to controls. PCB congener 118 has antiestrogenic activity and no estrogenic activity; congener 99 has estrogenic activity (Hansen 1998). Although the number of subjects was too small to produce unequivocal results, a different association between organochlorine concentrations and breast cancer, depending on the ER status of the tumor, would be of interest. In this study, with a small sample size and many analytes being studied, some associations, either positive or negative, are likely to appear by chance alone.

Two studies with stronger epidemiologic design and analytic strategies—and larger numbers of subjects—are those of Wolff et al. (1993) and Krieger et al. (1994). Both were case-control studies nested within cohorts. Blood was drawn and a questionnaire was administered at the time of cohort inception. Questionnaire data were used in multivariate models as potential confounders or as effect modifiers of an organochlorine-breast-cancer association. In each study, DDE and PCBs were measured by gas chromatography with electron-capture detection. All PCB congeners were reported as a combined value, and although not specified, the DDE value presumably represents primarily *p,p′*-DDE.

In the study by Wolff et al. (1993), the cohort (n = 14,290) consisted of women living in the New York City area who attended a mammographic screening clinic between 1985 and 1991 and who agreed to have blood drawn at the time of clinic attendance. Within 6 mo of blood draw, 58 breast-cancer cases were diagnosed. A control group was formed of cancer-free women matched with respect to age, date of blood draw, and menopausal status. There were two

controls for each postmenopausal case and a 4-to-1 ratio for premenopausal cases. The median age was 51, and 80% of the women were white. Higher serum concentrations of PCBs and DDE were observed in cases than were found in controls. Using conditional multiple-logistic regression analysis and retention of first-degree family history, months of lactation, and age at first full-term pregnancy in the model, the odds ratio (OR) was 3.7 (95% confidence interval (CI) 1.01-13.50) for the top versus the bottom quintile of DDE. The OR for PCBs was elevated in all quintiles, 2-5, relative to the lowest. However, when PCB concentrations were included in the regression equation for DDE effect, the OR for DDE was further increased, and the PCB effect became nonsignificant.

The short interval between blood draw and case diagnosis could be considered a disadvantage of the study, if the carcinogenic process altered lipid and organochlorine concentrations. If such a phenomenon occurred, it would imply that the disease was altering the amount of exposure rather than the exposure causing the disease. Alternatively, organochlorine concentrations present during the period shortly before diagnosis might represent a fairly accurate dose measure of the integrated effects of all prior events and factors influencing dose. That hypothesis is based on the premise that the relevant window of exposure is a few months or years before diagnosis. Because this exposure window has been observed with pharmacologic sex steroid hormones in women, it could also be postulated that the effects of organochlorines could be observed quickly, rather than after a prolonged lag period. If this were the case, adjustments made in the Wolff et al. (1993) study to account for organochlorine excretion via lactation (which has been noted to reduce risk of premenopausal breast cancer (Newcomb et al. 1994)), might have been inappropriate, because lactation would already have been integrated into the biologic dose marker.

The study by Krieger et al. (1994) was based on a cohort of women who received a multiphasic examination and had blood drawn as participants of a California medical-group-practice plan during 1964-1971. Serum specimens were frozen and stored, and the women were followed for the diagnosis of breast cancer until the end of 1990. From among the cancers that developed, 150 cases and 150 controls were selected such that there were 50 cases and 50 matched controls in each of three racial groups: Asian, black, and white. Cases and controls were matched on race and ethnicity, age at multiphasic exam, date of joining the medical-care group, year of multiphasic exam, and length of follow-up. The mean age of the subjects was 45 at blood draw, and the mean follow-up was 14.2 yr. Blood was analyzed for DDE and PCBs.

For the combined group, there was no difference in mean DDE or PCB concentration for cases versus controls. When the racial groups were analyzed separately, white and black cases had higher DDE concentrations than did white and black controls, and Asian cases had lower concentrations than did Asian controls. Multivariate models controlling for body mass index, age at menarche,

pregnancy, and menopausal status at diagnosis were used to provide risk estimates for tertiles of the organochlorine distributions.

Blacks and whites in the study by Krieger et al. (1994) exhibited some elevations in OR for the second and third tertiles of DDE relative to the first, but all 95% confidence intervals overlapped unity and none of the trend tests was statistically significant at $p \leq .05$. When the analysis of PCBs was separated by racial group, Asian and white cases had somewhat lower mean values than did their respective controls. ORs based on tertiles of the PCB distribution were below unity for whites and above unity for blacks, but at nonsignificant levels. Based on the data presented, one can agree with the authors' conclusion that there was no association between the specific serum organochlorine concentrations measured and the risk of breast cancer. However, it is also true that, had the trend in the data persisted with larger numbers of subjects in each racial group, ORs of two- to three-fold could have been interpreted as a positive finding (Savitz 1994).

Blood for the study was drawn before either DDT or PCBs were withdrawn from the U.S. market, so blood concentrations were high by comparison with the study by Wolff et al. (1993). The geographic locations of the studies also might have figured into the different concentrations found. Whereas in the study by Wolff et al. (1993) the controls' mean serum DDE was 7.7 ng/mL, the comparable numbers from Krieger et al. (1994) were 35.0 for whites, 43.4 for blacks, and 50.8 for Asians. Black women and Asian women had higher concentrations than did white women.

Overall, these studies published prior to 1995 do not support an association between DDT metabolites or PCBs and risk of breast cancer. Additional studies, published since that time, are summarized in Table 9-2. The table is divided into two groups. In the first group, organochlorine compounds were measured in adipose tissue; in the second group, concentrations were obtained from serum. Except for the small cohort by Sutherland et al. (1996), the studies are all case-control in design.

The first of the studies employing adipose tissue was a large case-control study from five European countries in which DDE concentrations were related to risk of breast cancer (van't Veer et al. 1997). Postmenopausal breast-cancer cases were identified from hospitals, and age-matched controls were obtained from population registries or from the register of the general practitioner of the case; 265 and 341 controls were included. Adipose tissue was removed by fine-needle aspiration from the buttocks within 1 wk of the case's diagnosis. Gas chromatography and electron-capture methods were used to measure DDE. Conditional logistic regression allowed for matching of age and sex, adjustment for body mass index, age at first full-term pregnancy, and alcohol consumption. ORs were calculated by quartiles of the controls' DDE distribution, with the lowest quartile being the reference group. The OR for the highest relative to the lowest quartile was 0.73 (95% CI 0.44-1.21) in a model, without adding covariates.

TABLE 9-2 Summary of Studies of Breast Cancer Published After the Review by Adami et al. (1995)

Reference, Location	Exposure	Date of Blood Draw or Tissue Sampling	Data Source for Cases and Controls	Date of Case Diagnosis	Case	Controls	Case Mean ± SD[a]	Control Mean ± SD	Odds Ratio (95% CI)[b] (Notes)
Adipose Tissue									
van't Veer et al. 1997, Europe (5 countries)	DDE	Within 7 d after hospital admission	Participants in EURAMIC[c] study with breast cancer (cases) and population and noncancer patients (controls)	1991-1992	265	341	1.35 µg/g of fatty acids	1.51 µg/g of fatty acids	Highest to lowest quartile: 0.48 (0.25-0.95) *p* for trend 0.02 (1,2,3)
Liljegren et al. 1998, Sweden	Nonco-planar PCBs	During surgery	Patients operated for invasive breast cancer (cases) and patients operated for benign breast disease (controls)	Between 1993 and 1995	43	35	1205 ng/g of lipid	1149 ng/g of lipid	0.7 (0.1-2.4) (1,4)
	DDE						767 ng/g of lipid	1026 ng/g of lipid	0.4 (0.1-1.2) (1,4)
	HCB						72.6 ng/g of lipid	48.1 ng/g of lipid	1.3 (0.3-4.5) (1,4)

(table continues)

TABLE 9-2 Continued

Reference, Location	Exposure	Date of Blood Draw or Tissue Sampling	Data Source for Cases and Controls	Date of Case Diagnosis	Case	Controls	Case Mean ± SD[a]	Control Mean ± SD	Odds Ratio (95% CI)[b] (Notes)
Guttes et al. 1998, Hesse, Germany	Chloroganic pesticides and PCBs[d]	1993 or 1994 immediately after patho-logic diagnosis	Hospital patients with breast cancer (cases) and with benign breast disease (controls)	1993-1994	45	20			NA[e] (5,6)
	p,p′-DDE						805 µg/kg of lipid (p = 0.017)	496 µg/kg of lipid	
	PCB No. 118						81 µg/kg of lipid (p = 0.042)	65 µg/kg of lipid	
	PCB No. 153						624 µg/kg of lipid (p = 0.083)	505 µg/kg of lipid	
Serum									
Hunter et al. 1997, United States	DDE	1989 or 1990	Participants in Nurses' Health Study with breast cancer (cases) and without (controls)	Between date of blood draw and June 1, 1992	236	236	6.01 ± 4.56 ng/mL[f]	6.97 ± 4.56 ng/mL[f]	Highest to lowest quintile: 0.72 (0.37-1.40) (7,8,9)
	PCBs				230	230	5.08 ± 2.51 ng/mL[f]	5.16 ± 2.26 ng/mL[f]	Highest to lowest quintile: 0.66 (0.32-1.37) (7,8,9)

Lopez-Carrillo et al. 1997, Mexico	DDE	During diagnostic workup, but before any treatment	Hospital patients with breast cancer (cases) and without (controls)	Mar. 1994-Apr. 1996	141	141	4.75 ng/mL (p = 0.211)	4.07 ng/mL	Highest to lowest tertile: 0.76 (0.41-1.42) (6,7,10)
Schecter et al. 1997, Hanoi, Vietnam	DDT	1-mo period in 1994	Newly diagnosed patients with invasive adeno-carcinoma of the breast (cases) and patients with fibro-cystic breast disease	1-mo period in 1994	21	21	2.33 ± 0.46 ng/mL	2.37 ± 0.58 ng/mL	Highest to lowest tertile: 1.21 (0.15-9.65) (7,11)
	DDE						12.17 ± 2.41 ng/mL	16.67 ± 4.14 ng/mL	Highest and lowest tertile: 1.14 (0.23-5.68) (7,11)
Høyer et al. 1998, Copenhagen, Denmark	Organo-chlorines[g]	1976	Participants in Copenhagen City Heart Study with breast cancer (cases) or without (controls)	Between 1976 and 1993	240	477			
	Dieldrin						24.42 ng/g[f,h] (cases and controls)		Highest to lowest quartile: 2.05 (1.17-3.57) (p = 0.01) (12,13,14)

(table continues)

TABLE 9-2 Continued

Reference, Location	Exposure	Date of Blood Draw or Tissue Sampling	Data Source for Cases and Controls	Date of Case Diagnosis	Case	Controls	Case Mean ± SD[a]	Control Mean ± SD	Odds Ratio (95% CI)[b] (Notes)
Moysich et al. 1998, Western New York	DDE	Within 3 mo. of surgery	Postmenopausal breast cancer patients (cases) and postmenopausal community subjects (controls)	Between 1986 and 1991	154	192	11.47 ng/g[f]	10.77 ng/g[f]	Highest to lowest tertile: 1.34 (0.71-2.55) (15,16,17)
	HCB						0.41 ng/g[f]	0.42 ng/g[f]	Highest to lowest tertile: 0.81 (0.43-1.53) (15,16,17)
	Mirex						0.043 ng/g[f]	0.037 ng/g[f]	Between detectable and nondetectable: 1.37 (0.78-2.39) (15,16,17)
	PCBs						4.29 ng/g[f]	4.12 ng/g[f]	Highest to lowest tertile: 1.14 (0.61-2.15) (15,16,17)
Olaya-Contreras et al. 1998, Colombia	DDE	Before chemotherapy	Hospital patients with breast cancer (cases) and without (controls)	Between July 1995 and February 1996	153	153	3.30 ± 4.12 ng/mL (p = 0.025)	2.50 ± 3.60 ng/mL	Highest to lowest tertile: 1.95 (1.10-3.52) p for trend 0.09 (6,7,18)
Sutherland et al. 1996, Charleston, S. Carolina	DDE	1974 or 1975	Participants in Charleston Heart Study with breast cancer (cases) and without (controls)	Between 1974/75 and 1994	20	385	32.0 ng/mL (cases and controls)		NS[i] (19,20)

[a] SD, standard deviation.
[b] CI, 95% confidence interval.
[c] European study on antioxidants, myocardial infarction, and cancer of the breast.
[d] *p,p′*-DDT, *p,p′*-DDE, *p,p′*-DDD, HCB, α-HCH, β-HCH, γ-HCH, 12 PCB congeners.
[e] Not applicable.
[f] Lipid adjusted.
[g] Mirex, dieldrin, aldrin, endrin, α-chlordane, γ-chlordane, heptachlor, heptachlor epoxide, oxychlordane, transnanochlor, γ-hexachlorocyclohexane (HCH), β-hexachlorocylcohexane, hexachlorobenzene (HCB), *o,p′*-DDT, *o,p′*-DDE, *p,p′*-DDT, *p,p′*-DDE, *p,p′*-DDD, and 28 PCB congeners.
[h] 78% of the blood samples had detectable concentrations of dieldrin.
[i] Not stated.

Notes:
[1]Multivariate logistic regression models.
[2]Matched for age and center.
[3]Adjusted for body mass index, age at first birth, and current alcohol consumption.
[4]Adjusted for age and parity.
[5]Regression analysis and single-factor analysis of covariance.
[6]Adjusted for age.
[7]Conditional logistic regression model.
[8]Matched for age, menopausal status, month in which blood sample was returned, time of day blood sample was obtained, fasting status at blood sampling, and hormone use for postmenopausal women.
[9]Adjusted for family history, previous benign breast disease, age at menarche, number of children and age at first birth, duration of lactation, and body mass index.
[10]Adjusted for Quetelet index, breast feeding with first birth, parity, family history of breast cancer, and time elapsed.
[11]Matched for age and place of residence.
[12]Conditional multiple logistic regression model.
[13]Matched for age, date of examination, and vital status at diagnosis.
[14]Adjusted for weight and number of full-term pregnancies.
[15]Unconditional logistic regression analysis.
[16]Matched for age and date of blood draw.
[17]Adjusted for age, education, family history of breast cancer, parity, Quetelet index, duration of lactation, age at first birth, years since last pregnancy, fruit and vegetable consumption, and serum lipids.
[18]Adjusted for first-child breast-feeding, family history of breast cancer, body mass index, parity, and menopausal status.
[19]Logistic regression and proportional hazards regression.
[20]No adjustments.

Addition of relevant covariates to the equation resulted in an OR of 0.48 (95% CI 0.25-0.95) with a *p* for trend in ORs of .02. Although DDE concentrations varied by country, overall they were low—1.9 μg/g in controls. The authors (van't Veer et al. 1997) equate this level with a serum concentration of 3.1 ppb. The latter number is less than half the mean serum value in the study by Wolff et al. (1993), and it is more than 10 times lower than the mean control value for white women in the study by Krieger et al. (1994). DDT use has been restricted in Europe since the early 1970s. These data could be interpreted to suggest an inverse association between body burden of DDE and risk of breast cancer. The authors stated that the data provided no support for the suggestion that exposure to DDE increased the risk of breast cancer.

The Swedish (Liljegren et al. 1998) and German (Guttes et al. 1998) studies were similar in that various compounds in breast adipose tissue were analyzed from women with invasive carcinoma (cases) and women with benign breast lesions. In both studies multiple compounds were measured in a small number of subjects. Whereas Guttes et al. (1998) found higher concentrations of DDE and PCBs numbers 118 and 153 in the fat of cases than controls, Liljegren et al. (1998) found no such associations. After reviewing case-control studies from numerous countries in which total DDT and PCBs concentrations had been analyzed in breast fat, Guttes et al. (1998) concluded that "there is no correlation between concentrations of these substances in the human body and breast cancer."

Studies based on serum concentrations of various compounds include the Charleston Heart Study cohort of 405 white and black women who had blood drawn and analyzed for DDE in 1974-1975 (Sutherland et al. 1996). During follow-up that lasted until 1994, 20 women developed breast cancer. Proportional hazard regression models, including relevant covariates, provided no evidence of increasing breast-cancer risk with increasing serum concentrations of DDE. Although the numbers are small, the data are of interest because the average DDE concentration in 1974-1975 was 32.0 ng/mL—approximately the same as for white subjects in the study by Krieger et al. (1994).

More recently a publication appeared from the Nurses Health Study, a cohort of about 120,000 nurses followed since 1976 (Hunter et al. 1997). The authors compared blood levels of DDE and PCBs in 240 incident cases of breast cancer and equal numbers of paired, matched control women. Bloods were drawn in 1989 or 1990 and cases were diagnosed through May 1992. The results can be summarized briefly. For DDE, mean values (ng/ml) in cases were 6.01 and in controls 6.97. For PCBs, the mean values were 5.08 and 5.16 in cases and controls, respectively. The multivariate adjusted relative risk for breast cancer comparing the fifth (highest) with the first (lowest) quintile of DDE was 0.72, 95% CI 0.37-1.40. The analogous risk estimate for PCBs was 0.66, 0.32-1.37. Those findings are convincingly consistent with no association between plasma organochlorine levels and risk of breast cancer, with the best estimate of risk for both compounds being less than one.

Because most reports have been based on U.S. or European populations, a publication from Mexico, where DDT is still used for malaria control, should reflect the breast-cancer experience in a more highly exposed population (Lopez-Carrillo et al. 1997). A case-control study based on 141 newly diagnosed cases and equal numbers of controls from three referral hospitals in Mexico City between 1994 and 1996 produced findings very similar to those reported in other recent studies. Mean serum DDE levels (ng/mL) were 4.75 in cases and 4.07 in controls, not a statistically significant difference. When DDE levels for all subjects were divided into tertiles, the multivariate adjusted odds ratio for breast cancer for the highest relative to the lowest tertile was 0.76 and for the second versus the first tertile was 0.60. Confidence intervals of 95%, including unity, surrounded each estimate. The unexpected and not fully explained finding in this study was the relatively low blood levels of DDE among all study subjects. The levels were similar to those reported in the Nurses Health Study, which encompasses many geographic areas in the United States and the Wolff et al. (1993) study from New York City.

The small hospital-based case-control study by Schecter et al. (1997) examines DDT/DDE concentrations in the serum of women in Vietnam where DDT has been heavily used in the recent past. The *p,p′* DDE levels were more than twice as high (16.7 ng/mL in controls) as those characteristic of United States or European women. Little can be said about the lack of case-control differences given the small sample size.

The recent report by Høyer et al. (1998) was based on the population of women in the Copenhagen City Heart Study. From the sample of 10,317 women identified in 1967, bloods were drawn and stored on 7,712. This cohort of women was followed until 1993 through linkage to the Danish Cancer Registry to identify the occurrence of breast cancer. The 268 women who developed invasive breast cancer provided the cases for a nested case-control study in which two controls were matched to each case on age, date of examination, and vital status at the time of the case diagnosis. Serum samples were available for 240 cases and 477 controls (Høyer et al. 1998).

Using gas chromatographic techniques, serum samples were analyzed for 18 different pesticides or their metabolites and 28 different PCB congeners. The data analysis provided odds ratios estimated by conditional multiple logistic regression, with the reference group based on the lowest quartile of the frequency distribution of the particular compound in the controls. Data were presented on four of the 46 individual compounds that were subjected to laboratory analysis in addition to total PCBs and total DDT. There were no associations between any of the specific DDT isomers or metabolites or any of the individual PCB congeners and breast cancer. Of the 46 compounds analyzed, however, only dieldrin showed a positive association with breast cancer risk: odds ratios of 1.96 and 2.05 in the top two quartiles of the distribution of values. Whether this is a biolgically significant or a chance occurrence, given the 46 different compounds analyzed and the multiple compari-

sons, is difficult to know. However, the observation is sufficiently interesting to warrant additional epidemiologic and laboratory studies.

The authors did not show the actual concentrations of dieldrin for either cases or controls. But for all subjects combined, dieldrin was not detectable in 22% of samples, a higher percentage than for any of the other compounds shown. Median blood concentrations for dieldrin were also significantly lower than those for other compounds.

Several studies have been conducted of workers employed in the production of dieldrin, settings that would allow for higher exposures than those experienced by the Copenhagen City Heart Study population. In these occupational studies, no type of cancer has been reported in excess (Van Raalte 1977; Ribbens 1985; de Jong 1991). Furthermore, the evidence for estrogenic activity of dieldrin is limited to testing in in vitro systems (the E-screen).

