EPA/630/R-96/012
February 1997

SPECIAL REPORT ON ENVIRONMENTAL ENDOCRINE DISRUPTION: AN EFFECTS ASSESSMENT AND ANALYSIS

Prepared for the
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U.S. Environmental Protection Agency
Washington, D.C. 20460

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CONTENTS

LIST OF ACRONYMS/ABBREVIATIONS

α-NE	alpha-noradrenergic
Ah	aryl hydrocarbon
AIS	androgen insensitivity syndrome
AMH	anti-Mullerian hormone
APE	alkylphenol-polyethoxylates
AR	androgen receptor
BKME	bleached Kraft mill exposure
cAMP	3', 5' cyclic AMP
ChAT	choline acetyl transferase
CNS	central nervous system
DDD	tetrachlorodiphenylethane
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DES	diethylstilbestrol
DHT	dihydrotestosterone
E/T ratio	estradiol/testosterone ratio
ETU	ethylene thiourea
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
hCG	human chorionic gonadotropin
LH	luteinizing hormone
N-OH-DMAB	N-hydroxy-3,2'-dimethyl-4-amino biphenyl
NOEL	no observed effects level
PAHs	polycyclic aromatic hydrocarbons
PCBs	polychlorinated biphenyls
PCDD	polychlorinated dibenzodioxin
PTU	propylthiouracil
Q*	carcinogenic potency factor
RfD	reference dose
SHBG	sex/steroid hormone-binding globulin
T_3	triiodothyronine
T_4	thyroxine
TBG	thyroxine-binding globulin
TBT	tributyltin
TCDD	tetrachlorodibenzo-p-dioxin
TCDF	2,3,7,8-tetrachlorodibenzo-furan
TEBG	testosterone-estrogen-binding globulin
THC	tetrahydrocannabinol
TSD	temperature-dependent sexual determination
TSH	thyroid-stimulating hormone
UV	ultraviolet

PREFACE

The U.S. Environmental Protection Agency (EPA) Risk Assessment Forum was established to promote scientific consensus on risk assessment issues and to ensure that this consensus is incorporated into appropriate risk assessment guidance. To accomplish this, the Risk Assessment Forum assembles experts throughout EPA in a formal process to study and report on these issues from an Agencywide perspective. For major risk assessment activities, the Risk Assessment Forum has established Technical Panels to conduct scientific reviews and analyses. Members are chosen to assure that necessary technical expertise is available.

Recently, a number of ecologists, epidemiologists, endocrinologists, and toxicologists have called attention to the potential hazardous effects that estrogenlike and antiandrogenic chemicals and certain other environmental chemicals may have on human health and ecological well-being. A hypothesis has been proposed that certain chemicals may disrupt the endocrine system. These chemicals have been called "endocrine disruptors" because they are thought to mimic natural hormones, inhibit the action of hormones, or alter the normal regulatory function of the immune, nervous, and endocrine systems. Possible human health endpoints affected by these agents include breast cancer and endometriosis in women, testicular and prostate cancers in men, abnormal sexual development, reduced male fertility, alteration in pituitary and thyroid gland functions, immune suppression, and neurobehavioral effects.

In addition to potential human health effects, reports have accumulated that many chemicals released into the environment can disrupt normal endocrine function in a variety of aquatic life and wildlife. Some of the deleterious effects observed in animals have been attributed to some persistent organic chemicals such as polychlorinated biphenyls, DDT (dichlorodiphenyl-trichloroethane), dioxin, and some pesticides. Adverse effects include abnormal thyroid function and development in fish and birds; decreased fertility in shellfish, fish, birds, and mammals; decreased hatching success in fish, birds, and reptiles; demasculinization and feminization of fish, birds, reptiles, and mammals; defeminization and masculinization of gastropods, fish, and birds; decreased offspring survival; and alteration of immune and behavioral function in birds and mammals. It has been proposed that the above adverse effects may be due to an endocrine disrupting mechanism.

The EPA has followed closely the recent reports dealing with the potential effects of environmental endocrine disruptors on human health and ecological well-being. EPA's Science Policy Council requested that the Risk Assessment Forum prepare a Technical Panel report that would provide an overview of the current state of the science relative to endocrine disruption. It is intended that this report serve as an interim assessment to inform Agency risk assessors of the

major findings and uncertainties and to serve as a basis for a Science Policy Council position statement.

Science Policy Council's Interim Position

The EPA is aware of and concerned about information indicating the possibility of adverse impacts on human health and the environment associated with exposure to endocrine disruptors. At the present time, however, there is little knowledge of or agreement on the extent of the problem. Based on the current state of the science, the Agency does not consider endocrine disruption to be an adverse endpoint *per se*, but rather to be a mode or mechanism of action potentially leading to other outcomes, for example, carcinogenic, reproductive or developmental effects, routinely considered in reaching regulatory decisions. Evidence of endocrine disruption alone can influence priority setting for further testing and the assessment of the results of this testing could lead to regulatory action if adverse effects are shown to occur. This position could change as additional data become available on the mechanisms and role of endocrine disruptors.