The Moysich et al. (1998) study is based on a subset of women from a case-control study of breast cancer conducted in western New York State from 1986 to 1991. DDE, HCB, mirex, and 73 PCB congeners were analyzed by gas chromatography with electron capture. Although the overall findings for individual and grouped compounds were essentially negative, a subgroup analysis of parous women who had never lactated revealed small elevations in the odds ratios for several compounds including mirex. Lactation appears to provide a means for reducing the body burden of various fat-soluble chemicals including the organochlorines under study.

Olaya-Contreras et al. (1998) conducted a hospital-based case-control study in Bogota, Colombia and, unlike the other studies in Table 9-2, found an association between DDE levels and risk of breast cancer. Mean and median concentrations in both cases and controls appeared low, and there is little information provided that helps to clarify this discrepant observation.

Occupational Studies

Studies are often conducted in occupational settings where workers are exposed to relatively high concentrations of chemicals used in the products and processes of manufacturing. Occupational studies are sometimes designed to use retrospective cohorts of employees working at a plant or in another occupational setting who are identified from employment records and characterized for likely exposure to the agent of interest. Exposure measurement is usually based on job title, industrial hygiene surveys of the ambient environment, measurements of air concentrations of chemicals, and, occasionally, sampling of blood concentrations. The cause-specific mortality experience of the cohort can be identified through death certificates and medical records up to a recent point in time. An unexposed reference population must be chosen from which to obtain the expected number of deaths or disease events. Analyses are then performed to identify differential mortality rates, or observed-to-expected deaths, by amount

of exposure. The advantage of occupational studies over studies of the general population is the higher exposure encountered and the fact that some of the subjects will have been employed at the study site for long periods. Studies on occupational PCB and TCDD exposures are reviewed below.

PCBs

Studies of occupational PCB exposures have been reported from Italy and the United States. Bertazzi et al. (1987) reported on the cancer mortality of workers engaged in the manufacture of capacitors impregnated with PCBs. Workers employed for at least 1 wk between 1946 and 1978 were enrolled and followed for the occurrence of cancer deaths from 1946 to 1982. PCB exposure was high, as shown by monitoring of air and surface areas. Blood concentrations in a selected sample of 37 workers averaged 282.8 ppb or 142.8 ppb, depending on the chlorine content of the PCBs. Several cases of chloracne were reported in 1954 and 1977, indicating TCDD exposure as well. Among the 1,556 women in the cohort, the 12 deaths from cancer and four deaths from hematologic neoplasms were significantly higher than the number expected, based on local population rates. It was later reported (Adami et al. 1995) that two of the cancer deaths were from breast cancer, where 1.99 would be expected in the local population.

In a retrospective cohort mortality study from two plants manufacturing electrical capacitors in New York and Massachusetts, 1,318 women were followed through 1982 for a total of 30,492 person-years (Brown et al. 1987). Forty-five deaths from malignancies occurred, compared with 48.3 expected based on U.S. general population rates. Of those deaths, nine were breast cancer deaths; 11.6 deaths would have been expected. The four deaths attributed to biliary tract cancers were noted to be in excess.

A cohort of 3,588 electrical-transformer and capacitor workers was followed from 1957 to 1986 (Sinks et al. 1992); 846 were women. Estimates of exposure were based on environmental sampling and the distance employees worked from the impregnation ovens. For cancer deaths of men and women combined, the standardized mortality ratio (SMR) was 0.8, using U.S. population rates as the reference. There was a suggested increase in deaths from malignant melanoma and brain cancer. No deaths from breast cancer were noted.

TCDD

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin functions by binding the aryl hydrocarbon (Ah) receptor and regulating the induction of specific forms of cytochrome P450, specifically aryl hydrocarbon hydroxylase (Skene et al. 1989). Ah receptor agonists, including TCDD, inhibit E2-induced responses in rodent models and in human mammary cell lines, suggesting antiestrogenic rather than estrogen agonist effects. TCDD is frequently a contaminant in other complex chemical mix-

tures and the production of phenoxy herbicides. It has been shown to be carcinogenic in some laboratory animal species (IARC 1997).

Manz et al. (1991) reported on cancer mortality of a cohort of workers in a West German herbicide plant that was heavily contaminated with TCDD. A cohort of 1,184 men and 399 women hired between 1952 and 1984 was followed until 1989, during which time malignant neoplasm was the underlying cause of death for 93 men and 20 women. Individuals were classified into three exposure groups (high, medium, and low), according to the production departments in which they had worked. German national data provided the reference rates for calculating SMRs. Among women, the SMR for all malignant neoplasms was 0.94 (0.58,1.45); the SMR for carcinoma of the breast was 2.15 (0.98,4.09), based on nine deaths. Interpretation of this possible excess is difficult: Only 7% of the women worked in high-exposure departments, compared with 40% of the men. Furthermore, some processes in the plant involved exposure to other carcinogens, such as benzene. No data are provided for women to indicate whether risk was associated with duration of employment or inclusion in the high-exposure group. In an occupational cohort of phenoxy herbicide workers in Denmark, which included 1,069 women, there was no excess of breast cancer (Lynge 1985). However, the herbicide (4-chloro-2-methyl-phenoxyacetic acid) was not thought to be contaminated with significant concentrations of TCDD.

An international registry of workers occupationally exposed to chlorophenoxy herbicides, chlorophenols, and dioxins has been established by the International Agency for Research on Cancer (IARC) and the National Institute of Environmental Health Sciences (NIEHS). Most reports have concentrated on men or on men and women combined (Saracci et al. 1991). Kogevinas et al. (1993) used data from the registry to report on incidence and mortality from malignant neoplasms in 634 exposed women with an accumulated 10,782 person-years at risk. Cause-specific national death rates and cancer-incidence rates were used as the referents. Exposure classification was based on job history; no blood concentrations of any chemicals were available for women. Cancer risk for all neoplasms combined and for breast cancer specifically was not increased; the standardized incidence ratios (SIRs) were 96 and 91, respectively. Only one of the seven incident breast-cancer cases had probable (versus unlikely) exposure to TCDD. The SMR for all malignant neoplasms combined was also low (66). The SIR and SMR were elevated for the subset of workers who were most probably exposed to TCDD. Attribution of risk is tenuous, however, because these workers were exposed to many other toxic chemicals. Three of the nine cancer deaths were malignant melanomas among New Zealand residents, for whom ultraviolet radiation was likely to be a prominent exposure.

The occupational cohorts add little information either to support or to refute an association between PCBs or TCDD and risk of breast cancer. Primarily, they are unsatisfactory because of the small cohort sizes and few deaths, resulting in low power to detect true effects and a significant opportunity for reporting effects

that are merely chance events. In the years covered by these studies, 1950s to 1980s, few women were employed in manufacturing, and of those, fewer still were in the most heavily exposed occupations.

Other problems pervade the studies to make them less informative than the case-control studies previously reviewed. The exposures are generally complex. Although the analysis is focused on PCBs or TCDD, many organic solvents and other toxicants, including carcinogens, are involved in the manufacturing processes. Therefore, even if an excess cancer risk were identified, it might be impossible to attribute it to a single chemical. Exposure assessment is frequently sketchy, based on ecologic information about the workplace rather than on individual exposure measurements. Interindividual variability in metabolism and excretion of chemicals is rarely measured. Information on potential confounders of the association between exposure and disease is usually lacking. For breast cancer, that is probably less critical than it would be for a disease such as lung cancer, for which smoking has a large effect on disease risk that could be differentially distributed among the exposure groups. The choice of reference population from which the expected number of cases is derived will influence the SMR or SIR. Use of general population rates tends to reduce the magnitude of the SMR because of the "healthy-worker" effect: People who are employed and able to work are, in general, healthier and at lower risk of death than the population at large, which includes many ill and less-fit persons. Also, the use of different reference populations for obtaining rates makes it impossible to compare SMRs or SIRs directly among studies.

Natural Experiments

TCDD

An industrial accident at a chemical plant near Seveso, Italy, in 1976 provides information on cancer effects in a population exposed to TCDD (Bertazzi et al. 1989). The area surrounding the plant was divided into zones (A, B, and R) based on decreasing mean concentrations of TCDD soil contamination. Subject exposure classification was based on area of residence. The referent cohort lived in an uncontaminated area. A 10-yr mortality analysis revealed no clear association between site-specific cancer deaths and area of residence. In men and women, the risks of death from all malignancies combined were not elevated—irrespective of zone or time after the accident. None of the 193 people with confirmed chloracne died during the study period. Residents of the contaminated zones appeared to be at decreased risk of death from breast cancer.

A report on cancer incidence after the Seveso accident appeared in 1993 (Bertazzi et al. 1993). As with the mortality study, relative risks and 95% confidence intervals were reported separately for each of the three exposure zones. The authors stated that there was a deficit of breast cancer, because the relative

risk declined with increasing exposure. In zone R, with 106 cases, the relative risk was 1.1 (95% CI 0.9-1.3); zone B had ten cases, and relative risk was 0.7 (0.4-1.4); zone A had one case, and relative risk was 0.5 (0.1-3.3). For zones A and B, the estimates were imprecise because of the small number of events.

Increased relative risk was observed for several neoplasms: hepatobiliary cancers; hematologic malignancies including lymphosarcoma, multiple myeloma, myeloid leukemia, and non-Hodgkin's lymphoma; and soft tissue tumors. Most of those diagnoses were elevated in only one sex or exposure zone. However, the pattern of results supports the prior literature on TCDD, showing an excess of soft tissue sarcomas and lymphomas, and possibly of malignancies, at other sites. No mention is made of other chemical contaminants released during the accident or their possible role in causing cancer.

PBBs

An accidental poisoning of the food supply with polybrominated biphenyls (PBBs) occurred in Michigan in 1973. A cohort of 3,653 individuals who consumed contaminated foodstuffs was followed until 1991 for cause of death (Sinks et al. 1996). PBB blood concentrations were assayed from blood samples drawn in 1976. Mortality from all causes and all cancers was less than expected. The SMR for breast cancer also was reduced, based on the five deaths observed. Only for cancer of the stomach was there a suggested increased risk of death. An earlier incidence study based on the same cohort of subjects had reported higher risks of breast cancer among women with PBB blood concentrations ≥2 ppb compared with those with blood concentrations <2 ppb. The analysis was based on 20 breast-cancer cases, and 95% CIs for all risk estimates encompassed unity (Henderson et al. 1995).

Ecologic Data

Overall, existing epidemiologic data provide little support for a positive association between the HAAs discussed in this chapter and the risk of breast cancer (Longnecker et al. 1997). Any positive association suggested in earlier studies becomes less tenable in view of more recent data from large, well-designed investigations. Although the analytic epidemiologic data previously presented provide little support for this supposition, ecological analyses could be useful to indicate whether the hypothesis is plausible. Trends in exposure and disease should be positively correlated, taking into account a biologically relevant lag time between them. Human adipose concentrations of DDT, *p,p′*-DDE, and PCBs were at their peak in the United States in the 1970s. Since then, serum and adipose concentrations have declined, consistent with the declining concentrations in the environment and in food sources (Kutz et al. 1991). The decline has been 5-fold or more for serum concentrations of *p,p′*-DDE, a stable DDT

metabolite (Krieger et al. 1994; Wolff et al. 1993). Products that contain PCBs are still in use, although they are not newly manufactured, so human exposure to PCB congeners is declining more slowly. It seems unlikely that a declining exposure would be responsible for an increasing incidence of cancer.

There are other uncertainties in establishing a relationship between specific HAAs measured in the above studies and breast cancer. Specifically, after age 40, breast-cancer incidence rates are consistently higher in whites than they are in blacks, and the disparity increases with age. Conversely, serum concentrations of *p,p′*-DDE and PCBs are consistently reported to be higher in blacks than in whites (Krieger et al. 1994; Sutherland et al. 1996; Schildkraut et al. 1999). However, those differences might reflect differences in sensitivity, perhaps due to genetic or phenotypic differences. Differences in the distributions of the polymorphisms for various metabolizing enzymes by racial group that could affect serum concentrations are not well characterized (Millikan et al. 1995).

ENDOMETRIAL CANCER

It was estimated that in 1997 there would be 34,900 new cases and 6,000 deaths from cancer of the uterus (Ries et al. 1997). The 1990-1994 average age-adjusted incidence rate was 21.5 per 100,000 women. Between 1973 and 1994, the incidence of uterine cancer decreased by 27.9%.

If some HAAs have either an estrogenic or an antiestrogenic effect in humans, that effect should be most easily observable in the endometrium. Unfortunately, there are few data on the topic. One case-control study of endometrial cancer reported on women from five geographic regions of the United States (Sturgeon et al. 1998). A subset of 90 cases and 90 matched community controls had blood drawn for analysis of serum organochlorine concentrations. Laboratory analyses were conducted to identify four DDT-related compounds, 27 PCB congeners, and 13 other organochlorine compounds. Results, corrected for serum lipid concentrations, were shown for a portion of the compounds tested; three of four DDT metabolites, PCBs grouped by their likely estrogenic, antiestrogenic, or enzyme-inducing properties, and six of the 13 other compounds. Many of the compounds were non-detectable in 40% or more of the subjects tested. Relative risks were calculated based on quartiles or tertiles of the distribution of values in the control subjects. No statistically significant increases in relative risks were observed for "high" versus "low" levels of any individual compound or group of compounds.

The retrospective cohort studies in occupational settings are uninformative. Neither Sinks nor Bertazzi mentioned uterine cancer with respect to PCBs (Bertazzi et al. 1987; Sinks et al. 1996). Brown and CDC (1987) reported an SMR for "female genital organs" of 85. The most common cancer of the female genital organs arises from the uterine cervix, and it has a epidemiologic risk profile that is different from that for carcinoma of the endometrium.

The TCDD occupational data are only slightly more informative. Manz et al. (1991) provided no information on cancer-specific causes of death in women except for breast cancer. Saracci et al. (1991) noted an SMR of 94 for "female genital organs." The Lynge (1985) study of phenoxy herbicides in Denmark provided a corpus uteri relative risk of 0.67 for exposed women based on two observed cases. However, this cohort was thought not to be heavily exposed to TCDD. In white populations, more than 95% of cancers of the corpus uteri are endometrial; the remainder are sarcomas of the uterine muscle and supporting tissues. None of the 9 reported cancer cases or deaths in the combined international cohort of women exposed to TCDD were endometrial or "corpus uteri" cancers (Kogevinas et al. 1993).

In the Seveso industrial accident, cancer of the uterine corpus was reported only from zone R, the most heavily populated but the least heavily exposed of the three exposure zones (Bertazzi et al. 1993). In zone R, the relative risk for corpus cancer was 0.5 CI (0.2-1.0), based on 9 cases. That apparent risk reduction is consistent with the antiestrogenic effects of TCDD described by in vitro assays and animal models.

ENDOGENOUS AND EXOGENOUS HORMONES AND THEIR EFFECTS IN WOMEN

The relevance of data on therapeutic exogenous hormones (e.g., oral contraceptives and HRT) to HAAs in the environment is not known. Although exposure to HAAs could be continuous, analogous to the administration of hormonal drugs, the potency of the HAAs is orders of magnitude lower than that of the endogenous steroid hormones present in premenopausal women or of exogenous HRT exposure in postmenopausal women (Adami et al. 1995).

If chemicals exert hormonal actions in humans, one of the most responsive tissues that would demonstrate the effects of such actions is the lining of the uterine cavity. Endometrial tissue proliferates, becomes secretory, and then sloughs as the result of stimulation by ovarian estrogens followed by progesterone in the normal menstrual cycle. Glandular tissue of the breast also undergoes changes in response to cyclical endogenous hormones. However, the endometrium is distinctive in its response to exogenous sex steroid hormones. Estrogens alone, when given as replacement therapy after the menopause, have been shown to cause cellular proliferation, hyperplasia, and carcinoma. The time required for these changes to occur could be a few months or years. One to 3 yr of unopposed estrogen use increases risk for the development of carcinoma; after 10 yr of use, the relative risk estimate approaches 10-fold. For this reason, progestins have been added to the hormone replacement regimen for women with intact uteri. Progestins block the induction of cell proliferation by estrogens and cause cellular differentiation in the uterus, thereby protecting the endometrium from cancer development. Progestins can be given continuously with estrogen or

for 10 d or more of a monthly cycle. With the latter regimen, bleeding occurs monthly and the endometrial cells sluff. Combined treatment reduces endometrial cancer risk, although a small elevation above nonuser concentrations still remains (Weiss et al. 1979; Hulka et al. 1980; Brinton and Hoover 1993).

Modern oral contraceptives contain both an estrogen and a progestin in every dose. In the past, sequential oral contraceptives, which contained only estrogen in a portion of the monthly pill supply, were available. When endometrial cancer occurred in women of reproductive age using these products, sequential products were taken off the market (Piper and Kennedy 1987).

The latency period between exposure to a cancer-inducing agent and cancer diagnosis must be considered. Most of the experimental data from in vitro systems and animal models indicate that estrogen agonists have a proliferative effect on cells and tissues. In models of carcinogenesis, this is considered a promotional action and its effect on cancer production is rapid. Thus, one would expect to see the effects of these compounds on human cancer risk within a few years.

A human example of this model system exists for estrogen-replacement therapy and endometrial cancer. In the late 1960s and early 1970s, endometrial cancer incidence rates had taken an abrupt upward turn (Weiss et al. 1976). Although not recognized at the time, the rise was consistent with the increasing use of estrogens, unopposed by progestins, for HRT. Publication of the first epidemiologic studies showing a strong association between estrogen-replacement therapy and endometrial cancer appeared in December 1975 (Smith et al. 1975; Ziel and Finkle 1975). Those reports had a rapid effect on sales of noncontraceptive estrogens, which dropped by about one-third between 1975 and 1980 (Kennedy et al. 1985). The decline was associated with a rapid decline in the frequency of endometrial-cancer reported to the SEER registries. In California, the decline in endometrial cancer incidence was almost concurrent with the decline in prescribing estrogen for replacement therapy (Austin and Roe 1982). After the estrogen-induced epidemic of the 1970s, age-adjusted incidence rates for endometrial cancer declined from 28.4 per 100,000 women in 1973 to 21.6 per 100,000 in 1992 (Kosary et al. 1995). If HAAs in the environment are acting as estrogen agonists, their cancer-inducing effects should be evident in the endometrium.

Hormonal effects on breast tissue are different from those observed in the uterus. Endogenous estrogens cause glandular cells to proliferate, but the addition of progesterone in the latter half of the cycle not only produces maturation effects but causes structural changes in the glands such that they become more complex and extensive. The cancer-causing effects of exogenous hormones on the breast have been studied extensively in epidemiology studies. Still, major studies of HRT are not in full agreement on its effect. If one accepts the data from the Nurses Health Study (Colditz et al. 1995), the relative risk of breast cancer is 1.3 with estrogen alone and 1.4 with estrogen plus progestin. These

risks are higher for women in their 60s, who are current hormone users of at least 5 yr duration. It seems likely that both recency of use and duration of use affect the risk estimates. Most researchers agree that adding a progestin to the estrogen does not reduce the risk of breast cancer as it does for endometrial cancer.

The risk of breast cancer from the use of oral contraceptives has been studied extensively (IOM 1991). One recent publication compiles information from almost all of the epidemiology studies on this topic published in recent years (Collaborative Group on Hormonal Factors in Breast Cancer 1996). A complete reanalysis was done from the original data, providing more than 50,000 cases and 100,000 control women. Small increments in risk (1.2-1.5) were observed for current and recent use; no increase in risk was observed after 10 yr or more since last use.

The use of diethylstilbestrol (DES) to reduce spontaneous abortion is frequently cited as a model for the effects of HAAs in the environment. Adenocarcinoma of the vagina has been documented in the female offspring of treated mothers, occurring at a rate of about 1 per 1,000 treated pregnancies. However, follow-up of the mothers themselves for the development of breast cancer has revealed surprisingly little. Relative risks of up to 1.5 have been reported, and some studies have shown no risk elevation (Colton et al. 1993). It should be noted that DES is an extremely potent synthetic estrogen; it was given in doses ranging from 5 mg/d in the first trimester to 150 mg/d in the third trimester of pregnancy (Mittendorf 1995). It is not clear how this unfortunate human experience, based on high doses of a potent estrogen taken orally for a limited time, relates to exposure to HAAs in the environment.

TESTICULAR CANCER

It was estimated that in 1997 there would be 7,200 new cases and 350 deaths from testicular cancer in the United States (Parker et al. 1997). The disparity between cases and deaths indicates the effectiveness of treatment and the excellent prognosis for those diagnosed with the disease. Testicular cancer is uncommon in white men and rare in black men. The 1990-1994 average age-adjusted incidence rates were 5.3 per 100,000 for white men and 0.7 per 100,000 for black men in the United States. Secular trends in incidence rates differ for whites and blacks. Between 1973 and 1994, white men exhibited an average annual increase of 2.4%, whereas black men experienced an annual decrease of 0.2%. Both groups showed a decline in mortality rates that averaged 5.6% annually (Ries et al. 1997). Figure 9-1 shows incidence and mortality trends for white and black men in the United States. The peak incidence rates are in the 25- to 39-yr age groups: for whites ages 30-34, 16.7 per 100,000; for blacks ages 25-29, 1.9 per 100,000, and for blacks ages 34-39, 1.9 per 100,000. Testicular cancer is unlike most major cancers with respect to its rarity (particularly in blacks), its excellent response to treatment, and its age incidence curve, where young adults are at

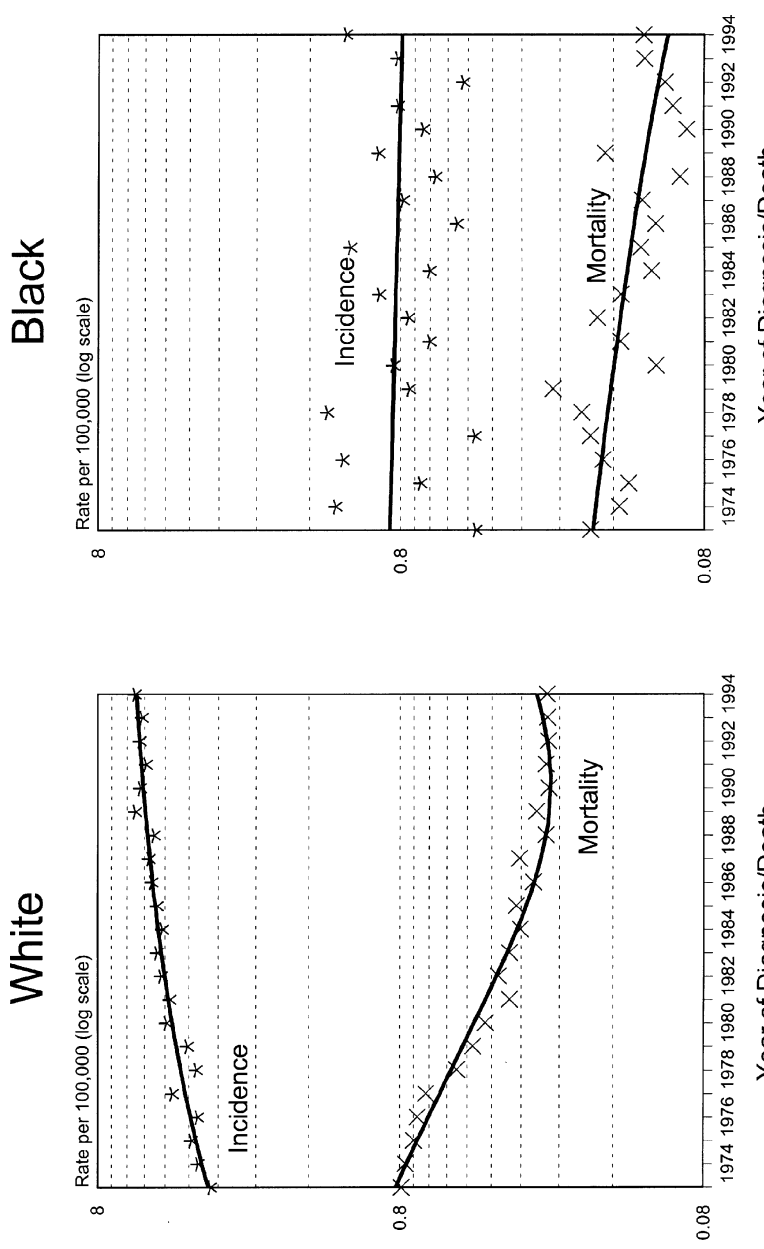

FIGURE 9-1. SEER incidence and U.S. mortality for testicular cancer in men under 65 yr of age by race, 1973-1994. SOURCE: Ries et al. 1997.

highest risk. The latter pattern is suggestive of etiologic factors operating in utero or in childhood, or from genetically transmitted susceptibility.

Most analyses of blood concentrations of PCBs, DDT, and DDE have shown significantly higher concentrations in blacks than in whites (Krieger et al. 1994; Sutherland et al. 1996; Schildkraut et al. 1999). If these particular chemicals were etiologically related to testicular cancer, one might expect the racial differences in incidence rates to be consistent with the differences in their concentrations. Just the opposite was observed, with very low rates of testicular cancer in blacks relative to whites and reasonably stable rates among blacks over time. Interpretation of these results is complicated by genetic differences between populations.

Epidemiologic studies of testicular cancer have identified a single well-established risk factor: cryptorchidism—a condition marked by the failure of the testes to descend into the scrotum—is consistently elevated among cases relative to controls. It has been estimated that cryptorchidism accounts for up to 10% of all cases (Chilvers and Pike 1992). The data suggest that exposure to DES in utero does not increase the risk of testicular cancer in male offspring (Leary et al. 1984).

Incidence rates for testicular cancer are available from cancer registries in ten northern European countries for the past 3-4 decades. Analyses of these data show a large variation in testicular-cancer incidence rates among countries for each decade. However, all countries were consistent in showing a secular trend of continuously increasing rates (Adami et al. 1994). The reason for the increase is not known. Data were available on concentrations of *p,p′*-DDE in breast milk from four of the Scandinavian countries in this study. The DDE values were similar in all four countries and showed similar declines from 1965 to 1992 (Ekbom et al. 1996). Breast milk is a relevant medium for assessment of exposure to the fetus and to the infant, because it indicates the presence of xenobiotics in blood to which the fetus is exposed and actual intake during breast-feeding. The decline in *p,p′*-DDE concentrations in breast milk during the same period that testicular-cancer rates have been increasing, including a biologically plausible time lag, seems inconsistent with a causative role for DDT.

Several occupational cohorts, including workers exposed to herbicides contaminated with TCDD or to PCBs in manufacturing processes, have reported site-specific cancer mortality as an outcome. Only from the International Cohort of Workers Exposed to Phenoxy Herbicides and their Contaminants was testicular cancer reported: SMR = 225 (95% CI, 90-464) (Saracci et al. 1991). Testicular cancer was not reported from other occupational studies—either because it is a rare disease that does not occur in excess or because it is diagnosed and successfully treated. Incident testicular-cancer cases were not in excess among individuals exposed to TCDD in the Seveso accident (Bertazzi et al. 1993).

There are no published case-control or cohort studies of testicular cancer in which blood concentrations of any HAAs have been measured. Such studies are clearly needed because exposure assessments derived from estimation procedures

could be poorly correlated with serum concentrations. This was found to be the case for TCDD among Vietnam veterans (CDC 1988). Blood concentrations provide a more accurate assessment of internal dose and potential biologic response.

In a recent ecologic study, a regression analysis was conducted using *p,p′*-DDE concentrations from human adipose tissue obtained in 1968 under the U.S. EPA Human Monitoring Program to predict testicular cancer mortality among white males in 22 states. (African-American males could not be analyzed because of their low testicular cancer mortality rates (Cocco and Benichou 1998)). Testicular cancer mortality rates were averaged for each 5-yr interval from 1971 to 1990. The coefficients from the regression analysis were negative in five of the six models, indicating no positive association between DDE and testicular cancer mortality some 2 to 22 yr later. Limitations of this study are the lack of exposure information on individual subjects (characteristic of ecologic studies), the limited statistical power, and the ad hoc nature of the population samples for adipose tissue. However, the findings are of interest since *p,p′*-DDE has been shown to bind to the androgen receptor in male rats producing an anti-androgenic environment.

PROSTATE CANCER

Prostate cancer is the most commonly occurring cancer among men in the United States, with 334,500 new cases and 41,600 deaths estimated for 1997 (Parker et al. 1997). Incidence rates, averaged over 1990-1994, were 155.8 per 100,000 for whites and 229.3 per 100,000 for blacks (Ries et al. 1997). The age-adjusted mortality rates were 24.3 and 55.5 per 100,000 for whites and blacks, respectively. Age-specific rates in both racial groups start to increase in the late 40s and continue to increase exponentially through the oldest age groups. The reasons for the higher incidence and mortality rates in blacks than in whites have been studied extensively but not fully explained.

Since the mid-1980s age-specific and age-adjusted incidence rates have increased abruptly, and this shift is superimposed on an existing secular trend of gradually rising rates (Ries et al. 1994). An abrupt increase in mortality rates has not occurred, although the steady upward trend continues for blacks and whites. These changes are shown in Figure 9-2. The increase in incidence rates coincides with and is attributed to the increasing clinical acceptance of prostate specific antigen (PSA) as a test for the early detection of prostate cancer. Screening for prostate cancer is a problematic issue because autopsy studies suggest that about 10% of men ages 50-59 harbor latent prostate cancer, and that percentage rises with age (Sheldon et al. 1980): By age 70, the figure is nearer 30%. Although the majority of the latent lesions never progress to clinical cancer, many are detectable through PSA. Thus, the epidemic of prostate cancer arises both from earlier diagnosis of cancers that would otherwise appear at a more advanced stage later in life and from those that would never surface during a lifetime in the absence of

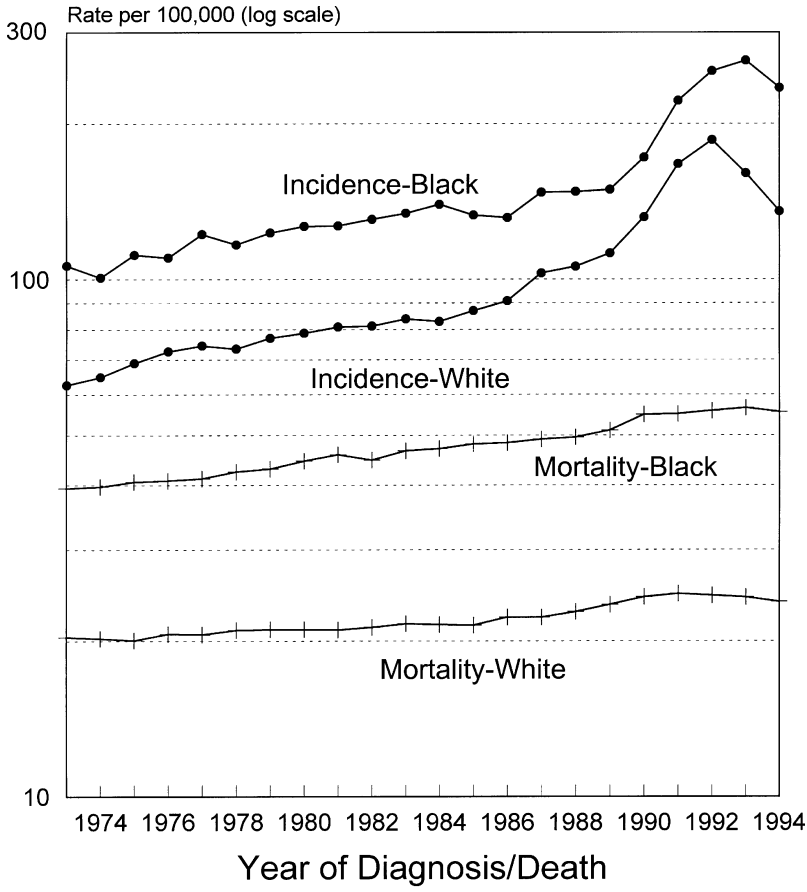

FIGURE 9-2. SEER incidence and U.S. mortality for prostate cancer, 1973-1994. SOURCE: Ries et al. 1997.

screening. The abrupt rise in incidence of prostate cancer since the mid-1980s can be attributed to screening. The 1993-1994 decline may be attributed to the depletion of prevalent cases. But what accounts for the long-term rise in both incidence and mortality is not known. Many factors have been considered, including diet, exposure to infectious agents, reproductive patterns, sexual behavior, lack of exposure to sunlight (inadequate vitamin D), and, currently, exposure to HAAs or other environmental chemicals.

Occupational studies of PCB-exposed workers give no hint of an association between PCBs and prostate cancer. Of the studies reviewed, none provided data on prostate cancer deaths (Bertazzi et al. 1987; Brown and CDC 1987; Sinks et al.

1992). Had deaths occurred, they would most likely have been reported along with the other site-specific cancer deaths.

In the Seveso incidence study, prostate cancers were reported only from zone R (the low-TCDD-exposure zone), and their numbers were consistent with expectations (Bertazzi et al. 1993). However, incidence studies of prostate cancer require special design considerations because of the potential for detection bias due to screening or diagnostic and therapeutic procedures undertaken for benign prostatic hyperplasia.

Mortality studies of prostate cancer can avoid many potential biases. Detection bias is unlikely to occur unless the diagnosis is made at autopsy where the number of histologic sections reviewed correlates with the chances of finding occult cancer. Also, a prostate cancer diagnosis on a death certificate is not unequivocal; it requires verification by medical records and a physician's report.

In the 10-yr mortality follow-up of the Seveso population, three deaths from prostate cancer occurred in zone B, and 16 occurred in zone R. In each area, the numbers were slightly greater than expected, but the variation was not statistically significant (Bertazzi et al. 1989).

Fingerhut et al. (1991) reported on cancer mortality among workers in U.S. chemical companies that made TCDD-contaminated products between 1942 and 1984. Exposure assessments based on years of exposure were validated for a sample of 253 workers from whom serum TCDD concentrations were obtained. In this highly exposed population, the correlation between the logarithm of years exposed to contaminated products and the logarithm of serum concentrations was good ($r = 0.72$). Among the subcohort that had worked more than a year with at least a 20-yr latency, the SMR for prostate cancer was 152 with 95% CIs of 70-290.

In the cohort study based on the international registry of workers exposed to TCDD-contaminated chlorophenoxy herbicides, there was no increased prostate cancer mortality (Saracci et al. 1991). Worker populations reported from Germany (Becher et al. 1996; Manz et al. 1991) showed a small, statistically insignificant excess in prostate cancer deaths based on seven cases. (There appeared to be duplication in the populations appearing in the two reports.)

These occupational studies were not designed to address the question of which chemicals (if any) are associated with prostate cancer or whether any of the chemicals acts through a hormonally related mechanism. They do provide an incentive to undertake studies with more accurate exposure assessment (serum or adipose-tissue analyses) focused on prostate cancer.

The ecologic study noted previously under Testicular Cancer (Cocco and Benichou 1998) also evaluated prostate cancer mortality rates for the 22 states from which the U.S. EPA's Human Monitoring Program had human adipose tissue. Adipose tissue was analyzed for *p,p′*-DDE concentrations in 1968. Separate models were produced to predict both African-American and white male mortality rates in each 5-yr time period from 1971 to 1990. The regression coefficients for adipose tissue *p,p′*-DDE were negative for each race and time period, indicating no positive association between *p,p′*-DDE and prostate cancer mortality. The usual constraints

of ecologic studies need to be recognized in interpreting these data. In addition, because the primary known mode of action of *p,p′*-DDE is antiandrogenic these results are not surprising. However, the lack of support for any positive association makes the theory of a positive association less tenable.

SUMMARY AND CONCLUSIONS

This chapter focuses on breast, endometrial, prostate, and testicular cancers, since these are known to arise in hormonally sensitive issues. If HAAs have a role in cancer formation in humans, their activity should be most evident in tissues that are known targets for endogenous and exogenous sex steroid hormones. The data that exist for evaluating the postulated relationship between HAAs and human cancers are mostly limited to studies involving exposure to DDT, DDE, TCDD, and various PCBs. Other compounds with potential hormonal activity have received little attention. Furthermore, exposure to HAAs and their possible effects during susceptible periods such as fetal life or pregnancy and transgenerational effects have not been evaluated in human studies.

Several recent studies have added to our knowledge concerning a possible association between HAAs and breast cancers. These studies have included large numbers of women and employed internal dose measurements of exposure. In these studies, DDE and PCBs were measured in blood or adipose tissue collected from the subjects at varying time intervals before the diagnosis of breast cancer, thus accounting for varying exposure windows and latency periods, which may be relevant to breast cancer development. Individually and as a group, these studies do not support an association between DDE and PCBs and cancer in humans. However, little is known about the effect of other compounds with hormonal activity in in vivo and in vitro systems.

Endometrial tissue is extremely responsive to the actions of estrogenic and antiestrogenic compounds. Therefore, it should be a sentinel target tissue for HAA actions. The actions should be most readily observable in postmenopausal women in whom endogenous steroid hormone concentrations are low. Decreased frequency of endometrial cancer was suggested in one study of environmental exposure to high TCDD concentrations. No association was found between a variety of organochlorine compounds and risk of endometrial cancer in the one published case-control study.

There are no analytic epidemiologic studies that examine a connection between exposure to HAAs and testicular cancer. Increasing testicular cancer incidence rates in northern European countries and in North America are unlikely to be related to environmental DDT because exposure, as measured by concentrations of DDT metabolites in both serum and breast milk, has declined significantly over the past 40 yr. Although testicular cancer incidence rates have been increasing in the United States in white men, they are extremely low and declining in black men, whereas most analyses of blood concentrations of PCBs, DDE, and DDT are higher in blacks than in whites. Ecologic data alone cannot confirm

an etiologic hypothesis. However, lack of consistency between ecologic data and the hypothesis reduces the plausibility of the hypothesis. Interpretation of these results is complicated by genetic differences between populations.

Epidemiologic data on prostate cancer are derived almost exclusively from occupational and environmental studies that do not include measurements of dose in body fluids or tissues. In occupational studies, exposure to the individual is estimated from job classification, years worked, and environmental monitoring. These studies show no association between PCB exposure and incidence of prostate cancer. However, several studies show small increases in prostate cancer mortality in relation to TCDD exposure, although none of the risk estimates was statistically significant. The antiestrogenic activity of TCDD could alter the functional balance between estrogen and androgen in the prostate resulting in a hormonal environment conducive to cancer formation. However, the currently available data are not adequate to evaluate this possibility.

RECOMMENDATIONS

Based on the committee's evaluation of the available human data on carcinogenicity, the following is recommended:

—Case-control and retrospective cohort studies that use biologic samples (fat or serum) to assay for HAA exposure are needed. If appropriately designed and conducted, such studies can document the presence or absence of associations between HAAs and cancer and whether the associations found are likely to be causal.

—Design considerations in epidemiologic studies must take into account the relevant latency period between exposure and disease, the timing of the exposure with respect to susceptible life stages, and the role of potential confounders that could distort any associations identified.

—Exposure assessment based on biologic markers and/or chemical concentrations in blood or adipose tissue stores should be included in epidemiologic studies. Exposure estimation based on occupational classification, environmental pollution, or dietary intake is rarely accurate for evaluating cancer risks.

—Biologically relevant hypotheses based on HAA toxicologic profiles and activities in animal models need to be incorporated into the epidemiologic studies.

—The biologic potency of HAAs must be related to that of endogenous hormones in premenopausal and postmenopausal women and in men. Additional comparisons should be made with pharmacologic estrogens (hormone-replacement therapy and hormonal contraceptives) and phytoestrogens because large segments of the population are exposed to these compounds.

—Compounds in addition to DDE and PCBs need to be evaluated in human studies of cancer risk.

10

Ecologic Effects

ECOSYSTEMS PROVIDE ECOLOGIC GOODS and services—including clean water, air, and soil—for wildlife and human activities such as agriculture, hunting, fishing, recreation, and aesthetic values (SCOPE 1996). In addition to their own intrinsic value, wildlife species are critical parts of the structure and functioning of ecosystems that support human activities. Evidence has been accumulating that environmental contaminants are affecting their populations (Colborn and Clement 1992). Ecotoxicologic studies have been the primary field providing data on ecologic effects of hormonally active agents (HAAs), particularly from laboratory-based studies examining effects of HAAs. Other chapters in this volume focus on the effects of HAAs on individual animals and humans; this chapter examines their effects on populations, communities, and ecosystems.