The Agency thinks that identification of environmental agents that cause adverse effects as a result of endocrine disruption, as well as enhancement of our understanding of how these agents exert their effects, will improve the EPA's ability to reduce or prevent risks, particularly to children and vulnerable ecosystems. These considerations become increasingly important as we expand our risk assessment activities to incorporate a wider range of susceptible populations, multiple pathways of exposure, and mixtures of chemical substances.

Further research and testing are needed to address existing gaps in knowledge concerning the consequences of endocrine disruption. Such knowledge will reduce uncertainties in the assessment of hazard, exposure, and risk. The Agency is working with other federal agencies, as well as academic, international, and industry groups to expand the body of defensible and credible information and data on this issue. Several major activities are underway that address these needs. Some of these are listed below.

Examples of activities:

1. EPA is co-sponsoring the detailed review and interpretation of the existing literature on endocrine disruption currently underway at the National Academy of Sciences' National Research Council. This study is expected to be completed later this year;

2. EPA has developed and is implementing a multi-year endocrine disruptors research strategy;

3. EPA chairs the workgroup convened by the President's Office of Science and Technology Policy tasked to document and then coordinate research on endocrine disruptors

across the federal government. Also, this activity serves as the basis for pursuing coordination of research on an international level;

4. Under the mandates of the Food Quality Protection Act (FQPA) of 1996 and the 1996 amendments to the Safe Drinking Water Act (SDWA), EPA has established an advisory committee to assist in developing a screening and testing strategy for evaluating chemicals for their potential to cause effects via endocrine disruption. The FQPA requires that the strategy be developed and peer reviewed within two years, implemented during the third year, and that a progress report be submitted to the Congress by the end of the fourth year.

EPA continues to stay abreast of scientific developments and will take regulatory action whenever sound scientific information and prudent public policy dictate. We are currently committed to pursuing domestic and international opportunities for exposure/risk reduction related to endocrine disruptors.

I. EXECUTIVE SUMMARY

A. PURPOSE OF DOCUMENT AND AREAS CONSIDERED

This document provides an overview of the current state of the science relative to environmental endocrine disruption in humans, laboratory testing, and wildlife species. It is intended to serve as an *interim assessment and analysis of the environmental endocrine disruption hypothesis* until a more extensive exploration of environmental endocrine disruption can be completed by the National Academy of Sciences (NAS). The present document is *not* intended to address all of the endocrine glands that might be disrupted by environmental insult. Furthermore, the document does not address high occupational or accidental human exposures. Rather, this document focuses on those reports of adverse human and ecological effects where a common theme of endocrine system disruption by environmental agents has been hypothesized or demonstrated.

An environmental endocrine disruptor is defined as an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior. Background information is presented on the field of endocrinology, the nature of hormones, and potential sites for endocrine disruption, with specific examples of chemicals affecting these sites. An attempt is made to present objectively the issue of endocrine disruption, consider working hypotheses, offer opposing viewpoints, analyze the available information, and provide a reasonable assessment of the problem. Emphasis is placed on disruption of central nervous system-pituitary integration of hormonal and sexual behavioral activity, female and male reproductive system development and function, and thyroid function. In addition, the potential role of environmental endocrine disruption in the induction of breast, testicular, and prostate cancers, as well as endometriosis, is evaluated. The interrelationship of the endocrine and immune system is documented. Finally, some data gaps in our understanding of the environmental endocrine disruption issue are identified and a few future research needs are recommended. A research strategy dealing with this issue is being developed within EPA.

With respect to endocrine-related ecological effects, specific examples in the peer-reviewed literature are presented and discussed. For each topic area, data gaps and areas of uncertainty are discussed, conclusions are drawn, and general research needs are suggested.

B. GENERAL BACKGROUND

1. Nature of Hormones

Hormones are natural, secretory products of endocrine glands (ductless glands that discharge directly into the bloodstream). Hormones travel in the blood in very small concentrations and bind to specific cell sites called receptors in distant target tissues and organs, where they exert their effects on development, growth, and reproduction in addition to other bodily functions.

2. Role of the Endocrine System

The endocrine system is one of at least three important integrating and regulatory systems in humans and other animals. The other two are the nervous and immune systems. Hormones influence important regulatory, developmental, growth, and homeostatic mechanisms, such as reproductive structure and function; maintenance of normal levels of glucose and ions in blood; control of general body metabolism; blood pressure; and other glandular, muscle, and nervous system functions. Some of the major endocrine glands include the pituitary, thyroid, pancreas, adrenal, and the male and female gonads (testes and ovaries).

C. TECHNICAL PANEL'S MAJOR FINDINGS

1. Human Health Effects

a. Types of Effects

i. Female Reproductive System

A variety of chemicals have been shown to disrupt female reproductive function throughout the lifespan in laboratory animals and humans (e.g., diethylstilbestrol). These effects include the disruption of normal sexual differentiation, ovarian function (i.e., follicular growth, ovulation, corpus luteum formation and maintenance), fertilization, implantation, and pregnancy. Only a few agents are associated with direct interference with the endocrine reproductive axis. Examples are those with estrogenic activity or the potential to interact with the aryl hydrocarbon (Ah) receptor. Exposure to toxicants during development is of particular concern because many feedback mechanisms functioning in the adult are absent and adverse effects may be noted at doses lower than those observed in the adult. Although there are a limited number of endocrine-disrupting studies evaluating reproductive function in the female, it is important that each stage of the life cycle be examined thoroughly. Appropriate, validated in vitro and in vivo tests to determine the endocrine-disrupting potential of agents with clearly defined endpoints are needed. Additionally, studies that include multigenerational exposure should be conducted, followed up by time of exposure and dose level required for effect.

Endometriosis is a painful reproductive and immunologic disease of women characterized by aberrant location of uterine endometrial cells. It affects approximately 5 million women in the United States from 15 to 45 years of age and often causes infertility. The etiology of this disease is unknown. In a single study with a small number of animals, research has suggested a link between dioxin exposure and the development of endometriosis in rhesus monkeys. The severity of this lesion was dependent on the dose administered. Recently, a small pilot study to test the hypothesis that serum dioxin concentrations have an association with human endometriosis has been reported. No statistically significant correlations between disease severity and serum levels of halogenated aromatic hydrocarbons were found. These preliminary data, admittedly on a limited population, suggest that serum dioxin concentrations may not be related to human endometriosis. There is a need to develop and validate nonprimate laboratory animal endometriosis models for testing chemicals and xenobiotics. Several novel models for predicting potential causative agents of endometriosis are available.

Human breast cancer is a major health problem in the United States. While considerable information is available on risk factors for human breast cancer, the mechanisms of mammary gland carcinogenesis and the precise role played by chemical carcinogens, physical and biological agents, varied lifestyles, genetic susceptibility, and developmental exposures have yet to be elucidated. It has been hypothesized that exposure to organochlorines, some pesticides, and/or polyaromatic hydrocarbons might play a role in the etiology of mammary gland neoplasms via an endocrine disruption pathway, perhaps via an estrogen-mimetic route or alternate estrogen pathways. With respect to the possible role of hormone disruption by chemicals in the occurrence of breast cancer, there is a lack of sufficient evidence implicating organochlorines in this disease. Clearly, there is a need for research on the etiology of breast cancer. With respect to chemically induced breast cancer, there is the need to develop and validate both in vitro short-term tests and in vivo animal testing models for predicting human breast cancer risk following chemical insult. Finally, given the wealth of human epidemiologic data on human breast cancer, but limited specific agent exposure data, it is not possible to assign a specific chemical or physical cause and effect at this time. Further epidemiologic investigations to address the issue are needed as well as complementary mechanistic studies.

ii. Male Reproductive System

Convincing evidence exists in rodents that exposure to chemicals that have estrogenic activity, reduce androgen levels, or otherwise interfere with the action of androgen during development can cause male reproductive system abnormalities that include reduced sperm production capability and reproductive tract abnormalities. Results obtained from observation of men exposed to DES in utero demonstrate a limited potential of exogenous estrogens to disrupt

the reproductive system during development in human males as compared with that observed in rodents. Further intense research on the population exposed to DES might allow stratification of effects by timing and level of exposure. Apparently, no increased incidence of reproductive system cancer has been found in those men.

Controversy persists as to the allegation that human sperm production has decreased over the past 50 years. However, the firm data indicating an increase in human testicular cancer, as well as apparent occurrence of other plausibly related effects, support the concept that an adverse influence has occurred or still exists. Whether these effects in humans can be attributed to an endocrine disruption by environmental chemicals is unknown at present, and research into the cause(s) is needed. It is possible that the mechanism by which estrogenic chemicals impair development of the male reproductive system is via antiandrogenic properties rather than or in addition to activity related to estrogen receptor activation.

Leydig cell hyperplasia and tumors are a significant problem in testing with laboratory species. Participants at a workshop on the topic concluded that human incidence of the tumors is low relative to rodents and that not all modes of induction in test species are relevant to humans. However, occurrence of Leydig cell tumors in test species may be of concern with certain modes of action, provided the potential exists for sufficient exposure.

Testing for endocrine-disrupting potential of environmental chemicals should include the ability to detect antiandrogenic activity in addition to estrogenic activity. Testing also should be able to detect alteration in androgen receptor and Ah receptor function as reflected in genome expression.