In this ecologic perspective, the committee has focused more on effects than on mechanisms. This is because ecologic effects are usually the aggregate of effects on individuals and, therefore, they are even harder to pin down than are effects on individuals. For individuals, controlled laboratory experiments with many replicates are often possible, whereas for wild populations, and especially for biologic communities and ecosystems, multiple replication is usually very difficult. Thus, ecologic studies can elucidate effects of suspected HAAs on populations, communities, and ecosystems, but cannot usually determine whether a chemical is an HAA.

A useful way to understand and model ecologic effects is through life-history dynamics. Although ecologic mechanisms might be difficult to pin down, a known effect on individuals can be extrapolated to populations through life-history models. The result of such modeling can then suggest what population (and even community-level) effects might be expected and, thus, could be valuable guides to research.

In the first section, the theoretical and science-based conceptual framework for studying the effects of contaminants is examined, and criteria for determining whether those agents do affect the structure and functioning of communities and ecosystems are discussed. In the second section, case studies illustrate what is known about the ecologic effects of contaminants on fish, birds, alligators, and mammals. The criteria established in the first section are used to evaluate evidence that ecologic effects have occurred as a result of environmental contaminants, especially HAAs. Although the mechanism of action has not been established for some of the adverse effects, and it is uncertain if or how they involve the endocrine system, it is clear that some synthetic hydrocarbons have had adverse effects on some populations of some species. The population effects often have been due to the effects of small concentrations of persistent and bioaccumulative synthetic halogenated hydrocarbons on reproduction and development, such as embryo lethality and deformities (Giesy et al. 1994a). Finally, conclusions and recommendations are presented.

NATURE OF ECOLOGIC EFFECTS

Ecologic Effects in Humans and Wildlife

Preceding chapters have devoted much attention to humans, but ecologically, humans are only one of many species. With respect to human health, the unit of concern is usually the health and well-being of individuals, including individuals who are past their reproductive years. Occurrence of cancer and other diseases in individuals is an end point commonly used, and the human-health risks of exposure to toxic agents are often assessed in terms of the increase in probability that an individual will contract a disease after exposure to a given agent. Epidemiologists do study populations, but the motivating concern is the health of individuals. Wildlife are more than sentinels because their physiology is similar to that of humans. Their well-being is an important end in itself.

Ecologic effects are those that manifest themselves at the population level or higher. Effects on individuals of wildlife species are important, but they fall under the heading of wildlife toxicology or physiology and are considered elsewhere in this report (Chapters 5-9). Indeed, the potential for HAAs and other agents to produce ecologic effects is mainly through the aggregation of their effects on the physiology of individuals. Although effects on individuals are measured, and the experimental unit is the individual organism, when statistical measures are applied and rates or probabilities of responses are reported, effects are manifested at the population level of organization.

For wildlife species, the most common unit of concern and study is the population. The end points of concern are changes in population size or reproductive capacity. Although it is often necessary to examine adverse effects in individuals to understand population changes, the population is the most impor-

tant level of organization (NRC 1993; Burger and Gochfeld 1996). Ecologic effects can also manifest themselves at higher levels of organization: biologic community, ecosystem, and landscape. A biologic community is an assemblage of species that interact or at least co-occur. An ecosystem consists of one or more biologic communities and the physical and biologic environment. Ecosystems can be as small as a pond or as large as one of the Great Lakes—indeed the entire planet could be considered a single ecosystem (Keeton 1972). A landscape is an array of biologic communities and ecosystems (Forman and Godron 1986). Each of these levels of organization embodies information that is not included in lower levels; thus, they have emergent properties that cannot necessarily be predicted from information on the lower levels. Because one ecosystem can include hundreds or thousands of species and habitats, evaluating the effects of a single disturbance is complex and more difficult than determining the effects of a chemical or chemicals on an individual organism (NRC 1981).

Population effects are usually more difficult to detect than are effects on individuals, and it is usually difficult to identify their causes. Because there is much background variability in population numbers and structure and because it is usually more difficult to obtain accurate population data for most species than it is to get information about the physiology of individuals, detection of effects at higher levels of organization is more difficult still, although changes in populations of key predator or prey species, for example, can have effects on some other species in a community. For example, contaminants in fish have affected fish-eating birds in the North American Great Lakes, as described below.

Differences between how humans and wildlife species are viewed can influence the kinds of studies and analyses needed to evaluate evidence that HAAs in the environment cause harm. To be sure, the potential for population-level effects in humans would cause great concern, but the potential for individual abnormalities and increased incidence of disease is a much greater cause for concern with respect to humans than it is for wildlife. Because of these differences, this chapter focuses on population- and higher-level effects on nonhuman species. But the methods and analyses described for evaluating effects of HAAs on individual humans described in other chapters of the report can be applied to individuals of wildlife species as appropriate.

Effects and Measurement End Points

Ecologists have used many measures to examine the structure and functioning of populations, communities, and ecosystems (Table 10-1). Population-level measures are perhaps the most useful for determining adverse effects because they are the most direct; the population is the level of organization at which effects are likely to occur first. The measures include population size, age structure, sex ratios, recruitment, and biomass (NRC 1981). In most cases, knowing population characteristics before and after a disturbance will suggest hypotheses

TABLE 10-1 Measures of Structure and Functioning of Ecologic Systems

Level of Organization	Measurement
Population	Population size
	Age structure
	Sex ratio
	Recruitment
	Biomass (of particular species)
Community	Keystone species
	Sentinel species
	Predator number
	Predator–prey ratio
Ecosystem	Species diversity
	Species richness
	Species abundance
	Species assemblage
	Primary productivity
	Erosion rate
	Decomposition rate
	Biomass change
	Nutrient cycling time
	Energy flow

SOURCES: Burger and Peakall 1995; Linthurst et al. 1995.

regarding the ecologic significance of the event. Understanding changes in population metrics as a function of contaminants might provide warning of potential population changes (NRC 1986a, 1995).

Several measurement end points can be used to determine the effect of any perturbation or chemical exposure on the structure and functioning of communities and ecosystems (NRC 1981, 1986a, 1993). Unlike measures of individual and population abnormalities, most of these require greater interpretation before cause-and-effect relationships can be demonstrated and effects of contaminants on wildlife understood. For example, species diversity is generally a useful measure of the structure of a community or ecosystem. In its simplest form, species diversity refers to the number of species in a given community or ecosystem. However, simply knowing of the species before and after a disturbance is not enough information by itself to decide whether an adverse effect has occurred (NRC 1986a).

Ideally, there should be some easily measurable end points to determine whether specific chemicals, such as HAAs, adversely affect the structure and functioning of communities and ecosystems. However, major problems are involved in identifying ecologic hazards, such as determining geographic bound-

aries, identifying target populations, choosing end points at the community and ecosystem levels, designating indicators or indices, recognizing temporal and spatial scales, identifying the effects of successional stages, and identifying appropriate reference points or systems for comparison (NRC 1986a; Norton et al. 1992; Burger and Gochfeld 1996).

Pyramid of Effects

Because communities and ecosystems are composed of many species and habitats, pyramid effects can occur, including bioaccumulation, biomagnification, cascading, keystone effects, and matrix effects.

Bioaccumulation is the storing of chemicals over time, leading to increasingly greater concentrations in the tissues of an organism. Given the same relative exposure, organisms that live longer usually accumulate more (have a greater body burden) than will organisms that have a shorter life span (Phillips 1993).

Biomagnification is the increase in exposure and accumulation that is observed as one advances up the food chain (Maedgen et al. 1982; Calabrese and Baldwin 1993; Genoni and Montague 1995). Chemical accumulations in organisms at high trophic levels can be far greater than they are in low trophic-level organisms (e.g., Buckley 1986).

Cascading effects are effects that follow from one effect on components of an ecosystem (Carpenter et al. 1985; Lipton et al. 1993). HAAs that selectively harm top carnivores can affect predator-prey relationships, and thus can indirectly change a species' reproductive performance by changing the effectiveness of its predators. Related is the idea of the keystone species (Paine 1966), which posits that removal of one predator can change the population sizes of several other species in a community. Other predator populations might increase because of lack of competition; prey populations might increase (at least initially) because of a lower predation rate and prey composition could shift because of increases in competition among prey organisms.

Matrix effects are those that occur in adjacent communities or ecosystems by virtue of their proximity.

Although the statements above are general, the processes they describe have been abundantly demonstrated. The committee has not provided a catalog of specific examples of bioaccumulation and biomagnification because many factors affect those processes. The factors include abiotic ones, such as seasonal climatic variations, and biotic ones, such as the animal's lipid content. It has thus been difficult to quantify bioaccumulation and biomagnification in most cases.

Susceptibility, Variability, and Evolution

Nearly all traits (anatomic, physiologic, or behavioral) that have been examined in humans and in wildlife show intraspecific variations (Darwin 1859; Fal-

coner 1960). These variations can lead to differences in susceptibility to chemicals, including HAAs. In terms of human-health risks, susceptibility refers to differences in genetically and nongenetically mediated susceptibility, and it can be affected by lifestyle and the environment (Omenn 1982; Woodhead et al. 1988). Ecologists have lagged behind in examining genetic and epigenetic (for example, hormonal) susceptibility of wildlife populations. Susceptibility differences could be important because they could be another form of selection within populations; that is, individuals that are less susceptible to toxicants would be more likely to contribute to the next generation (Fox 1995). Such an effect, although possibly important, would be virtually impossible to attribute to any particular cause, and so would not be useful in evaluating evidence of ecologic effects of HAAs. However, various techniques of genetic analysis, including those of molecular biology, could be useful for identifying potentially significant changes in population genetics and gene function (such as differential methylation and thus, gene expression) before their effects become apparent in such measures as population size or reproductive capacity. Moreover, because of differences in structure, some ecosystems are more susceptible than others (Burger 1997).

The generation time of organisms affects their response to selective pressures (evolution) (Lewontin 1965). For example, organisms with short generation times—insects and bacteria, for example—can rapidly evolve resistance to pesticides and antibiotics (NRC 1986b). They also should have the potential to adapt to HAAs. On the other hand, long-lived organisms—including turtles, elephants, and some bird and fish species—would be much slower to evolve adaptations to toxic pollutants; and they also are more susceptible because of bioaccumulation and biomagnification. Thus, in the absence of specific information, more attention should be paid to long-lived species in looking for ecologic effects of HAAs than to very short-lived species at low trophic levels.

Temporal and Spatial Scales of Effects

Temporal and spatial scales of exposure and effects are critical in evaluating evidence on the effects of environmental contaminants. Species sometimes are distributed evenly over the available and suitable habitat or they can occur in patches or discrete populations. The distribution pattern influences both their exposure and the possible effects of any toxicant. Some species experience continuous exposure; others have periods of low exposure, either because of physiologic mechanisms (hibernation) or behavior (migration). One aspect bears special comment with respect to HAAs in the environment. Many purported HAAs are distributed unevenly regionally and globally (see Chapter 3). Uneven distribution makes evaluation of the evidence more difficult, and it provides the potential for widespread and variable effects.

Risk Assessment and Ecologic Effects

Risk assessment is one method of evaluating the effects of HAAs on ecologic systems, from the individual to the entire ecosystem. Although in the study of ecology the method borrows the four-part paradigm used for human-health risk assessment (NRC 1993), it differs significantly in that the problem-definition stage is far more complicated because of the inherent complexity of ecologic systems (Bartell et al. 1992; Norton et al. 1992; Burger and Gochfeld 1996) and because of the large variety of life-history patterns (Burger 1994). The choice of end points to evaluate is difficult because ecologic-risk end points include those for individuals, populations, communities, ecosystems, and even the biosphere (Sheehan 1984a; Suter 1990; Peakall 1992).

Use of Indicators

Because of the inherent complexity of ecologic systems, it is sometimes useful to identify biologic indicators of exposure and ecologic effects (Hunsaker et al. 1990a,b; Suter 1990; Kendall and Akerman 1992). Several investigators have addressed this topic for communities and ecosystems, including Sheehan (1984a,b), Cairns et al. (1992), Heimbach et al. (1992), Kendall and Akerman (1992), Peakall (1992), Weber et al. (1992), and Linthurst et al. (1995). Biologic indicators are often used to see whether toxicologic effects that occur in the laboratory mirror what happens in nature.

Framework for Evaluating Ecologic Effects

The preceding discussion leads to the framework that the committee used to evaluate information on ecologic effects of HAAs. Ecologic effects can be manifested at different population levels and can be preceded by changes in the population and genetic structure of various species. It is important (but not required) first to establish whether an agent is present in the environment, at what concentration, and how it is distributed. If the agent is not present or the ecologic systems of interest are not exposed to the agent, then one can conclude that agent is not a factor in that system. However, care must be taken when analytic techniques for detecting and measuring chemicals are less sensitive to disruption by the chemicals than are organisms.

It is important to establish plausible modes of action for a suspected agent. This, usually done through laboratory studies, is especially important if the presence or concentration of the suspected agent in the environment or organisms' exposure to it cannot be established easily, as might be the case if a suspected chemical can have an effect at extremely low concentrations.

If changes in population size, structure, growth rate, or other aspects of population dynamics are established, then it is necessary to link the suspected

agent to those changes. It is also useful to rule out other possible causes of the changes. Next, changes in higher-level ecologic factors, such as community composition or primary productivity, must be plausibly related to established population-level effects or directly to the suspected agent. Whether the mode of action of the suspected agent is through a hormonal mechanism must be established. It is rarely, if ever, possible to establish all of the above relationships. Thus, one must use judgment, based on a weight-of-evidence approach, as described in the case studies below.

EFFECTS ON POPULATIONS AND COMMUNITIES

Studies of selected communities and individual populations can be used to illustrate what is known about the ecologic effects of environmental contaminants on fish, birds, alligators, and mammals. The studies discussed here were selected because they are the ones for which the committee found the strongest evidence for the potential of HAAs to disrupt community and ecosystem functioning.

Fish

Fish are good indicators of contamination for several reasons. Runoff and erosion carry chemicals from land to water, so aquatic organisms are exposed to all the contaminants in their drainage basin. Because fish are food for wildlife and humans, they are monitored for concentrations of toxic chemicals. Fish thus serve as good indicators of toxic substances in the aquatic environment. Because it is a long-lived top predator, the lake trout (*Salvelinus namaycush*) has been suggested as an indicator of ecosystem functioning (Edwards et al. 1990).

Great Lakes Fish

Observations of fish from the Great Lakes have been instrumental in shaping much of what is understood of the potential for persistent, organic pollutants to accumulate in and have adverse health effects on wildlife. The lessons learned have been of much broader use than only for the lakes themselves (Gilbertson 1992). The Great Lakes are typical of many ecologic systems in that they have been subjected to a great deal of human incursion, including commercial and sport fishing (often overfishing); the introduction of native and alien species, such as forage fishes, Pacific salmon (*Oncorhynchus* spp.), and sea lampreys (*Petromyzon marinus*), and various genetic strains of those species (Strittholt et al. 1988); habitat loss; and chemical pollution.

For nearly 2 centuries the Great Lakes have received industrial and municipal wastewater. The effects of that contamination were not suspected until population effects were observed in the top predators of the ecosystem—birds (Giesy et al. 1994a). It was not until the late 1960s that analytic methods (gas-liquid

chromatography) were available to measure the presence of synthetic halogenated hydrocarbons (Reinert 1970). Even so, it was not yet understood that small concentrations (parts per trillion or parts per quadrillion) of the compounds could harm the Great Lakes biota (Thomann and Connolly 1984) or that the chemicals could persist, bioaccumulate, and biomagnify. Since the 1960s, many sources of major contaminants, such as polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-equivalent (TCDD-EQ) have been eliminated or greatly curtailed, and much of the Great Lakes ecosystem is recovering (Makarewicz and Bertram 1991). However, contaminant residues, especially from petroleum hydrocarbons and metals, remain in sediment and could continue to harm fish in some locations (Fitchko 1986). For example, the chinook salmon (*Oncorhynchus tshawytscha*) population in Lake Michigan crashed in 1989 as the result of an outbreak of bacterial kidney disease (BKD), and populations have not yet recovered to their former size—despite an increased rate of stocking (Garling et al. 1995). The bacteria that cause this disease are normally present in the kidneys of the salmon, but they generally do not harm the fish. It is only when the fish are subjected to additional stress that BKD is expressed to the point of affecting survival and growth. PCB concentrations in chinook salmon (Williams and Giesy 1992; Williams et al. 1992) are lower than those found to cause immune suppression in rainbow trout (*Oncorhynchus mykiss*) (Arkoosh et al. 1994).

Lake trout (*Salvelinus namaycush*) also have suffered a population decline because of poor reproductive success (FWS 1981; Mac and Seelye 1981b). Annual rearing mortalities in lake trout fry of as much as 97% were described for hatchery-reared fish between 1978 and 1981 (Mac et al. 1985). Several studies have attributed this to toxic organic residues in eggs (Mac and Seelye 1981a; Seelye and Mac 1981; Mac and Schwartz 1992; Mac et al. 1993). In addition, some of the fish produced fry that developed "blue sac" disease, an edematous condition that results in fluid filling the yolk sac, causing a bluish color, and leading to death of the egg.

In the early 1980s, PCBs and dichlorodiphenyltrichloroethane (DDT) were believed to be responsible for the effects. However, efforts to correlate the degree of fry mortality in lake trout with relatively high concentrations of PCBs (11 mg/kg bw) and DDT (7 mg/kg bw) were unsuccessful (FWS 1981; Seelye and Mac 1981). Subsequent studies identified the total concentrations of TCDD-EQ to be a more plausible causative agent (Symula et al. 1990; Walker and Peterson 1994).

The failure of recruitment of the lake trout populations could be the result of several factors—behavior, predation, genetics, changes in diet caused by changes in prey species composition, or inappropriate stocking practices—but the evidence supports the hypothesis that toxic chemicals in eggs were, at least historically, responsible for some of the population decline (Walker and Peterson 1991; Walker et al. 1991). Current concentrations of TCDD-EQ in lake trout fry are near the threshold for mortality (Walker and Peterson 1994). Thus, it is likely

that the concentrations of TCDD-EQ in lake trout eggs were well above the threshold in the recent past, and that the current concentrations might continue to exert adverse effects on survival.

The effects of contaminants on fish populations of the Great Lakes are difficult to demonstrate because of the complex interactions among species and their environment. For example, during the 1970s and 1980s, populations of alewife (*Alosa pseudoharengus*) also were declining because of predation (Dunstall 1984) and die-off (Brown 1984). Salmon switched to other prey, such as bloaters (*Coregonus hoyi*) and smelt (*Osmerus mordox*) (Elrod 1983; Swedberg and Peck 1984; Muth and Busch 1989; Miller and Holey 1992). Thus, changes in reproductive performance and population size in salmonids might result from complex changes in predator-prey relationships instead of or in addition to the effects of exposure to halogenated hydrocarbons. The fish have been exposed to several compounds at once, so it has been difficult to correlate exposure to any one compound with the observed effects. Many contaminants have been identified as hormonally active, but it is not known whether hormonal or other endocrine disrupting actions promote all of the observed health and population-level effects. However, the data indicate that environmental contaminants can affect fish, especially the reproductive capacity (see Chapter 5), of fish species in the Great Lakes and probably have affected their populations.

M-74 Syndrome in the Baltic Sea

A syndrome analogous to the reproductive impairment observed in Great Lakes salmon has been observed in Baltic Sea fish. The number and fertility of eggs are normal, but the fry become listless and settle to the bottom and die. This phenomenon has been seen in Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) in the Baltic Sea, and the condition has been called M-74 syndrome because it was first reported in these two species in the Baltic Sea in 1974. The M is an abbreviation of the Swedish word for environment, *miljö.* The effect is observed in wild and feral fish captured from the Baltic Sea for spawning in hatcheries. The intensity of the phenomenon varies among individuals and from one year to another (Norrgren et al. 1993). Several causes of the syndrome have been suggested, including genetics and nutrition, but most researchers believe the phenomenon was attributable to exposure to toxicants, particularly the dioxinlike polychlorinated diaromatic hydrocarbon (PCDH) (Norrgren et al. 1993). The syndrome has not been observed every year or to the same extent from one occurrence to the next, and tagged individuals have produced fry that exhibit the syndrome one year but not in others. Some scientists postulate that M-74 syndrome was not caused by exposure to contaminants; others believe it might be a combination of effects, due in part to contaminants, but that some accessory factor might be necessary for the phenomenon to be observed.