Little is known about the causes of human prostatic cancer, but age, genetics, diet, endocrine status, and environmental risk factors have been proposed. With respect to the cause(s) of human prostate cancer, a single retrospective epidemiology study has linked a weak but statistically significant association between acres sprayed with herbicides and prostate cancer deaths. Furthermore, an occupational study of coke oven workers has found an association of coke oven emission with significant excess mortality from cancer of the prostate. Whether herbicide or polyaromatic hydrocarbon exposure contributes to the increasing incidence of human adenocarcinoma of the prostate and whether the mechanism is by way of an endocrine disruption remain to be determined. More research is required before assigning a specific endocrine disruption (or any other) mechanism as a specific cause of human prostate cancer.

iii. Hypothalamus and Pituitary

There are a number of ways that environmental agents may alter neuroendocrine function both during development and in the sexually mature organism. Exposure during development may be of particular concern because many of the feedback functions of the endocrine system are

not operational during this period, permanent changes in endocrine function may be induced at levels of exposure to a toxicant that may have no effect in the adult animal, and compounds that may be considered antiestrogenic in the adult (i.e., tamoxifen, dioxin) may act as estrogens in the developing organism. Similarly, exposure to such agents in the adult can modify the feedback of endogenous hormones as well as behavior (i.e., libido, appetite, aggression) of the individual. Because of the complex role that the central nervous system plays in regulating endocrine function, cells within the brain are a potential target for environmental chemicals that have no impact on steroid hormones directly but yet will lead to a disruption of endocrine function. There is a substantial need to better characterize the role of the brain and pituitary when evaluating suspected reproductive toxicants in both the male and female.

iv. Thyroid

Numerous environmental agents have been found to alter thyroid hormone levels (e.g., urea derivatives, polyhalogenated biphenyls, and chlorinated dibenzo-p-dioxins). Thyroid hormones are critical to normal growth and development; thus, developmental exposures may have lasting adverse effects.

b. Strengths and Weaknesses of the Data

The observation that humans have experienced increased incidences of developmental, reproductive, and carcinogenic effects, and the formulation of a working hypothesis that these adverse effects may be caused by environmental chemicals acting to disrupt the endocrine system that regulates these processes, is supported by observations of similar effects in aquatic and wildlife species. In other words, a common theme runs through both human and wildlife reports. The hypothesis also is strengthened by the fact that cancer and noncancer effects, at least with respect to published reports, are related in large part to reproductive structure and function (e.g., vaginal and breast cancer in women, testicular and prostatic cancers in men, endometriosis, cryptorchidism, sperm counts and quality, and infertility).

In contrast, the hypothesis that the reported increased incidence of human cancers and reproductive anomalies and infertility can be attributed to an endocrine disruption phenomenon is called into question by the following. First, secretion and elimination of hormones are highly regulated by the body, and mechanisms for controlling modest fluctuations of hormones are in place via negative feedback control of hormone concentrations. Therefore, minor increases of environmental hormones following dietary absorption and liver detoxification of these xenobiotics may be inconsequential in disrupting endocrine homeostasis. Second, low ambient concentrations of chemicals along with low affinity binding of purported xenobiotics to target receptors probably are insufficient to activate an adverse response in adults. Whether the fetus and the young are

capable of regulating minor changes to the endocrine milieu is uncertain. Finally, the data are not available for mixtures of chemicals that may be able to affect endocrine function. At the same time, in the case of environmental estrogens as endocrine disruptors, it is known that competition for binding sites by antiestrogens in the environment may moderate estrogenic effects of some chemicals. Clearly, more research to fill data gaps and remove the uncertainties in these unknowns is needed.

c. Conclusions

With few exceptions (e.g., DES), a causal relationship between exposure to a specific environmental agent and an adverse effect on human health operating via an endocrine disruption mechanism has not been established. However, in view of the Agency's concern that certain persistent chemicals might be responsible for some of the recently-reported reproductive, developmental, and carcinogenic effects operating through an endocrine disruption mechanism, new epidemiologic and laboratory testing studies could be undertaken to better define the scope of the problem. Short-term screening studies could be developed and validated in an effort to elucidate mechanisms. Biomarkers (indicators) of exposure could be defined and their concentrations related to degree of actual exposure. Studies of absorption, distribution, metabolism and elimination are essential for improving risk assessments by allowing extrapolation between species and assessing other routes of exposure. The reader is advised to refer to the report of the April 1995 endocrine disruptor workshop recommending specific high-priority research (Kavlock et al., 1996).

2. Ecological Effects

a. Types of Studies

A number of laboratory and field investigations have been reported that provide information from which the potential effects of certain chemicals on the normal endocrine function of invertebrates, fish, reptiles, birds, and mammals can be evaluated. Based on these studies, it has been suggested in the literature that both synthetic and naturally occurring compounds may have the potential to disrupt reproductive and developmental events associated with hormonally mediated processes. In some cases, compounds have been deliberately synthesized for their potential to disrupt endocrine systems. For example, several classes of insecticides have been developed to selectively disrupt the endocrine system of specific insect species without creating substantial risk to nontarget vertebrates due to direct toxic effects, although adverse responses in nontarget arthropods, especially crustaceans, have been observed. Certainly in most other instances, suspect synthetic compounds were either not intentionally designed to have biological activity or their primary mode of toxic action in a short-term exposure is not attributed to effects

on the endocrine system. Several examples, highlighted below, illustrate the range of information currently available for a wide spectrum of species.

A series of field and laboratory investigations with marine invertebrates suggest that tributyltin compounds, which are used as antifouling paints on ships, can have significant hormonal effects on some snail species at sublethal exposure concentrations. Through controlled dose-response studies, it appears that these compounds can induce irreversible induction of male sex characteristics on females (imposex), which can lead to sterility and reduced reproductive performance. In turn, field investigations in numerous locations around the world suggest this class of compounds may be responsible for localized reductions in specific snail populations. The possibility that other mollusks (e.g., oysters) could be sensitive to tributyltin compounds also raises ecological concerns, as does the fact that these compounds bioaccumulate in the food chain, leading to questions as to whether or not effects in fish, wildlife, or humans are possible.

A wide variety of compounds and environmental settings also have been associated with potential reproductive and developmental anomalies in fish. For example, hermaphroditic fish have been observed in rivers below sewage treatment plants, and masculinization, altered sexual development, and decreased fertility have been noted for some fish species near pulp and paper plant discharges. It is unclear from these studies, however, as to the extent to which these observations are associated with significant changes in population dynamics. In addition, it is generally unclear as to the primary causes of these perturbations, which could include synthetic chemicals as well as naturally occurring plant-derived compounds. However, correlative data, supported in some cases by controlled laboratory studies, suggest that alkyl phenol ethoxylates and their degradation products, chlorinated dibenzodioxins and difurans, and polychlorinated biphenyls (PCBs), among other compounds, could be contributing causative agents.

Perhaps the most fully documented example of putative ecological effects caused by a disruption of endocrine function has been reported for alligators in Lake Apopka, Florida. Through a series of detailed field and laboratory investigations, it appears very likely that a mixture of dicofol, dichlorodiphenyltrichloroethane (DDT), and dichlorodiphenyldichloroethylene (DDE) associated with a pesticide spill in 1980 is responsible for a variety of developmental effects that indicate a demasculinization of male alligators and "super-feminization" of females. In addition, the effects of the spill also have been reported to include detrimental effects on hatching success and population levels.

Instances of potential effects of chemicals on the endocrinology of warm-blooded wildlife also have been reported. For example, a variety of organochlorine insecticides have been implicated in eliciting feminization of male gull embryos and has led to the suggestion that these effects may contribute to locally observed population declines and skewed sex ratios in Western gulls in California and Herring gulls in the Great Lakes. Although numerous controlled laboratory

studies have been undertaken that demonstrate a variety of compounds can elicit hormonally mediated effects on reproduction and development in rodents, the establishment of credible cause-and-effect relationships in wild mammalian populations has not been reported in the scientific literature to date, although the extreme sensitivity of mink, seals, and related species to adverse reproductive effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and PCBs is well established.

b. Strengths and Weaknesses of the Data

Numerous effects in aquatic life and wildlife have been hypothesized to be elicited by chemicals that disrupt hormonally mediated events underlying reproduction and development. The strongest evidence available suggesting that specific compounds, or classes of compounds, could potentially affect the endocrinology of aquatic life and wildlife is typically associated with controlled laboratory investigations. However, although these suborganismal and organismal level studies help to establish a mechanistic potential for specific compounds, it is generally not clear if these effects would be observed in environmentally relevant exposure scenarios or to what extent changes in these in vitro and in vivo processes can reasonably be projected to cause declines in populations. In addition, while several well-designed investigations are reported in the literature that establish a sound mechanistic framework for specific effects, the amount of comparative interspecies data is limited. For example, there is comparatively little information available for amphibian species, and the majority of studies available for fish are restricted to teleosts (bony fish).

Several intensive field studies also have been reported that clearly document a wide variety of physiological abnormalities in invertebrates, fish, reptiles, birds, and mammals. In some instances, these abnormalities also have been observed within declining populations. Further, in many of these studies, trends in adverse effects have been correlated with environmental concentrations of synthetic and/or naturally occurring endocrine modifying chemicals. However, as with most uncontrolled field studies, it is difficult in most cases to establish clear cause-and-effect relationships.