Several researchers have investigated the concentrations of contaminants in herring oil (Falandysz 1986; Cooper et al. 1991) and the effects on herring (*Clupea harengus harengus)* (Hansen et al. 1985) and on salmonids fed oil extracted from the herring (Andersson et al. 1993). They demonstrated that there were contaminants in the herring oil, but they could not cause M-74 syndrome in the fish fed the herring oil. Several researchers surmised that a nutrient deficiency caused by the change in diet from a mixture that included shrimp to an almost exclusive herring diet was responsible for the reproductive effects (Norrgren et al. 1993).

Adult fish exhibiting M-74 syndrome are characterized by flesh that is white or grey rather than the normal pink or orange. The eggs produced from those adults are less colored. Some of the researchers suggested that a carotenoid deficiency was responsible (Andersson et al. 1993; Norrgren et al. 1993). This was supported by the color of the adult flesh and eggs, which, when analyzed for carotenoid pigments, were found to be deficient in astazanthine. That discovery led to several studies (Andersson et al. 1993; Norrgren et al. 1993) in which the diet of adult salmon was augmented with astazanthine or eggs were dipped in a mixture containing astazanthine. The treatments increased the carotenoid content of the eggs, but they did not prevent M-74 syndrome.

A great deal of research into the diet of the salmon as well as general chemical and physical conditions in the Baltic Sea has been conducted (Holm et al. 1993; Henriksson et al. 1996). Those studies show that, depending on the amount of fresh water entering the Baltic Sea, the degree of oxygen saturation in the benthic water varied from year to year. In fact, in some years, large areas of the benthic Baltic Sea were anaerobic. When this occurred, the shrimp (*Crangon crangon*) population was much reduced. It was thought that shrimp could be an important prey item for Atlantic salmon and brown trout. It was postulated that, in the years when shrimp populations were depressed, the two fish that exhibited M-74 switched their diet to one of mostly herring without consuming any shrimp. However, although shrimp make up a significant proportion of the diet of Atlantic salmon in the Atlantic Ocean, they are not a significant proportion of the diet of salmonids in the Baltic Sea (P. Vuorinen, Finnish Game and Fisheries Research Institute, Helsinki, Finland, personal communication, 1997). Thus, it does not seem likely that this is an alternative to the hypothesis that M-74 is caused by contaminants.

Working independently on salmon populations in the Finger Lakes of New York, Fisher et al. (1994) observed a syndrome in salmon (*Salmo salar*) that was similar to M- 74. They named the syndrome “Cayuga Syndrome” for the lake in which it was observed. A number of instrumental analyses were conducted to determine the concentrations of chlorinated insecticides, PCBs, and dioxin equivalents in the lake, but none of the compounds seemed to be occurring at sufficiently high concentrations to cause the syndrome. However, the investigators were able

to demonstrate that the syndrome was caused by the presence of thiaminase (an enzyme that degrades thiamine) in the herring of the salmon's diet. When the fry from adults exhibiting the syndrome were fed diets supplemented with thiamine, the syndrome was eliminated. Thus, the investigators concluded that xenobiotics were not involved in the syndrome, but rather it was a thiamine deficiency caused by the consumption of herring in the diet. However, it should be noted that interactions between PCBs, TCDD, and thiamine metabolism have been reported in laboratory studies with rats (Yagi 1979; Yagi et al. 1979; Pelissier et al. 1992).

Birds

Birds are perhaps the most studied group of organisms with respect to environmental contamination because they are conspicuous and relatively easy to observe and collect; they were among the first groups in which the effects of environmental pollutants were observed. Most of the effects observed have been on piscivorus (fish-eating) birds, which are exposed to pollutants in contaminated fish. Two significant population effects have been changes in sex ratios in gull populations and population declines among gulls, cormorants, and terns.

Skewed Sex Ratios

The effects of exposure to environmental pollutants have been demonstrated in studies of skewed sex ratios in gulls exposed to organochlorines, such as DDT, PCBs, and TCDD. Skewed sex ratios in favor of females were documented by the increased number of nests containing an abnormally large number of eggs, or supernormal clutches. Nests containing five or more eggs are considered to be the result of female-female pairing, because a single female gull normally lays one to three eggs. It is believed that females associate with one another when males are unavailable because reproductive success relies on the presence of a pair of nest attendants. One adult must remain at the nest to guard chicks from predation while the other forages for food. It is also possible that females associate with one another to avoid aggression directed toward unmated birds (Shugart et al. 1988). Some studies have found that the reproductive success of supernormal clutches was less than normal (Schreiber 1970; Hunt and Hunt 1973), but other researchers have documented successfully fledged young from supernormal clutches (Kovacs and Ryder 1983).

The most dramatic and best-documented example of a skewed sex ratio occurred between 1968 and 1978 in the western gull (*Larus occidentalis*) population on Santa Barbara Island in California (Hunt et al. 1980). The adult sex ratio in that population was measured by laparotomy of 856 captured birds to be 0.26 males to females. The investigators also calculated the male-to-female ratio by estimating the number of nests on the island (896), the number of nonbreeding birds (200), and, based upon the number of nests with more than three eggs, the

percentage of female-female pairs (15%). Using those estimates, the male to female ratio was 0.67. Because many birds laid fewer than normal numbers of eggs in 1978, the investigators believe that the estimate for female-female pairs might have been too low. Therefore, the real ratio of males to females was probably between 0.26 and 0.67.

A supernormal clutch incidence of 0.6% to 1% also was documented in northeastern Lake Michigan herring gulls (*L. argentatus*) between 1978 and 1981 (Shugart 1980; Fitch and Shugart 1983). Both the California population and the Great Lakes populations of gulls were exposed to large concentrations of organochlorine— particularly DDT—contamination from the 1950s to the 1970s (Fry and Toone 1981). Historical studies of supernormal clutches in gulls to determine whether incidences have actually changed between the pre- and post-DDT eras indicate that the number of supernormal clutches has decreased significantly for many species of tern throughout the United States (Conover 1984b). However, three gull species have experienced a significant increase in supernormal clutches since the 1950s: western gulls, herring gulls nesting in the Great Lakes, and Caspian terns (*Sterna caspia*) breeding in the United States.

The decrease in the number of males in these populations could be attributable to several factors, some related to persistent organochlorine contaminants. For example, differential mortality between male and female gulls could occur because of differences in body burden of the toxicants. Male western gulls weigh about 25% more than females and they feed higher on the food chain (Pierotti 1981), which makes them more likely to accumulate pollutants throughout their lifetimes. It also has been suggested that estrogenic contaminants could be causing feminization of male embryos with resultant chemical sterilization and failed recruitment into the breeding population (Fry et al. 1987). However, behavioral studies of western gulls (Hunt et al. 1984) and attempts to correlate gonadal feminization with organochlorine contamination in a glaucous-winged gull (*L. glaucescens*) population in Puget Sound, Washington (Fry et al. 1987), have been inconclusive.

Thus, although environmental contamination has been correlated with the skewed sex ratio observed in several North American gull populations, it is still not known how pollutants cause the differential effects. The explanations presented above are just a few ways in which pollutants could affect populations, and there is no indication that effects are caused by a hormonal mechanism. There might be alternative causes that do not involve environmental contaminants.

Population Declines

Several species of fish-eating birds of the Great Lakes region have experienced population declines, which have been attributed to exposure to environmental contaminants (Keith 1966; Anderson and Hickey 1976; Fox et al.

1991a,b). Keith (1966) found that reproductive success in herring gulls in the early 1960s around Lake Michigan was approximately one-third of normal; Gilbertson (1974) found that it was about 10% of normal for birds nesting near Lake Ontario. Nesting colonies of Forster's terns (*Sterna forsteri*) at Lake Michigan experienced hatching success as low as 26%, and no fledgling success for several years (Hoffman et al. 1987). Although the effects observed with the gulls were partly attributable to eggshell thinning (see Chapter 5), 30% of eggs that were incubated to term failed to hatch. Egg-transfer experiments with herring gulls (Peakall and Fox 1987) and Forster's terns (Kubiak et al. 1989), in which eggs were transferred between nests in "clean" and "contaminated" sites, indicated that the declines in hatching success were the result of toxicants in the eggs and abnormal parental behavior. Environmental contaminants have been shown to affect egg and embryo survival (see Chapter 5), but it is not known whether pollutants are the cause of the behavioral changes observed in adults. This is important to consider because changes in parental behavior could have consequences for other aspects of avian survival outside of the breeding season.

Eggshell thinning has contributed to the decline of several bird species in the Great Lakes region and elsewhere. For example, Great Lakes colonies of double-crested cormorants (*Phalacrocorax auritus*) suffered widespread reproductive losses due to accidental egg breakage caused by eggshell thinning associated with 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) (Anderson and Hickey 1972), and the populations declined by 80% (Postupalsky 1978). Although there are no studies on community-level effects that result from population effects, it is likely that they were present because cormorants are strictly piscivorus, and lowering their populations by 80% must have had an effect on the fish populations they prey upon (and potentially on fish size and species composition). However, the effects of these declines on the populations of fish that are the prey of cormorants is almost impossible to discern, because so many other factors have changed as well (e.g., introduction of exotic species, stocking of predatory fish, sport and commercial fishing, other effects of pollutants, and lamprey predation).

In Georgian Bay, 95% of cormorant eggs broke or disappeared before incubation was complete (Weseloh et al. 1983). Dissection of nearly full-term eggs indicated that half of the eggs contained chicks with deformities (Ludwig et al. 1996), and the deformities were typical of those caused by exposure to organochlorine compounds (Fox et al. 1991a,b; Gilbertson et al. 1991). Chicks with deformities often die soon after hatching, resulting in reduced fledgling success in the colony, and ultimately, in changes in age ratios and overall population size.

Another notable incident occurred in 1986, when there was a severe flood in the Saginaw River ecosystem that released PCBs from the sediments in that area (Ludwig et al. 1993). This exposure led to the occurrence of deformities in Caspian tern chicks at a frequency 168 times greater in 1987-1989 than was seen in 1962-1987. Increases in deformities and lessened hatching success for a 2 yr period are likely to have effects on local tern population demography for years,

but it might not be detectable by simply enumerating the total population because immigration occurs from other colonies. The evidence that there continues to be toxicant-related embryonic death, deformities, and immune dysfunction in chicks strongly suggests that there are still population-level effects in the Caspian terns (Mora et al. 1993), even though there are no changes in the number of individuals. Populations of cormorants also declined substantially (see Table 10-2 for references).

TABLE 10-2 Population and Community Effects Associated with HAAs in the Great Lakes Ecosystem[a]

Species	Reproductive Effects	Behavioral Deficits	Population Declines	Community Effects	Reference
Human		X			Rogan et al. 1986b; Jacobson and Jacobson 1996; Lonky et al. 1996
Mink	X		X		Allan et al. 1991
Double-crested cormorant	X		X	X	Anderson and Hickey 1972; Allan et al. 1991; Fox et al. 1991a,b; Giesy et al. 1994a; Ludwig et al. 1995, 1996; Larson et al. 1996
Black-crowned night heron	X	X	X		Allan et al. 1991
Bald eagle	X	X	X		Allan et al. 1991; Bowerman et al. 1994, 1995; Giesy et al. 1994a
Herring gull	X	X	X		Fox et al. 1978, 1991a; Peakall and Fox 1987; Allan et al. 1991; Giesy et al. 1994a; Grasman et al. 1996
Forster's tern	X	X	X		Hoffman et al. 1987; Kubiak et al. 1989; Allan et al. 1991; Fox et al. 1991a
Caspian tern	X	X			Mora et al. 1993
Snapping turtle	X		X		Allan et al. 1991
Fish (several species)	X		X	X	Allan et al. 1991

[a] Chapter 2 of this report describes some of the mechanisms involved with these effects.

Cormorant embryos exposed naturally to environmental concentrations of PCBs, polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) have a statistically significant increased incidence of brain (intercerebral) asymmetries correlated with the exposure (Henshel et al. 1997).

Bald eagle (*Haliaeetus leucocephalus*) populations also declined in the 1950s and early 1960s, primarily because of exposure to DDT and its metabolites (Wiemeyer et al. 1984). Although many populations have increased in much of the Great Lakes region since then, some populations have not recovered. Others have increased because of migration from other areas and not from reproduction (Best et al. 1994; Bowerman et al. 1994). Craniofacial malformation in eagle embryos and chicks is attributable to contaminated eggs derived from the parents' consumption of contaminated fish and closely resembles abnormalities observed in cormorants and terns. However, the effects of exposure to other pollutants, such as mercury (Giesy et al. 1995) cannot be ruled out. Although there is an inverse correlation between contamination with DDE and PCBs in eggs and plasma of nesting eagles and reproductive success (Bowerman et al. 1994, 1995), it is impossible to decide the degree to which each contaminant affects the birds or the degree to which effects are additive.

Populations of some bird species have been monitored in the Great Lakes region for the past half century, and there is clear evidence for population decline, reproductive impairment, or both, in several fish-eating species. There is also convincing evidence for community-level effects: Many of the declines in bird populations seem to be caused by eating of contaminated fish. Although the declines initially were believed to be caused by DDE-induced eggshell thinning and pollutant-induced adult toxicity, the populations still exhibit subtle effects, such as deformities probably caused by dioxin-like PCBs (Giesy et al. 1994a,b). As populations of some species have recovered, a significant incidence of teratogenicity is still observed. In several species, the effects have been associated with putative HAAs, such as coplanar PCBs, DDT and its metabolites, and other organochlorine compounds. Laboratory experiments with birds indicate that several agents can alter reproduction and development (see Chapter 5) and that the toxic effects of exposure to multiple compounds can be additive. However, a range of other factors can cause declines in population numbers, such as human disturbance and exploitation, loss of habitat, inclement weather, predation, disease, and competition for space or food (Burger and Gochfeld 1996). Any or all of those factors could contribute to lowered reproductive success. Nonetheless, it is remarkable that so many species of fish-eating birds in the Great Lakes have experienced the same individual and population problems. Such changes have not been observed so consistently in such a wide geographic area, over such a span of years, in colonial fish-eating birds of other regions.

Alligators

The effects of exposure to pollutants on the health and reproductive biology of the American alligator (*Alligator mississippiensis*) of Lake Apopka, Florida, have been extensively studied (see Chapter 5), and anomalies in genital and gonad morphology and alterations in plasma sex-steroid concentrations have been the reason for declines in its population size in this area over the years. The most significant decline occurred after the Tower Chemical spill into the lake in 1980 (Woodward and Moore 1990), when the pesticide dicofol (contaminated with DDT and its metabolites 1,1-dichloro-2,2-bis(*p*- chlorophenyl)ethane (DDD), DDE, and chloro-DDT) was accidentally released. There was a rapid, 90% decline in the population of juvenile alligators between 1980 and 1984 (Figure 10-1), and, beginning in 1984, the viability of alligator eggs from this lake decreased significantly (Figure 10-2). Recent data suggest that the juvenile population on Lake Apopka is increasing slowly, and egg viability has continued to vary, with most years exhibiting an average below that observed on other lakes (Rice et al. 1996). It has been suggested that the initial decline in the alligator population was a direct

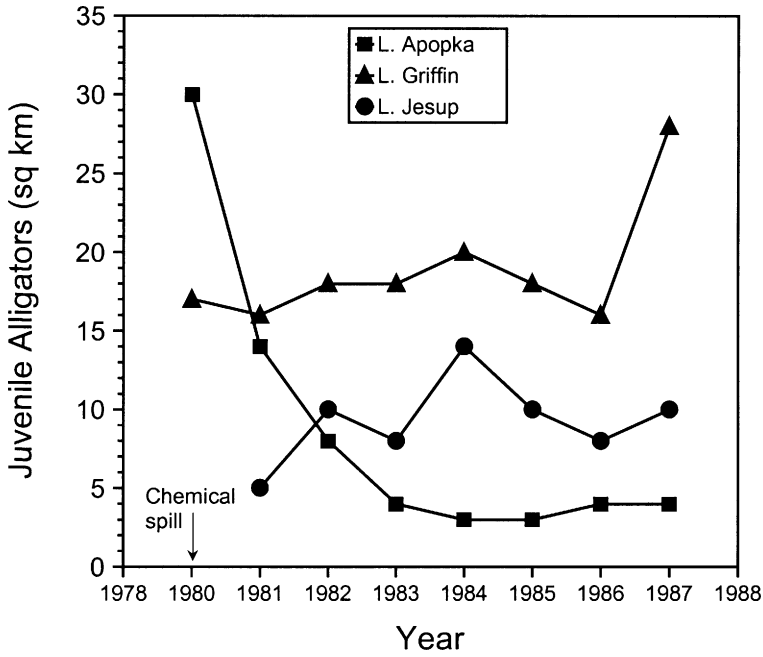

FIGURE 10-1 Representative estimates of juvenile population density of American alligators from three lakes in central Florida. SOURCE: Data from Woodward et al. 1993.

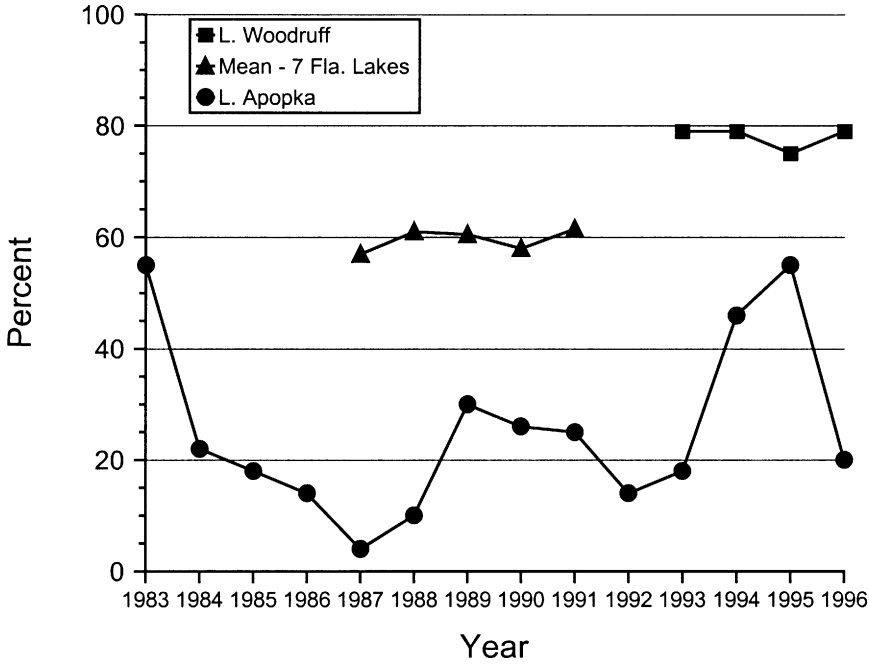

FIGURE 10-2 Viability of alligator eggs obtained from various lakes in Florida and incubated under controlled conditions. SOURCES: Data from Woodward et al. 1993; Guillette et al. 1994; Masson 1995; Rice et al. 1996.

consequence of the lethal effects of high concentrations of dicofol-DDT (or its metabolites) on hatchlings and juveniles living in the lake, whereas the current reduced population density of those age groups is a result of poor clutch viability (Guillette and Crain 1996).

An analysis of embryonic mortality in alligator eggs suggests that more than 80% of all embryonic mortality takes place during the first month after fertilization of the egg (Masson 1995). There have been reports of greater concentrations of *p,p′*-DDE in alligator eggs collected between 1984 and 1985 from Lake Apopka compared with eggs from two other lakes (Heinz et al. 1991), but greater concentrations of organochlorine compounds could not be correlated directly with poor egg viability. The mean concentrations of *p,p′*-DDE observed—5.8 ppm weight-to-weight (w/w) (1984: $n = 3$ eggs; range, 3.4-7.6 ppm) and 3.5 ppm w/w (1985: $n = 23$ eggs; range, 0.89-29 ppm)—were above the concentrations known to harm avian eggs and embryos (Cooper 1991). In addition to *p,p′*-DDE, alligator eggs ($n = 23$) collected in 1985 from Lake Apopka had detectable concentrations of *p,p′*-DDD (≤1.8 ppm), dieldrin (0.02-1.0 ppm), and *cis*-chlordane (≤0.25 ppm) (Heinz et al. 1991).

As described in Chapter 5, contaminants of Lake Apopka are believed to be the cause of abnormal gonadal morphology and altered sex-steroid concentrations in juvenile alligators. It has been suggested that the gonads of male and female alligators were permanently modified in ovo, and that normal sexual maturation was unlikely (Guillette et al. 1994). Studies by the U.S. Environmental Protection Agency (EPA) (1994b) have shown that juvenile and hatchling alligators from Lake Apopka have high concentrations of *p,p′*-DDE, primarily in fat (1.6-8.5 ppm) and in liver tissues (0.013-0.17 ppm). That also has been observed in hatchling alligators exhibiting developmental abnormalities.

Because alligators take 12-15 yr to reach sexual maturity (Ferguson 1985; Joanen and McNease 1989), the community and long-term population effects of changes in the current population size and age ratios in Lake Apopka will not be known for many years. It is likely that a reduced population size, coupled with the gonadal and sex-steroid abnormalities observed in juveniles, will affect the reproductive success of these alligators in the future and could pose significant long-term population and community threats.

Mammals

Data on the effects of mammalian exposure to environmental contaminants are limited and come mainly from work with ranch mink (*Mustela vison)*, river otter (*Lutra lutra*), and harbor seal (*Phoca vitulina*). Cause-and-effect relationships are difficult to establish for any toxic chemical in wildlife because of the many difficulties inherent to field studies. Therefore, subtle effects often must be inferred using epidemiologic criteria in concert with laboratory studies.

Mink

In the 1960s and 1970s, mink fur production decreased from ranches that used Great Lakes fish in the diet. Studies of ranch mink fed a diet of carp (*Cyprinus carpio*) from Saginaw Bay, Michigan, have suggested that population declines in the ranches were caused by impaired reproduction and reduced kit survival caused by PCB and TCDD in the diet (Heaton et al. 1995a), because fur production decreased at the time when pollutant concentrations were highest in the region and subsequently increased slightly in the 1980s, when concentrations of PCBs and other pollutants were lower. It is believed that pollutants could also be the cause of observed population declines in the wild (Allan et al. 1991). For example, there are fewer mink in areas where Great Lakes fishes can be consumed than in areas where they cannot, such as above dams that limit the accessibility to lake-run salmon. Fish in Great Lakes-influenced sections of three Michigan rivers were shown to contain greater concentrations of PCBs and TCDD-EQ than did fish that did not have access to the Great Lakes (Giesy et al. 1994b).

Population declines in mink also have been reported in Georgia, North Carolina, and South Carolina (Osowski et al. 1995). A range of contaminants from the tissues of mink were analyzed, and concentrations of PCBs (0.216 μg/g) and mercury (3.5 μg/g) in liver tissue were in the range known to cause reproductive impairment, growth deficit, and behavioral impairment. Similarly, Foley et al. (1988) reported that PCB concentrations in wild mink from New York were in the range known from laboratory studies to cause reproductive damage (Platonow and Karstad 1973). A significant correlation between body burden in mink with body burden in fish collected from their home ranges (Foley et al. 1988) indicates that the food chain is being affected. These data, coupled with the evidence that exposure to PCBs impairs mink reproduction, reduces kit survival, and lowers body weights of kits (Platanow and Karstad 1973; Heaton et al. 1995a,b), suggest that environmental pollutants have affected mink populations, which could pose community threats.

River Otter

The river otter populations in Wales, England, and other regions in Europe declined in the 1950s, and home ranges were constricted (Mason and Macdonald 1986). The changes have been attributed to contaminants in the food chain, because pollution was great during that time, and as the contaminant concentrations in these areas have declined, the otter populations have begun to increase (Mason and Macdonald 1993a). However, recolonization (movement back into the areas) has been slower than expected, probably because of continued exposure to large concentrations of dieldrin, DDE, and PCBs (Mason and Macdonald 1993a, 1994). Using PCB concentrations in feces as an indication of contaminant concentrations in tissues, Mason and Macdonald (1993b) related dietary intake of PCBs to tissue concentrations, and related tissue concentrations to those that attend reproductive problems in otter. They concluded that exposure to PCBs was responsible for the slow recolonization rates in England and Wales (Mason and Macdonald 1993a,b).

In the Netherlands, the otter populations have become nearly extinct. In 1989, the Dutch government adopted a policy to restore their populations, and a project entitled "Development of Otter-based Quality Objectives for PCBs" was commissioned in 1993 to support this effort. The two-part project involved a critical review the field data from European otter-habitats (Smit et al. 1994) and an assessment of biomagnification of sediment-bound PCBs in otter habitats, biologic factors determining the bioaccumulation of PCBs in otters, induction of dose-dependent physiologic effects in material from captive and feral otters from different European countries, the health status of otters in Denmark in relation to PCB exposure, and the feasibility of monitoring of PCB exposure in feral otters (Smit et al. 1996). The project reported the following observations: significant biomagnification in the food chain of the otter in the Limfjord area in Denmark;

increased incidence of disease (viral infections, bacterial disease, pathologic deviations, and endoparasites) in otter populations, which was correlated with PCB-induced vitamin A deficiency; and current environmental PCB concentrations that are great enough to cause adverse effects (Smit et al. 1996).

Marine Mammals

Many marine mammals have undergone population declines in the past 40 yr. In some cases, the causes are well known (e.g., hunting of whales and some pinniped and collisions with boats and loss of habitat for manatees), but in others, such as the Steller sea lion (*Eumetopias jubatas*) in the Bering Sea, serious population declines are not fully understood (NRC 1996b). Another example is the decline observed in the beluga whale (*Delphinapterus leucas*) population of the St. Lawrence, which was estimated in the 1980s to be only 10% of the 1885 level (Reeves and Mitchell 1984) and to be lower than reference populations in the less contaminated Arctic (Sergeant 1986). Despite protection since 1980, the beluga whale populations have not recovered, due mainly to low calf production and low survival of young (Colborn and Smolen 1996). Low calf production and survival have been hypothesized to be due to exposure to HAAs (De Guise et al. 1995). Elevated concentrations of organochlorines, such as PCBs and DDT- and toxaphene-related compounds (Martineau et al. 1987; Muir et al. 1990a,b), have been found in the blubber of beluga whales since the early 1980s. Moreover, such organochlorines have been associated with a variety dysfunctions, which might provide an explanation for population declines.

Chapter 7 of this volume describes a correlation between feeding on PCB-contaminated fish and impaired immune response in seals and whales. It is believed that this could account for the drastic declines in the common harbor seal in the Wadden Sea off the north coast of the Netherlands and elsewhere in Europe (Reijnders 1981; Osterhaus and Vedder 1988; de Swart et al. 1996). These seals had high concentrations of PCBs in their tissues and experienced immunosuppression and subsequent viral infections. Similarly, a disease complex involving a primary lesion of the adrenal glands and secondary reactions in other organs has been observed in the Baltic grey seal (*Halichoerus grypus*) and the ringed seal (*P. hispida*) in the Baltic Sea and along Swedish west coast (Olsson et al. 1994). This complex is believed to be the cause of the dramatic decrease in these species in the 1960s and 1970s. The harbor seal population in these areas also suffered about a 60% decrease in 1988 because of epizootic disease caused by phocine distemper virus (Olsson et al. 1994). Historical skull bone material from these seals indicates the presence of unnatural stress factors associated with epizootic diseases. The tissue from the seals was contaminated with a variety of metal and nonmetal elements, and it has been suggested that these contaminants, particularly DDE and PCB methyl sulfones, might be the cause of the population declines (Olsson et al. 1994).

In a review of marine mammals, Colborn and Smolen (1996) reported that 13 species of cetacean and pinniped experienced recent population declines or die-off. Reproductive and endocrine impairments were reported for eight of the species, and immune problems were seen in four of them. However, such impairments are as likely to be the result of the conditions that caused the deaths as to be the cause of them, and, in general, we do not have good information on the causes of the population declines and die-offs.

SUMMARY AND CONCLUSIONS

Analysis of available data shows that environmental contamination with known HAAs has affected wildlife populations and, in some cases, communities. There is evidence that certain synthetic, persistent, bioaccumulative hydrocarbons have caused effects on wildlife reproduction, but the mechanism of action of the HAAs is generally not understood well enough to determine whether they act through hormone receptors or through other pathways. Ecologic problems are inherently complex. However, the potential damage to ecosystems and their components from HAAs is too severe to ignore. What is known is that certain chemicals that are released into the environment can cause population-level effects on wildlife. It is at least possible that changes in bird populations in the Great Lakes region (and perhaps elsewhere) and the declines of some seal populations in the North Sea are due in part to the hormonal activity of pollutants, although the evidence is not conclusive. The evidence that implicates HAAs in population declines of lake trout in the Great Lakes is less strong, but it still seems quite likely that HAAs have been among the several factors in those declines.

RECOMMENDATIONS

Long-term studies of populations subjected to HAA exposures are needed to assess the effects of these chemicals in altering population size, age structure, and dynamics. Observational and experimental studies of the linkages between chemical exposures and alterations of key aspects of life histories (e.g., fecundity, survivorship, longevity, and age of first reproduction) should be undertaken to understand how chemical exposures affect long-term ecologic attributes of natural systems. Ultimately, the physiologic and biochemical bases of these linkages, once established, should be determined.

11

Screening and Monitoring

THERE ARE NO GENERALLY ACCEPTED, validated methods to screen for or monitor exposure to chemicals that could cause adverse hormonal activity—largely because of the complexity of the endocrine system. Assays and end points for testing and monitoring have been proposed (Gray et al. 1997; OECD 1997), but many of them are specific for the effects of hormone mimics, especially estrogenlike compounds (ECETOC 1996), and they do not address the full range of putative hormonally active agents (HAAs). HAAs can act directly, at a specific receptor, or indirectly, and their effects can vary, depending on the target tissue, the timing of exposure, and interactions with other HAAs. Because HAAs can mimic or modulate the activity of endogenous hormones, it can be difficult to distinguish altered responses from the range of basal hormone-regulated responses. For this reason, various U.S. and European groups have urged that currently used tests be assessed for their adequacy to detect the effects of HAAs (Kavlock et al. 1996; Ankley et al. 1997; EPA 1997). EPA has organized the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to recommend screening and testing guidelines appropriate for chemicals with endocrine-disrupting activity. Congress has mandated a response from EPA by March 1998 (see Addendum).

No single assay can accurately predict all the effects of HAAs. In vivo assays are often unsuitable for large-scale screening because of their relatively high cost, low sensitivity, and labor intensiveness. Moreover, in vivo assays that assess highly complex responses can be modulated through other mechanisms and, therefore, might not be selective for the substances of interest. Screening and monitoring approaches for hazard assessment of HAAs should be designed to assess HAAs alone and in combination with other HAAs that modulate the response of the primary compound of interest (Kavlock et al. 1996; Patlak 1996;

Ankley et al. 1997). In vitro measurements can be made in screening (a priori) and in monitoring (a posteriori) modes to measure or predict the activity of HAAs, and several methods to do that have been proposed (ECETOC 1996).

The discussion presented here will not attempt to recapitulate all proposed methods; they are reviewed elsewhere (ECETOC 1996; Gray et al. 1997; OECD 1997; Zacharewski 1997). This chapter focuses on principles and strategies for selecting appropriate in vitro and in vivo testing systems. The discussion is restricted to general methods of screening and monitoring. Special attention is given to HAAs that mimic steroid sex hormones, generally defined as "estrogens" and "androgens," and the emphasis is on vertebrates, especially mammals. As additional information is obtained on the functions and dynamics of the endocrine system (particularly during development), new assays can be developed to identify biologic markers of exposure.

SCREENING TOOLS

The need to screen a large number of compounds for their potential to cause hormonal activity is mandated by law (Safe Drinking Water Act of 1997; Food Quality Protection Act of 1996). Several task forces have stressed the need for tests that predict in vivo responses in humans (Kavlock et al. 1996) and wildlife (Ankley et al. 1997). The methods available to screen for HAAs include biologic and instrumental chemical analyses. The former category includes assays that can be used to screen for potential hormonal activity in individual compounds, formulations, or environmental extracts. Extensive reviews of standard in vitro and in vivo tests to screen for estrogenic activity have been published (Reel et al. 1996; Zacharewski 1997). This section reviews mammalian and nonmammalian in vitro and in vivo assays and focuses on their advantages and limitations for identifying and assessing estrogenic substances.

In Vitro Assays

In vitro assays available for measuring receptor-mediated activities of HAAs include simple receptor-binding, cell-proliferation, gene-expression, and gene-product assays. Most have been developed for determining estrogenic activity, and there is a need for development and validation of in vitro assays for other hormone-receptor systems. In vitro systems are attractive as screening tools because they are rapid, inexpensive, and reproducible, and estimates of the relative potency of a large number of samples or compounds can be obtained rapidly. In vitro assays are also excellent models for investigating the mechanism of action of HAAs and the interactions between endocrine-response pathways.

In vitro assays are limited in that they cannot represent in vivo conditions. The pharmacokinetics, biotransformation, and binding to carrier proteins of a compound might or might not be represented accurately by in vitro systems.

There are methods to minimize some of the limitations and to increase the accuracy of in vitro assays. In addition, all in vitro assays that are based on the biologic properties of established cell lines require special care to maintain the original cell phenotype (Laursen et al. 1990; Ruedl et al. 1990; Zugmaier et al. 1991; Wiese et al. 1992; Villalobos et al. 1995). Some in vitro systems that are used to screen for HAAs are described below. Table 11-1 summarizes the advantages and disadvantages of those most commonly used. The selection of assays should be based on their responsiveness to the HAA of interest. There will never be a single in vitro assay capable of screening for a wide range of HAAs simultaneously.

Receptor Binding

Binding of agonists or antagonists to a receptor is required for direct-acting receptor-mediated HAAs to exert an effect (Clark and Mani 1994; Kramer and Giesy 1995). Therefore, the receptor-binding affinity of a compound could predict ligand potency relative to the endogenous ligand for the specific receptor. For this reason, receptor-binding assays have been used to screen for putative HAAs (R. White et al. 1994; Kelce et al. 1995).

Relative receptor-binding affinities of various ligands are routinely determined in competitive assays in which receptor preparations are coincubated with a high-affinity radioligand and with different concentrations of a test compound or mixture. Analysis of kinetic-binding data can be done to determine half-inhibitory concentration (IC_{50}) values, which define the concentration of the test compound required to displace 50% of the radioligand. Receptor preparations from diverse species and from estrogen-responsive tissues, as well as recombinant proteins, have been used for these assays. In vitro assays, such as estrogen-receptor (ER) binding, are invaluable as screening methods; however, they have several limitations: Structure-receptor binding might not always parallel structure-activity relationships; receptor binding is determined in the absence of pharmacokinetic or metabolic effects (Bitman et al. 1978; Bulger and Kupfer 1985; Kramer et al. 1997); receptor-binding assays have not been developed to distinguish between receptor agonists and antagonists; and the sensitivity of the assay is low (half-maximal effective concentration [EC_{50}] for estradiol is in the 300-pM to 1-nM range) (Wooge et al. 1992; Soto et al. 1995). Recent studies have identified a second form of the ER, ER_{β}, which exhibits a pattern of expression different from the ER pattern (Kuiper et al. 1996; Mosselman et al. 1996). There are significant structural differences between ER_{α} and ER_{β} proteins. However, Kuiper et al. (1998) evaluated the relative binding affinities of 60 estrogenic compounds to the two ER subtypes and found that only a few compounds exhibited major differences in binding affinity to ER_{α} and ER_{β}. The multiple and variant (Castles et al. 1995) forms of the ER, as well as different tissue-specific expressions of these forms, could be important determinants in the tissue-specific

TABLE 11-1 Selected In Vitro Assays

Assay	Advantages	Disadvantages	Reference
Receptor binding	Large-scale screening; direct receptor interaction; requires 1 d or less; yeast or produce baculovirus system can be used to milligram quantities of receptor	Cannot distinguish between agonists and antagonists; cannot detect proestrogens; requires radioligand; serum-binding proteins absent; no in vivo pharmacokinetics; artifactual competition by chemicals at high concentrations due to detergent-denaturation effects.	Jensen and Jacobson 1960; Ireland et al. 1980
Cell proliferation	Simple to perform; large-scale screening; animals not required; direct cell exposure; few false positives or negatives; sensitive detection; can distinguish between agonists and antagonists (partial and full); detects some proestrogens	Only a few cell lines are estrogen responsive; 4-6 d incubation period; cannot detect all proestrogens; no in vivo pharmacokinetics	Soto et al. 1990, 1991, 1992a, 1994, 1995; Villalobos et al. 1995; Welshons et al. 1990
Receptor-dependent gene expression	Large-scale screening; wide variety of cell-line origins can be used	Expression of some proteins and enzyme activities can be restricted to some cell lines; response can be susceptible to mechanisms that do not involve the estrogen receptor; compounds might not have consistent tissue- or species-specific responses; cannot detect proestrogens	Jobling and Sumpter 1993; Heppell et al. 1995; Jordan et al. 1985; Littlefield et al. 1990; Pelissero et al. 1993; Soto et al. 1995
Recombinant receptor–reporter gene constructs	Large-scale screening; direct cell exposure; internal control reporter gene to monitor toxicity; some require 1 d; animals not required; can use transfected mammalian or yeast cells; some can distinguish between agonists and antagonists	No standardized assay system generally available; cannot detect proestrogens; no in vivo pharmacokinetics; some cannot distinguish between agonists and antagonists; yeast cell walls can affect the uptake of xenoestrogens	Mayr et al. 1992; Miksicek 1993; R. White et al. 1994

SOURCE: Adapted from Reel et al. 1996.

action of ER agonists and antagonists. This could also be important for the different isoforms of other receptors of other HAAs.

Cell Proliferation

The ability of estrogens to induce cellular proliferation in target organs is considered a hallmark of estrogen action (Hertz 1985). Therefore, a reliable bioassay for assessing estrogenicity would measure cell proliferation as an end point. This measurement can be done in vitro by using established cell lines derived from estrogen-responsive target organs, including rat pituitary cells and several human breast cancer cell lines, such as MCF7 and T47-D cells.

MCF7 cells have been used extensively to screen for estrogen agonists and antagonists (Welshons et al. 1990; Soto et al. 1991, 1992a, 1994; Sonnenschein et al. 1994, 1995). MCF7 cells express the ER (Brooks et al. 1973), which regulates several critical genes as well as estrogen-dependent cell proliferation (Katzenellenbogen et al. 1984; Soto and Sonnenschein 1984). MCF7 cells become quiescent when placed in an estradiol-deficient culture medium supplemented with dextran-coated charcoal-absorbed serum. Estrogens release these cells from quiescence. The estradiol EC_{50} is in the 10- to 15-pM range (Soto et al. 1997). Results obtained using these cells were considered to be highly predictive of estrogenicity (Andersen et al. 1999).

Receptor-Dependent Gene Expression

Assays that test the ability of a compound to stimulate receptor-dependent responses (in genes or proteins) are routinely used to determine the estrogenic or antiestrogenic potency of HAAs in several cell lines. Genes or gene products that have been used include progesterone receptor, pS2, alkaline phosphatase, cathepsin D, prolactin, and vitellogenin (Jordan et al. 1985; Littlefield et al. 1990; Adlercreutz et al. 1992; Jobling and Sumpter 1993; Pelissero et al. 1993; Heppell et al. 1995; Soto et al. 1995). Although these genes and gene products are induced by estrogenic compounds, the induction responses are often specific to a target organ or cell and can be induced by several HAA classes.

Molecular biology techniques have been used extensively to develop an array of in vitro assays that are hormone-responsive promoter-reporter constructs that can be transiently or stably transfected into diverse mammalian cell lines. The cells also can be transiently or stably transfected with wild-type or variant hormone receptors or chimeric receptors (Pons et al. 1990; Gagne et al. 1994; Jausons-Loffreda et al. 1994; Mäkelä et al. 1994; Jobling et al. 1995; Miksicek 1995; Ruh et al. 1995; Zacharewski et al. 1995; Ramamoorthy et al. 1997). Various promoter sequences and reporter genes have been used for these assays, and some of the most sensitive constructs contain multiple or tandem estrogen-responsive-element (ERE) motifs, which are strong enhancer elements.