c. Conclusions

A challenging goal in assessing the ecological risks of endocrine-disrupting chemicals will be to establish the likelihood of adverse effects on populations and communities of aquatic life and wildlife as a result of toxic effects observed within species of concern. Equally challenging is the need to elucidate cause-and-effect relationships for responses observed in the field, where numerous chemical and nonchemical stressors could be responsible, either alone or in combination. While numerous reports indicate a variety of compounds can modulate the

endocrine system and affect reproduction and development in invertebrates, fish, and wildlife, few examples are currently available that establish the extent to which these insults at the organismal level have, or could, result in adverse population responses. To date, the most credible examples illustrating significant population declines as a result of exposure to endocrine-disrupting chemicals have been reported for alligators in central Florida and some local populations of marine invertebrate species. Because endocrine-disrupting chemicals can elicit a variety of hormonal responses and adverse effects in the reproduction and development of organisms, it can quite reasonably be hypothesized that these compounds could cause population level impacts in additional species or in other ecosystems. Certainly, from a problem formulation perspective within an ecological risk assessment, chronic exposures to compounds that can selectively affect reproduction and development raises a reasonably straightforward concern over potential population effects. However, toxicological effects observed within organisms do not necessarily all have the same potential to impact populations nor should it be expected that these varied effects would elicit population responses at the same exposure levels. In summary, prospective ecological risk assessments for compounds known or suspected to disrupt the endocrinology of aquatic life and wildlife are confronted with the need to establish the significance of observations at the suborganismal and organismal levels in the context of population and community responses. An understanding of linkages between these levels of biological organization also is required to help establish mechanistically plausible cause-and-effect relationships in retrospective risk assessments.

Based on the toxic mechanisms associated with xenobiotics, the collection and interpretation of organismal-level responses associated with reproductive and developmental processes are needed to better predict and interpret changes in populations and communities of aquatic life and wildlife. Unfortunately, endpoints derived from typically employed bioassays, which are based on short-term exposures, probably are not appropriate for identifying most reproductive or developmental effects or forecasting changes at higher levels of biological organization. However, because of the mechanisms associated with these compounds, it is reasonable to assume that the implementation of new techniques or the modification of existing approaches can appropriately quantify suborganismal/organismal responses (i.e., measurement endpoints) that can be readily linked to models and measurements designed to quantify changes in population dynamics (i.e., assessment endpoints).

d. Agency Actions

While the potential role of endocrine-disrupting chemicals in eliciting adverse ecological effects has heightened the need to develop and implement a more systematic examination of long-term chemical exposures, EPA has long recognized the importance of this issue in ecological

risk assessments. For example, chemicals such as tributyltin, DDT, and PCBs have been banned and heavily regulated, in part due to their effects in aquatic life and wildlife following long-term exposures. In addition, the ongoing reassessment of the effects of 2,3,7,8-TCDD and related compounds on ecological resources was initiated because of concerns associated with reproductive and developmental effects in fish and wildlife.

3. Further Research

An increasing concern over persistent bioaccumulative chemicals and appropriate techniques to assess their toxicological and ecological effects is evidenced in the Office of Pollution, Prevention, and Toxics' ongoing efforts to assess high-production-volume industrial chemicals, the Office of Water's development of sediment quality criteria, and the focus of the Great Lakes Water Quality Initiative. In addition, the Office of Research and Development has published the results of two workshops held in 1995 that specifically addressed the issue of environmental endocrine disruption (Kavlock et al., 1996; Ankley et al., 1996). The findings from these workshops discuss a broad range of short-term and long-term research objectives that are relevant for both prospective and retrospective assessments. The research needs range from improved techniques for rapidly screening untested chemicals for endocrine-disrupting potential to improved approaches to quantify the extent of current exposures and effects of suspected compounds in human populations, as well as aquatic life and wildlife. For risk assessment needs, a research strategy is under way that clearly addresses the causal linkage of observations at the subcellular through organismal levels of biological organization to responses of populations and communities. Such a research program, which will incorporate both intramural and extramural researchers (a call for research proposals was issued by EPA in February 1996), has been developed to support human health and ecological risk assessments for agents that may operate via an endocrine disruption mechanism.

II. INTRODUCTION

The purpose of this document is to provide an overview of the current state of the science relative to environmental endocrine disruption. The report pays particular attention to peer-reviewed published reports of adverse health and ecological effects attributed to specific environmental agents and to information in the Agency's pesticide registration and toxic substances databases. The document identifies gaps in our understanding of mechanisms of action for agents that disrupt the endocrine and endocrine-supported systems. It analyzes and interprets current hypotheses and specifies some of the uncertainties in our knowledge. Finally, the document recommends some general research needs. This document is *not* intended to address all components of the endocrine system that might be disrupted by environmental insult. Rather, this document emphasizes those reports of adverse human and ecological reproductive, carcinogenic, neural, and immune effects where a common theme of endocrine disruption has been hypothesized.