Estrogen responsiveness in cells that express wild-type ERs depends on the interaction of activation function 2 (AF-2), AF-1, or both domains of the ER with other nuclear proteins. The complex interactions of hormone receptors and nuclear coactivators, repressors, and accessory proteins are important in the ligand-dependent and cell-specific induction of various promoter-reporter constructs and their related genes (Katzenellenbogen et al. 1996). Ligand-dependent differences in the functions of wild-type and ER variants have been reported for different classes of ER agonists and antagonists (McDonnell et al. 1995), and it is likely that application of these techniques will demonstrate functional differences between estrogenic HAAs (Gould et al. 1998).

Chimeric-receptor and reporter-gene assays also have been developed to bypass the requirement for many nuclear factors. For example, Zacharewski et al. (1995) used a Gal4-HEGO chimeric receptor, which contains the ER ligand-binding domain fused to the DNA-binding domain of the Gal4 yeast transcription factor, and a Gal4-regulated luciferase reporter-gene construct, which contains copies of the Gal4 DNA-binding motif. These constructs have been used in transiently and stably transfected cells to detect estrogenic and antiestrogenic HAAs (Zacharewski et al. 1995; Connor et al. 1996; Moore et al. 1997). Recombinant estrogen-based yeast assays also can be used for rapid screening of hormone-receptor agonists (Klein et al. 1994; Routledge and Sumpter 1996; Ramamoorthy et al. 1997). Cotransfection of genes encoding various P450 enzymes involved in metabolic activation and inactivation of HAAs will increase the utility of these receptor assays.

Prediction of In Vivo Effects from In Vitro Assays

The usefulness of in vitro techniques is hampered by their inherent simplicity. In vivo, there are multiple cell types in tissues that communicate via extracellular signals, such as hormones, growth factors, and cytokines. However, in vitro assays use cloned cell lines; that rules out interactions among cell types (Kao et al. 1996) and thus modifies the potential of cells to respond to hormones. Additionally, the cell types used in in vitro systems might not have or might have lost the ability to metabolize certain compounds. In vivo, some compounds need to be activated to cause hormonal modulation, and others can be deactivated by being metabolized, congregated, and or excreted.

Most in vitro tests are based either on a cellular response, such as cell proliferation, or on gene expression. Historically, the understanding of steroid or thyroid hormonal function has been based on the genomic theory that hormones function through regulation of nuclear transcription complexes by intracellular steroid-binding proteins. That results in a steroid-effect cascade. However, recent studies have shown that nongenomic effects can occur (Wehling 1994). For example, *o,p′*-DDT is a weak ER agonist (i.e., it rapidly dissociates from the receptor) in vitro (Soto et al. 1994), and it has been difficult to reconcile the

estrogenlike effects of *o,p′*-DDT observed in wildlife (Fry et al. 1987; Fry 1995) with the concentrations that have been shown to elicit estrogenlike effects in vitro (Donahoe and Curtis 1996). Ligand-independent activation of ER-mediated responses via tyrosine-kinase pathways have been reported (Kato et al. 1995), and steroid hormonal action might also involve post-transcriptional regulation of mRNA (Hadcock and Malbon 1991; Martin et al. 1994). Another mechanism that can lead to differences between in vitro and in vivo potency of HAAs relative to estradiol is differential binding to plasma proteins. For example, *o,p′*-DDT shows little binding to estrogen-binding plasma proteins (Skalsky and Guthrie 1978), a factor that will increase the effective free fraction of *o,p′*-DDT in vivo relative to estradiol (Nagel et al. 1998).

Thus, because of the limitations of in vitro assays and because the precise mechanisms of hormonal action have not been determined, it is not always possible to predict in vivo effects from results of individual in vitro assays. Therefore, in vitro bioassays should utilize a battery of simple assays based on different mechanisms of action.

In Vivo Assays

Despite their limitations for use in large-scale screening of compounds or extracts, estrogen-responsiveness assays in rodents are important test procedures. In vivo assays measure several end points, including organ weight, cell differentiation, protein and gene expression, and enzyme activity (Lan and Katzenellenbogen 1976; Lyttle and DeSombre 1977; Dix and Jordan 1980; Cooper et al. 1992; Branham et al. 1993; Medlock et al. 1994; Sheehan et al. 1994; Heppell et al. 1995; Sumpter and Jobling 1995; Teng 1995). The advantages and disadvantages of various in vivo tests are discussed below.

In vivo tests for detecting HAA-induced effects on reproduction and development use a complete biologic system that accounts for pharmacokinetics (i.e., absorption, disposition, metabolism, and excretion) of a test compound. Moreover, in vivo testing is done in the presence of physiologically relevant types and concentrations of endogenous hormones, hormone-binding proteins, and accessory factors. Repair or defense systems that might be absent in an in vitro system will be present in an in vivo system. Thus, results of in vivo assays are relevant for extrapolating to wildlife or humans.

Many testing protocols have been standardized, validated, and in some cases, published as official guidelines (EPA 1996; OECD 1997). The regulatory protocols that relate particularly to HAAs are the two-generation reproduction, developmental toxicity, subchronic toxicity, and chronic toxicity studies (Stevens et al. 1997). These tests are designed to detect adverse effects on reproductive function or development, regardless of mechanism.

Response end points monitored in multigenerational reproduction studies include fertility; litter size; and weight, survival, and growth of offspring. All of

these responses can be altered by exposure to HAAs. Other end points, such as vaginal cyclicity, reproductive-organ weight, gonadal morphology, accessory-sex-organ weight, sperm count, and anogenital distance could be affected by HAA activity, but the changes are not necessarily specific to any particular hormone or portion of the endocrine system.

Hormone-Specific Tests

Hormone-specific assays determine whether a chemical acts through a particular hormonal mechanism (such as estrogenicity), regardless of whether the effect is thought to be harmful. Specific assays can be used to test the hormonal-agonist or -antagonist activity of a chemical. Mammalian estrogen biologic assays that measure reproductive-tract responses are summarized in Table 11-2. These assays use rodent vaginal and uterine tissues, and they are sensitive, reproducible, and biologically relevant for comparing the estrogenic potential of various HAAs. Typically, endogenous sources of estrogen are eliminated or reduced by using immature or ovariectomized animals, thus eliminating the problem of mistaking responses caused by endogenous estrogen for those caused by exogenous compounds. Measures of estrogenic activity include vaginal cornification, vaginal epithelial-cell proliferation and tetrazolium reduction, vaginal opening, vaginotrophic response, uterine fluid imbibition, uterotrophic response, uterine glycogen deposition, and uterine estrogen-withdrawal bleeding.

In the vaginal-cornification assay (Allen and Doisy 1923), estrogen agonists cause the vaginal epithelium to divide and differentiate from cuboidal cells to pseudo-stratified columnar cells to keratinized stratified squamous epithelial cells within 48-72 hr of exposure. Vaginal keratinization and cornification are among the most specific in vivo end points available for determining the estrogenic character of a compound (Edgren 1994). In addition, the vaginal-cornification assay requires only microscopic readings of vaginal smears.

Vaginal opening is an estrogenic response and a developmental end point. At birth, the external rat vagina is still a solid cord of cells (Allen and Doisy 1924). Before the first estrus, and about 5 d before ovulation, the vaginal lumen is formed. This process can be accelerated from the average of 40 d of age to 30 d, or even earlier, by exposure to estradiol or other estrogens (Edgren et al. 1966). It has been proposed that this end point be included routinely in the multigenerational reproduction study (EPA 1996).

Uterine fluid imbibition (Astwood 1938) is rapid (6 hr), but it is not a graded response. Therefore, this assay does not distinguish potent from relatively weak estrogens or short-acting from long-acting estrogens.

The uterotrophic assay typically uses a regimen in which animals are given daily doses of a compound for 3-4 d to measure true uterine growth (Bülbring and Burn 1935; Dorfman and Dorfman 1954; Edgren et al. 1966). Short-acting estrogens, such as estriol, do not produce the same dose-response curves, as do

TABLE 11-2 Mammalian Estrogen Biologic Assays

Assay	Advantages	Disadvantages	Reference
Vaginal cornification	Specificity; can detect proestrogens; multiple routes of exposure; in vivo pharmacokinetics, including bioaccumulation and repair; animals can be reused	Not for large-scale screening; requires 3 d or more; typically uses immature animals or ovariectomized adult animals	Allen and Doisy 1923
Vaginal epithelial cell proliferation and tetrazolium reduction	Specificity; can detect proestrogens; multiple routes of exposure; in vivo pharmacokinetics, including bioaccumulation and repair	Not for large-scale screening; labor intensive; typically uses ovariectomized adult animals; requires excision of the vagina for histologic examination	Martin and Claringbold 1958; Martin 1960
Vaginal opening	Can detect proestrogens; multiple routes of exposure; in vivo pharmacokinetics, including bioaccumulation and repair	Lacks specificity; not for large-scale screening; labor and time intensive; uses immature animals	Allen and Doisy 1924; Edgren et al. 1966
Vaginotrophic response	Can detect proestrogens; multiple routes of exposure; in vivo pharmacokinetics, including bioaccumulation and repair	Lacks specificity; not for large-scale screening; requires 3 d; typically uses immature animals or ovariectomized adult animals; requires excision of the vagina for weighing	Folman and Pope 1966
Uterine fluid imbibition	Simple, rapid response; can detect proestrogens; multiple routes of exposure; in vivo pharmacokinetics, including bioaccumulation and repair	Not for large-scale screening; uses immature animals; requires excision of the uterus for weighing and histologic examination; no graded response	Astwood 1938
Uterotrophic response	Sensitive; can detect proestrogens; multiple routes of exposure; in vivo pharmacokinetics, including bioaccumulation and repair	Lacks specificity; not for large-scale screening; requires 3 d; typically uses immature animals or ovariectomized adult animals; requires excision of the uterus for weighing	Bulbring and Burn 1935; Dorfman et al. 1936; Lauson et al. 1939; Evans et al. 1941; Rubin et al. 1951

Uterine glycogen deposition	Simple, rapid response; can detect proestrogens; multiple routes of exposure; in vivo pharmacokinetics, including bioaccumulation and repair	Not for large-scale screening; uses immature animals; requires excision of the uterus for biochemical analysis; specificity unknown	Bitman and Cecil 1970
Uterine estrogen-withdrawal bleeding	Can detect proestrogens; multiple routes of exposure; in vivo pharmacokinetics, including bioaccumulation and repair; and animals can be reused	Lacks specificity; not for large-scale screening; costly; typically uses ovariectomized rhesus monkeys; possible confounding by compounds with extremely long half-lives	Eckstein et al. 1952; Schane et al. 1972; Kassis et al. 1984; Tiemann and Tuchscherer 1995

SOURCE: Adapted from Reel et al. 1996.

such long-acting estrogens as diethylstilbestrol (DES), estradiol, and estrone. Weight increases are measured, and cell proliferation and hypertrophy are confirmed by histopathology. Although organ-weight responses are comparable for the vagina and uterus in these tests, vaginotrophic activity has not been used often as a quantitative end point (Folman and Pope 1966), because it is more difficult to excise and weigh vaginal tissue than uterine tissue. Estrogens also stimulate biochemical responses in the reproductive tract. For example, estrogens stimulate uterine glycogen concentrations within 6 hr of administration, and maximal concentrations are achieved within 24-48 hr of a single treatment (Galand et al. 1987).

BIOLOGIC MARKERS OF EXPOSURE AND EFFECT

Several functional biologic markers have been used or could be used to monitor exposures to HAAs in wildlife and human populations. Functional biologic markers include population-level responses, changes in secondary-sex characteristics, changes in concentrations of plasma steroid hormones, ex vivo responses, changes in enzyme activity, histologic changes in endocrine-responsive tissues, vitellogenin response, and zona-radiata-protein response.

Population-Level Responses and Secondary Sex Characteristics

There is no specific end point or group of end points that can be used exclusively to monitor exposure to HAAs and the effects of HAAs. Organisms are simultaneously exposed to many chemical stressors, and for many conditions, it is difficult to determine a single causative agent or group of agents (Ludwig et al. 1993). Biochemical markers of exposure are useful as an early-warning system, because they change before histologic, organism, or population-level effects are observed. Unfortunately, many of these biochemical markers are fairly specific to individual compounds or at least to classes of compounds.

In wildlife monitoring, a tiered approach has been used. In general, population-level responses are monitored, and if the populations are reproducing successfully it is inferred that they are healthy. If adverse effects are observed, then a combination of biologic and instrumental chemical techniques is often applied to determine the cause (Giesy et al. 1994a).

Because monitoring nonmammalian vertebrates for potential effects of HAAs is complicated by the limited information about what constitutes normal endocrine function for many species, surrogate species are used for laboratory testing and for field monitoring exposure to HAAs. Secondary sexual characteristics have been suggested as possible functional biologic markers of exposure to compounds that affect sex steroid hormones. These markers are best observed in cold-blooded vertebrates, such as fish and reptiles, and they include the size and

shape of sex organs, as exemplified in studies with male fish (Howell et al. 1980) and alligators (Guillette et al. 1995b).

Secondary sexual characteristics are greatly pronounced in some species, such as the fathead minnow (*Pimephales promelas*). Males have dark coloration and a body shape that is very different from that of females. In addition, the male has a fat pad on the top of its head and breeding tubercles on the snout. These characteristics can be altered by exposure to estrogen (estradiol). When males were exposed to 2 nM of estradiol in the water for 5 wk, the size of the breeding tubercles and fat pads was decreased (Miles-Richardson et al. in press). Changes in secondary sexual characteristics occurred at exposures that also caused significant histologic effects but at concentrations that were less than those required to cause significant decreases in fecundity. Thus, it is thought that such secondary characteristics could be used as indicators of exposure to some HAAs.

Other adverse effects on secondary sexual characteristics attributed to exposure to HAAs have been seen in mammals (Facemire et al. 1995), fish (Bortone and Davis 1994; Purdom et al. 1994; Harries et al. 1996), birds (Fry and Toone 1981; Fry et al. 1987), amphibians (Hayes 1997), and reptiles (Guillette et al. 1994). They include masculinization of females, feminization of males, deformities, and altered behavior (Colborn and Clement 1992; Giesy et al. 1994b). See Chapter 5 for more detail on these studies.

Compromised gonadal development in fish has been attributed to exposure to HAAs, primarily environmental estrogens (see Chapter 5). In the United Kingdom, a relatively high incidence of intersexing has been observed in fish living in the vicinity of wastewater treatment plants (Purdom et al. 1994). The gonad histologic effects were thought to be estrogenic in nature because vitellogenin was induced in males of the roach (*Rutilis rutilis*). Subsequent studies with caged rainbow trout (*Oncorhynchus mykiss*) indicated that indeed the compounds causing the effects were environmental estrogens (Jobling et al. 1996). It is believed that the most likely cause of the observed effects in these fish were steroidal estrogens from domestic sewage and alkylphenol ethoxylates from industrial effluent (R. White et al. 1994; Jobling et al. 1995; Nimrod and Benson 1996).

Histologic and Biochemical Responses

Exposure of animals to HAAs can result in pathologic tissue changes that can be observed histologically. For example, exposure of fathead minnows (*Pimephales promelas*) to 2 nM of estradiol causes histologic and ultrastructural changes that are related to impaired reproductive performance as seen in seminiferous tubules, hypertrophy and hyperplasia of Sertoli cells, and degeneration of spermatozoa (Miles-Richardson et al. in press). In females in the same study, the relative proportions of oocytes were altered to more primary cells and fewer graffian cells. These histologic effects were observed at exposures that did not

cause any induction of vitellogenin or decrease in the number of viable eggs produced.

Steroid hormonal concentrations in blood also could signal exposure to HAAs. Several pathways are possible, including inhibition or induction of key enzymes in steroidogenesis and in ovo exposure to HAAs during critical periods that results in abnormal steroidogenesis in offspring (Guillette et al. 1994).

Vitellogenin, a glycolipophosphoprotein synthesized by the liver in response to estrogen stimulation, is the precursor of egg yolk in oviparous animals, such as fish, birds, and crocodilians (Korsgaard and Petersen 1979). The term vitellogenin is applied to all such proteins, even though the exact structure can vary among species.

Vitellogenin is a good biologic marker of exposure to estrogenic substances because it is regulated by the ER, it is a plasma protein, and it can be readily detected and quantitated in plasma samples. Although exogenous estrogen agonists have minimal effects on plasma vitellogenin in female fish, male fish express low to nondetectable concentrations of the protein, which is readily induced by estrogenic compounds and secreted into plasma. Exposure to environmental estrogens has been shown to induce production of vitellogenin in the blood plasma of male fish (Jobling et al. 1995). Although it is not known whether such production has any adverse effect, measuring plasma vitellogenin does provide a useful biologic marker of exposure.

At least one study posited a connection between reproductive outcome and serum vitellogenin concentration (Kramer et al. 1997). The results suggest that induction in male fish is inversely correlated with the number of viable eggs produced by pairs of fish.

Based on tests with nonylphenol and effluent from an oil refinery treatment plant, there is some evidence that the zona-radiata-protein response could be as sensitive as the vitellogenin response in detecting exposure to estrogenic HAAs (Arukwe et al. 1997) and that it could be a more useful biologic marker for hormonal activity because critical population parameters, such as offspring survival and recruitment, are more directly affected. However, the advantages of the zona-radiata-protein as a biologic marker need to be further assessed through long-term exposure studies of fish exposed to low concentrations of xenoestrogens.

INSTRUMENTAL CHEMICAL TECHNIQUES

Hormonally active agents can occur everywhere in the environment, but the two primary vectors of exposure for wildlife and humans are water and food. The presence of known HAAs can be determined either by directly measuring concentrations of identifiable compounds or by screening for a range of possible HAAs. For compounds that have an established reference dose (RfD), a hazard quotient (HQ) can be calculated to determine whether adverse effects would be expected. The HQ is the ratio of the RfD to the measured concentration. The

RfD can be tissue-specific or dietary, and it can be related to concentrations in abiotic matrices by using appropriate transfer coefficients (Starodub et al. 1996).

In other cases, a combination of functional screening assays and instrumental analyses is applied in what has been defined as bioassay-directed fractionation and identification or toxicant identification and evaluation (TIE). In general, the process is an iterative process that uses fractionation methods and functional assays. Typically, the abiotic or biotic matrix of interest is separated into fractions based on polarity and molecular size. Functional assays are used to test the fractions for hormonal activity. Positive samples are further fractionated with instrumental methods, such as high-pressure liquid chromatography with both fluorescent and ultraviolet-visible detection, gas chromatography with flame-ionization detection, electron-capture detection, or mass-selective detection. Those methods are used repeatedly until the structure of the estrogenic compounds in the active fractions has been determined. Reference standards are used to quantify the mass of material in the sample. Relative potency factors (RPFs) are derived, and total equivalents are determined by multiplying the RPFs by the molar concentrations of the compound in the sample. Then, the predicted and measured activity in the assay are compared in a mass balance of potency. If the values are unequal, selective isomolar additions and selective removal of compounds can be used to confirm the presence or absence of interactions or unidentified compounds. These methods are repeated until all the active compounds are identified. Normally, the identity of the compound is confirmed by several instrumental techniques. A similar protocol has been proposed for use to separate the relative contributions of endogenous and exogenous hormones in plasma (Sonnenschein et al. 1995; Soto et al. 1997).

SUMMARY AND CONCLUSIONS

There are no generally accepted, validated methods to screen for or monitor exposure to HAAs, largely because the endocrine system is so complex. Relying on any single screening or monitoring method could result in uncertainty in the risk-assessment process and encourage the use of inappropriate or arbitrary safety factors. The use of simple models or screening methods can result in false-positive and false-negative results (Patlak 1996). Thus, efforts have been made to design a battery of simple tests that are based on different mechanisms of action or that are designed to assess the effects of activation or deactivation or the effects of accessory factors (Shelby et al. 1996). The design of such a battery should account for which mechanisms of action should be included, and it should consider the sensitivity and accuracy of the assays. In any case, it should always be recalled that a host of compounds with no hormonal activity can disrupt reproductive and other organismic functions.

RECOMMENDATIONS

On the basis of its evaluation of the available data on screening for and monitoring HAAs, the committee recommends the following:

—A battery of short-term assays should be developed for rapid and inexpensive screening for putative HAAs. The assays should detect diverse responses that depend on hormone receptors, should detect other indirect responses, and should be readily adapted for use in multiple laboratories.