A. GENERAL BACKGROUND

Investigators began expressing their concern for estrogenic effects of environmental xenobiotic chemicals more than 25 years ago (Bitman and Cecil, 1970; Nelson et al., 1978; McLachlan, 1980; McLachlan et al., 1984; McLachlan, 1985; Hertz, 1985; Richardson and Bowron, 1985). Within the past 4 years, this concern has become focused and intensified (Colborn and Clement, 1992; Colborn et al., 1993; Purdom et al., 1994; Rolland et al., 1995; McLachlan and Korach, 1995; Kavlock et al., 1996; Ankley et al., 1996). Attention has been called to the potential hazards that some chemicals may have on human health and ecological well-being (breast and reproductive tract cancers, reduced male fertility, abnormality in sexual development, etc.) (Birnbaum, 1994; Colborn et al., 1993; Davis et al., 1993; Kelce et al., 1994; Makela et al., 1994; Sharpe and Skakkebaek, 1993; Wolff et al., 1993; Davis and Bradlow, 1995; Colborn et al., 1996). There has been considerable controversy over the report (Carlsen et al., 1992) that human sperm counts have decreased over the past 50 years.

Clear evidence exists that in utero exposure to certain potent synthetic estrogens such as DES has an adverse reproductive effect in the sons (Gill et al., 1979) and daughters of women treated with DES during their pregnancy and that a rare adenocarcinoma of the vagina was seen some 20 years later in the daughters (Herbst et al., 1971). In female rats of the AEI strain, which has a low incidence of spontaneous mammary tumors, both prenatal and postnatal exposure to DES increased numbers of mammary tumors (Rothschild et al., 1987). Male rats treated from gestational day 14 to postnatal day 3 with the antiandrogenic fungicide vinclozolin exhibit varied reproductive dysfunction as adults (Gray et al., 1994).

Caged male rainbow trout exposed to effluent from 15 different sewage treatment facilities in the United Kingdom expressed elevated concentrations of vitellogenin, an estrogen-induced yolk protein precursor (Purdom et al., 1994). Furthermore, there is ample evidence that the pesticide DDT (1,1,1-trichloro-2,2-bis[4-chlorophenyl]ethane), now banned in this country, and its metabolites cause a dwindling bird population due to testicular feminization of male embryos leading to abnormal sex ratios of adult Western gulls in Southern California in the 1960s (Fry and Toone, 1981; Fry et al., 1987). More recently, the decline in birthrate and increasing male reproductive tract anomalies of alligators in Florida's Lake Apopka have been reported (Guillette et al., 1994).

For the past 25, EPA has been committed to the protection of human health and the environment and has ongoing research programs in these areas. The Agency has followed closely the recent reports dealing with environmental endocrine disruptors on human health and ecological well-being. EPA is particularly concerned with the possible role that xenobiotics, including endocrine disruptors, may have in the etiology of human cancers and adverse developmental, reproductive, immune, and neurological effects on human health. The Agency also is concerned with what possible adverse role these endocrine disruptors may have on growth and survival of animal species. Evidence for this concern is documented by the Office of Research and Development's (ORD's) ongoing research, a Risk Assessment Forum colloquium on environmental hormones held in April 1994, and two endocrine disruptor research needs workshops held in April and June 1995. Two reports, entitled "Research Needs for the Assessment of Health and Environmental Effects of Endocrine Disruptors: A Report of the U.S. EPA-Sponsored Workshop" (Kavlock et al., 1996) and "Development of a Research Strategy for Assessing the Ecological Risk of Endocrine Disruptors" (Ankley et al., 1996), have resulted from these meetings. In addition, an "ORD Research Plan for Endocrine Disruptors" has been written. Other Agency initiatives include a workshop on Leydig cell hyperplasia in the fall of 1995 (Clegg et al., 1996), the Office of Prevention, Pesticides, and Toxic Substances' revision of the developmental and two-generation reproductive toxicity test guidelines (for mammalian species), the EPA guidelines for reproductive toxicity risk assessment, the dioxin risk assessment document, the draft proposed guidelines for ecological risk assessment, and the new proposed carcinogenesis risk assessment guidelines.

The Agency is aware of three recent reports (two of them published) by European governments (United Kingdom, Denmark, and Germany) dealing with environmental endocrine disruption (Harrison et al., 1995; Toppari et al., 1995). An extensive exploration of environmental endocrine disruption is the subject of an NAS project supported by EPA, the Centers for Disease Control and Prevention, and the Department of the Interior (NAS, 1995).

B. HORMONES

Hormones are natural secretory products of endocrine glands and travel via the bloodstream to exert their effects at distant target tissues or organs. Chemically, hormones are glycoproteins, polypeptides, peptides, steroids, modified amino acids, catecholamines, prostaglandins, and retinoic acid. They are transported in blood at very low concentrations (ng or pg/ml, i.e., 10^{-9} or 10^{-12} g/ml) in the free state or attached to carrier proteins. They bind to specific cell surfaces or nuclear receptors and exert important regulatory, growth, or homeostatic effects. Steroid and thyroid hormones, bound to their protein receptors, regulate gene activity (expression) as DNA transcription factors; protein and peptide hormones function by transmitting a signal (intracellular second messenger) to regulate ion channels or enzymes. Some of the major endocrine glands include the hypothalamus, pituitary, thyroid, parathyroid, pancreas, adrenal, ovary, and testis. Other endocrine tissues include the placenta, liver, kidney, and cells throughout the gastrointestinal tract. The secreted hormones help regulate general body growth and metabolism, other endocrine organs, and reproductive function. Some target organs and tissues under endocrine control include the mammary glands, bone, muscle, the nervous system, and the male and female reproductive organs.