—Short-term assays should be validated, replicated, and deployed in a rational fashion. Investigations should also be conducted to determine whether short-term assays predict in vivo toxicity.

—Some potential biomarkers of exposure to HAAs in wildlife and humans are available and should be applied. Additional biomarkers should be developed and validated before application. In particular, assays should be developed that screen for embryonic and fetal events (markers) that predict long-term, delayed effects.

—Species- and tissue-specific effects resulting from exposure to environmental HAAs need to be investigated further.

—Differences in response to HAAs between adults and developing fetuses need to be investigated with regard to the possibility of unique effects due to exposure during critical periods in development when genetic imprinting is occurring.

—Wildlife can serve as environmental sentinels. Populations known to be exposed to HAAs should be monitored for both obvious and subtle responses to exposures. Studies of caged, pinioned, or telemetered animals could provide information about the location, duration, and magnitude of exposure, which could be used to interpret the results of field studies.

—Dose-response characteristics of recognized actions of various HAAs should be further investigated in in vitro and in vivo studies using concentrations found in the environment.

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APPENDIXES

A

Reproductive Effects Caused by Diethylstilbesterol

MUCH OF THE CONCEPTUAL BACKGROUND for the investigation of the actions of hormonally active agents (HAAs) is based on results of studies on the actions of diethylstilbesterol (DES), a potent synthetic estrogen (see Chapter 1). Because this compound is not an environmental toxicant and because to date, no known environmental toxicant has been demonstrated to be more potent than DES, its actions are not discussed extensively in the corpus of this report. Nevertheless, because some workers in the field believe that DES is an important model for the effects of other HAAs, the relevant aspects of the actions of this compound are discussed here.

There is extensive literature concerning the long-term effects of in utero exposure of humans to DES (Herbst and Bern 1981; Takasugi and Bern 1988; Mittendorf 1995) and of fetal and neonatal exposure in other animals (Vannier and Raynaud 1980; Bern et al. 1987; Brody and Cunha 1989; Newbold 1995). There is also evidence that developmental exposure to DES can alter the immune-system functioning of laboratory animals (Kalland et al. 1979; Kalland and Forsberg 1980, 1981; Ways et al. 1980; Blair 1981; Holsapple et al. 1983; Luster et al. 1984; Pung et al. 1984, 1985) and humans (Ways et al. 1987; Noller et al. 1988; Blair 1992). Effects on other tissues, such as bone, also have been noted (Migliaccio et al. 1992). However, the major concern has focused on prenatal exposure to DES and its effects on the reproductive system.

Table A-1 lists the various female and male reproductive-tract abnormalities in humans and rodents exposed prenatally to DES. Studies show that exposure to DES during the critical period of organogenesis can profoundly disturb differentiation of the reproductive organs. Some of the effects are not observed until adulthood, demonstrating the latent developmental effects of exposure to this potent estrogen.

TABLE A-1 Reproductive Tract Abnormalities in Humans and Rodents Exposed Prenatally to Diethylstilbestrol (DES)

Abnormality	Organ	Study	Details	Reference
Females				
Cancer	Vagina and cervix	Human	Clear cell adenocarcinoma	IARC 1979
		Rodent	Adenocarcinoma	McLachlan 1979; Newbold and McLachlan 1982
	Ovary	Human	Germ Cell Cancer	Walker et al. 1988
		Rodent	Not reported	
	Breast	Human	No increased risk of breast cancer observed	Hatch et al. 1998
		Rodent	Not reported	
Other genital tract changes	Uterus	Human	T-shaped; hypoplasia	Haney et al. 1979; Kaufman et al. 1980, 1986; Mittendorf 1995
		Rodent	Decreased muscle development; hyperplasia followed by hypoplasia	Medlock et al. 1988; Brody and Cunha 1989; Wordinger et al. 1991
	Cervix	Human	Adenosis; ectropion; ridging; hooding; incompetence	Herbst et al. 1972; Scully et al. 1974; Sherman et al. 1974; Sandberg 1976; Poskanzer and Herbst 1977; Kaufman and Adam 1978; Robboy et al. 1979
		Rodent	NA	
	Ovary	Human	Parovarian cysts	DeCherney et al. 1981
		Rodent	Intra and parovarian cysts	Newbold et al. 1983
	Oviduct	Human	Withered fimbria	DeCherney et al. 1981; Robboy et al. 1982

		Rodent	Developmental arrest	Newbold et al. 1983
	Vagina	Human	Adenosis; ridging, epithelial changes	Herbst et al. 1972; Scully et al. 1974; Sherman et al. 1974; Sandberg 1976; Poskanzer and Herbst 1977; Kaufman and Adam 1978; Johnson et al. 1979; Robboy et al. 1979
		Rodent	Adenosis Lesions	Newbold and McLachlan 1982; Bern et al. 1987
Pregnancy-related changes		Human	Infertility; ectopic pregnancy; premature delivery; spontaneous abortion	Barnes et al. 1980; Cousins et al. 1980; Kaufman et al. 1980; Herbst and Bern 1981; Mangan et al. 1982; Stillman 1982; Thorp et al. 1990
		Rodent	Infertility, abortion, stillbirths, malformations	Halling and Forsberg 1992; Walker 1983
Males[a]				
Cancer	Testis	Human	Inconsistent results	Henderson et al. 1979; Schottenfeld et al. 1980; Depue et al. 1983; Brown et al. 1986; Gershman and Stolley 1988
		Rodent	Adenocarcinoma of the rete testes, interstitial cell carcinoma	Newbold et al. 1985, 1987
	Prostate	Human	No data available	
		Rodent	Squamous cell of dorsolateral prostate	Arai et al. 1978
Other genital tract changes	Penis	Human	Reduced size; hypospadias	Gill et al. 1976, 1979; Henderson et al. 1976; Wilcox et al. 1995
	Testis	Human	Cryptorchidism; hypertrophy; capsular induration; epididymal cysts	Gill et al. 1976, 1979; Rothman and Louik 1978; Depue 1984; Newbold 1995; Wilcox et al. 1995

(table continues)

TABLE A-1 Continued

Abnormality	Organ	Study	Details	Reference
		Rodent	Cryptorchidism Epididymal cysts	Bullock et al. 1988; McLachlan et al. 1975
	Prostate	Human	Hyperplasia and metaplasia of the prostatic ducts	Driscoll and Taylor 1980; Blacklock 1983
		Rodent	Abnormal development; squamous metaplasia of prostatic and coagulating gland ductal epithelium	McLachlan et al. 1975; Turner et al. 1989; Prins 1992; Pylkkanen et al. 1993; vom Saal et al. 1997
Fertility-related changes		Human	Impaired semen quality and sperm concentration; impaired fertility inconsistent	Gill et al. 1976, 1979; Andonian and Kessler 1979; Leary et al. 1984; Shy et al. 1984; Newbold 1995; Wilcox et al. 1995
		Rodent	Impaired semen quality and sperm concentration; impaired fertility	McLachlan 1981

[a] Details of some of these studies are provided in Table A-4.

TABLE A-2 Incidence of Adverse Pregnancy Outcomes in DES-Exposed Daughters and Estimates of Their Relative Risk[a]

Outcome	Incidence in Controls	Incidence in DES Daughters			Estimate of Relative Risk[b] (95% Confidence Interval)	
		Abnormal DES[c]				
		Vagina–Cervix	Uterus	All DES	Abnormal DES[c]	All DES
Ectopic pregnancy	0.01	0.063	0.076	0.044	13.5 (2.1, 84.7)	8.6 (3.4, 21.9)
Premature live birth	0.02	0.75	0.38	0.13	9.6 (4.0, 23.4)	4.7 (2.8, 7.9)
Spontaneous abortion	0.13	0.19	0.36	0.23	2.6 (1.8, 3.8)	1.8 (1.5, 2.2)
Not full-term birth[d]	0.15		0.67	0.41	4.9 (3.1, 7.7)	2.7 (2.2, 3.0)

[a] Based on controlled studies by Herbst et al. (1980, 1981); Barnes et al. (1980); Kaufman et al. (1980); Cousins (1980); Mangan et al. (1982); Thorp et al. (1990).

[b] Mantel-Haenzel estimate of relative risk; Robins-Greenland estimate of 95% confidence interval.

[c] DES-associated abnormality.

[d] Includes ectopic pregnancy, premature birth, and spontaneous abortion.

SOURCES: Adapted from Swan 1992 and Stillman 1982.

Table A-2 provides estimates of adverse pregnancy outcomes in DES-exposed daughters. Because DES was used to treat women with histories of reproductive difficulties, it might be expected that their daughters also would have high-risk pregnancies independent of DES exposure. However, Barnes et al. (1980) has shown that the incidence of these unfavorable outcomes in DES daughters is not related to the obstetric history of the mothers. In fact, as shown in this table, the incidence is related to the presence of genital-tract abnormalities, which are in turn related to the gestational age of first exposure to DES.

Table A-3 presents case-control studies of testicular cancer in men in relation to prenatal exposure to DES and other hormones. Exposure assessment for these studies was problematic because none of the study protocols restricted exposure to the critical period of testicular development, and all combined prenatal exposure to all prenatal hormones, rather than to DES alone. Because prenatal DES exposure occurred in about 1% of pregnancies, the power of these studies to isolate a DES effect is limited.

Table A-4 shows the effects of exposure to DES on sperm concentration and on abnormalities of the male reproductive system.

Table A-5 presents observed effects on the mature reproductive system in workers exposed to DES and DES-like compounds.

TABLE A-3 Case–Control Studies of Testicular Cancer, Relation to Prenatal Exposure to DES and Other Hormones

Cases	Controls	Relative Risk	Reference
78	78	5.0[a] (p = .11) 4.3[b] (p = .01)	Henderson et al. (1979)
190[c]	166[d] 143[e]	1.8[c] (p = .20) 2.0[c] (p = .17)	Schottenfeld et al. (1980)
108	108	8.0[f] (p = .02)	Depue et al. (1983)[g]
225	213	0.8[h] (not significant)	Brown et al. (1986)
79	79	2 DES-exposed cases vs. 0 DES-exposed controls[c] (not significant)	Gershman and Stolley (1988)

[a] Hormone treatment not further specified.
[b] Hormone treatment for excessive nausea.
[c] Drug use for bleeding, spotting, and/or threatened abortion (DES, other hormones, or unknown).
[d] Hospital.
[e] Neighborhood.
[f] Exogenous hormones during first trimester of index pregnancy.
[g] Continuation of Henderson et al. 1979.
[h] Exogenous hormones during the index pregnancy.

TABLE A-4 Effects of DES on Abnormalities of the Male Reproductive System and Sperm Concentration

Exposed	Unexposed	Urogenital Abnormalities (Other Than Varicocele)	Cryptochidism	Impaired Sperm Concentration	Sperm count (10^6/mL)	Reference
163	168	25% vs. 6.5%[a] $p < 0.0005$		28% vs. 0%[b] $p < 0.05$		Gill et al. (1976)[c]
225	111	24.4% vs. 15.3%[d]	3% vs. 1%[e]			Henderson et al. (1976)
24	24	13% vs. 8% not significant		17% vs. 20%[b] not significant		Andonian and Kessler (1979)
307	308	32% vs. 7.8% $p < 0.0005$	17% vs. 1% $p < .005$	18% vs. 8%[b] $p < 0.05$	91 vs. 115 $p < 0.05$	Gill et al. (1979)
31	28	$p < 0.001$[f]				Driscoll and Taylor (1980)[g]
265	274	Not significant		12% vs. 15%[b] not significant		Leary et al. (1984)
51	29	35% vs. 4% $p = 0.0006$	8% vs. 0% $p = 0.07$	21% vs. 0%[h] $p < 0.02$	74 vs. 77 not significant	Shy et al. (1984)
253	241	15% vs. 5% $p < 0.01$[i]				Wilcox et al. (1995)[c]

[a] Epididymal cycts, hypertrophic testis, capsular induration, hypoplastic penis.
[b] Severely pathologic Eliasson score (>10).
[c] Dieckmann cohort.
[d] Problems passing urine ($p = .0006$) and penile stenosis or hypospadias ($p = .034$).
[e] Published in Cosgrove et al. 1977 (same population).
[f] For each of hypertrophy and squamous metaplasia of the prostatic utricle; high ratio of Leydig cells to spermatogenic cells in the testis.
[g] Autopsy findings in male perinates.
[h] Poor forward progression.
[i] Significantly higher rate of abnormalities among men exposed before week 11 of gestation ($p < .05$).

TABLE A-5 Effects of Occupational Exposure to DES or DES-like Compounds on the Mature Reproductive System

Compound	Sex of Workers	Observed Effects	Reference
DES	Male	Gynecomastia, decreased libido, decreased genital size	Shmunes and Burton 1981
4,4´-diaminostilbene-2,2´-disulfonic acid[a]	Male	Decreased total circulating testosterone	Quinn et al. 1990
	Male	Decreased total circulating testosterone, decreased libido, increased impotence	Grajewski et al. 1996
Estrogens	Females	Increased incidence of spontaneous abortion	Taskinen et al. 1986

[a] Stilbene derivative (DAS: CAS 81-11-8), similar in structure to DES.

B
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ADDENDUM

Endocrine Disruptor Screening and Testing Advisory Committee

THE 1996 FOOD QUALITY AND PROTECTION ACT (FQPA) and the Safe Drinking Water Act (SDWA) mandated that the U.S. Environmental Protection Agency (EPA) develop a screening and testing strategy for endocrine disruptors by August 1998 and to implement the plan by August 1999. In response to this mandate, EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to advise EPA on the screening and testing of pesticides and chemicals for their potential to disrupt the endocrine system. EDSTAC is composed of representatives from several federal agencies, industry, academia, and environmental groups. EDSTAC has held 10 public meetings to develop a screening and testing strategy for endocrine disruptors. A draft of EDSTAC's final report to EPA was released in August 1998. Below is a summary of the major recommendations in that report and discussion of how those recommendations compare with the recommendations of the committee.

SUMMARY OF EDSTAC'S RECOMMENDATIONS

The charge to EDSTAC was to develop recommendations for a screening and testing program for endocrine-disrupting chemicals. EDSTAC interpreted this charge to include the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act, the Toxic Substances Control Act, and the Federal Food, Drug, and Cosmetic Act, in addition to the 1996 FQPA and SDWA provisions. Because these acts require testing on a variety of chemicals for human health and ecological effects, EDSTAC recommends that the scope of EPA's screening and testing program for endocrine disruptors should

- address human and ecological (wildlife) effects;
- examine effects on estrogen, androgen, and thyroid hormone-related processes and on other hormones and their processes as more data and assays become available; and
- evaluate endocrine-disrupting properties of chemical substances and common mixtures.

EDSTAC estimates that approximately 86,000 chemicals and mixtures will need to be considered for endocrine-disruptor screening and testing. To handle the immense number of chemicals in the program, EDSTAC recommends an initial sorting and prioritizing of the chemicals, followed by a tiered testing approach to detect endocrine-disrupting chemicals and quantify their effects. Those core elements of the approach are described below.

Initial Sorting

Initial sorting involves an evaluation of existing data on a chemical. On the basis of that information, a chemical is classified into one of four categories: (1) chemicals (primarily polymers) that have sufficient data indicating that they are not likely to interact with the estrogen, androgen, and thyroid (EAT) hormone systems and therefore require no further analysis (they will be placed in a "hold box"); (2) chemicals that have insufficient data and therefore require tier 1 screening for hormonal activity; (3) chemicals that have sufficient evidence of hormonal interaction and therefore require tier 2 testing; and (4) chemicals that have sufficient evidence of hormonal interaction and hormone-related effects and therefore require hazard assessment.

Priority Setting

Chemicals that are placed in the second category of having insufficient data will be prioritized for screening on the basis of exposure-related information, effects-related information, and statutory criteria, and then phased into the screening program.

Tier 1 Screening

Chemicals and mixtures will be tested in a battery of in vitro and in vivo screening assays for their potential to interact with the EAT hormonal systems. If the weight of evidence from the screening assays indicates hormonal interaction, tier 2 testing will be required. If the weight of evidence indicates no hormonal interaction, the chemical will be placed in the hold box. EDSTAC recommends the following battery of assays for tier 1 screening:

- In Vitro Assays
  —estrogen-receptor binding and reporter-gene assay
  —androgen-receptor binding and reporter-gene assay
  —steroidogenesis assay with minced testis
- In Vivo Assays
  —rodent 3-day uterotrophic assay
  —rodent 20-day pubertal female with thyroid
  —rodent 5-7-day Hershberger assay
  —frog metamorphosis assay
  —fish gonadal recrudescence assay

Because those assays are designed to work as a whole, EDSTAC believes that all of them are necessary for EPA to make accurate decisions about chemicals that are screened. EDSTAC recommends that the assays be standardized and validated before final adoption.

Tier 2 Testing

Chemicals and mixtures will be tested to determine whether they exhibit endocrine-mediated adverse effects and to identify, characterize, and quantify those effects for EAT hormones. If endocrine-mediated effects are found, the data will be used in a hazard assessment; further testing might also be required to determine whether the identified effects are endocrine mediated. If endocrine-mediated effects are not identified, the chemical is placed in the hold box. EDSTAC recommends the following battery of assays for tier 2 testing:

- two-generation mammalian reproductive toxicity study or a less comprehensive test (e.g., alternative mammalian reproductive test)
- avian reproduction test
- fish life-cycle test
- mysid life-cycle test
- amphibian development and reproduction test

EDSTAC recommends that those assays be standardized and validated before final incorporation into the screening and testing program. As with the tier 1 tests, tier 2 tests are designed to work as a whole. However, the performance of the entire battery with multiple generations might not always be necessary and EDSTAC has provided some guidance in the selection of tier 2 tests.

EDSTAC recognizes that questions have been raised about the adequacy of conventional toxicology study designs for assessment of endocrine-active substances, particularly with regard to low-dose selection and identification of no-observed-adverse-effect levels (NOAELs). To address the questions, EDSTAC

recommends that a research program be conducted to resolve the underlying uncertainties and controversy about these issues.

In addition to the major recommendations described above, EDSTAC has provided EPA with more detailed guidance on all aspects of the screening and testing program. The reader is referred to EDSTAC's June 17, 1998 report for details on those recommendations.

COMPARISON OF NRC AND EDSTAC SCREENING RECOMMENDATIONS

Chapter 11 details the NRC committee's evaluation of the available data and methods for screening HAAs. The major conclusion of the committee is that no generally accepted, validated methods are available to screen for HAAs, and therefore a battery of different assays evaluating different end points will be necessary for reliable determinations of hormonal activity. The committee made the following general recommendations for screening HAAs:

—A battery of short-term assays should be developed for rapid and inexpensive screening for putative HAAs. The assays should detect diverse responses that depend on hormone receptors, should detect other indirect responses, and should be readily adapted for use in multiple laboratories.

—Short-term assays should be validated, replicated, and deployed in a rational fashion. Investigations should also be conducted to determine whether short-term assays predict in vivo toxicity.

—Some potential biomarkers of exposure to HAAs in wildlife and humans are available and should be applied. Additional biomarkers should be developed and validated before application. In particular, assays should be developed that screen for embryonic and fetal events (markers) that predict long-term, delayed effects.

— Species- and tissue-specific effects resulting from exposure to environmental HAAs need to be investigated further.

— Differences in response to HAAs between adults and developing fetuses need to be investigated with regard to the possibility of unique effects due to exposure during critical periods in development when genetic imprinting is occurring.

—Wildlife can serve as environmental sentinels. Populations known to be exposed to HAAs should be monitored for both obvious and subtle responses to exposures. Studies of caged, pinioned, or telemetered animals could provide information about the location, duration, and magnitude of exposure, which could be used to interpret the results of field studies.

—Dose-response characteristics of recognized actions of various HAAs should be further investigated in in vitro and in vivo studies using concentrations found in the environment.

These recommendations are consistent with EDSTAC's more specific recommendations that recognize the need for multiple assays evaluating diverse hormonal responses. Similar to EDSTAC, the committee recommends that validated and standardized assays be used to assess the effects of HAAs on various species and tissues and include a consideration of developmentally critical or sensitive life stages. The committee also recognizes the need to investigate further the action of HAAs in vivo and in vitro using concentrations of HAAs found in the environment.

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