In addition to the classical hormones found in higher vertebrates, including humans, there are hormones in invertebrates (e.g., ecdysone) and plants (e.g., auxins). Consequently, when environmental endocrine disruptors mimic or interfere with the action of endogenous hormones, they have the potential of influencing human health and exerting significant ecological effects globally.

For the purpose of this document, paracrine (secretions stimulating adjacent tissues) and autocrine (secretions targeted to the cell that synthesized the secretion) factors will not be considered here because little information is available as to their disruption by environmental agents.

C. ENDOCRINE/HORMONE DISRUPTORS

An environmental endocrine or hormone disruptor may be defined as an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior. For the purpose of this document, the term "endocrine disruptor" will be used as synonymous with hormone disruptor. Of importance here is the concept that endocrine disruptors encompass more than just environmental estrogens and include any agent that adversely affects any aspect of the entire endocrine system. Endocrine disruptors are usually either natural products or synthetic chemicals that mimic, enhance (an agonist), or inhibit (an antagonist) the action of hormones. Under some circumstances, they may act as hypertrophic

(stimulatory) agents and tumor promoters. Dose, body burden, timing, and duration of exposure at critical periods of life are important considerations for assessing adverse effects of an endocrine disruptor. Effects may be reversible or irreversible, immediate (acute) or latent and not expressed for a period of time.

The endocrine system includes a number of central nervous system (CNS)-pituitary-target organ feedback pathways involved in regulating a multitude of bodily functions and maintaining homeostasis. As such, there are potentially several target organ sites at which a given environmental agent could disrupt endocrine function. Furthermore, because of the complexity of the cellular processes involved in hormonal communication, any of these loci could be involved mechanistically in a toxicant's endocrine-related effect. Thus, impaired hormonal control could occur as a consequence of altered hormone: synthesis, storage/release, transport/clearance, receptor recognition/binding, or post-receptor responses.

1. Altered Hormone Synthesis

A number of compounds possess the ability to inhibit the synthesis of various hormones. Some compounds inhibit specific enzymatic steps in the biosynthetic pathway of steroidogenesis (e.g., aminoglutethimide, cyanoketone, ketoconazole). Estrogen biosynthesis can be inhibited by exposure to aromatase inhibitors such as the fungicide fenarimol (Hirsch et al., 1987).

Alterations in protein hormone synthesis can be induced by gonadal steroids and potentially by environmental estrogens and antiandrogens. Both estrogen and testosterone have been shown to affect pituitary hormone synthesis directly or by changes in the glycosylation of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Wilson et al., 1990). A decrease in glycosylation of these glycoproteins reduces the biological activity of the hormones. Any environmental compound that mimics or antagonizes the action of these steroid hormones could presumably alter glycosylation. The biopotency of pituitary hormones also may be altered by changes in glycosylation in response to treatment with biogenic amines (i.e., dopamine) and gonadotropin-releasing hormone (GnRH) (see Wilson et al., 1990, for review).

The synthesis of nonpeptide, nonsteroidal hormones such as epinephrine and melatonin, which also serve as CNS neurotransmitters, can be altered by environmental agents. Changes in the synthesis of norepinephrine and epinephrine have been observed following exposure to a number of dithiocarbamates, metam sodium, and carbon disulfide (CS_2) (Przewlocka et al., 1975; Goldman et al., 1994; Stoker et al., 1995). Exposure to these copper chelating compounds suppresses the activity of dopamine-β-hydroxylase, thereby inhibiting the conversion of dopamine to norepinephrine and subsequently to epinephrine.

2. Altered Hormone Storage and/or Release

Catecholamine hormones are stored in granular vesicles of chromaffin cells within the adrenal medulla and within presynaptic terminals in the CNS. This mechanism for storage is important to the maintenance of normal concentrations of the hormone so that they can be released quickly on demand. Without such a storage mechanism, the hormones are subject to deamination by monoamine oxidase. Reserpine and amphetamine are well-known examples of compounds that can affect this storage process. In contrast, steroid hormones do not appear to be stored intracellularly within membranous secretory granules. For example, testosterone is synthesized by Leydig cells of the testis and released on activation of the LH receptor. Thus, compounds that block the LH receptor or the activation of the 3',5' cyclic AMP (cAMP)-dependent cascade involved in testosterone synthesis can rapidly alter the secretion of this hormone.

The release of many protein hormones is dependent on activation of second messenger pathways such as cAMP, phosphatidylinositol 4,5-bisphosphate (PIP_2), inositol 1,4,5-trisphosphate (IP_3), tyrosine kinase, and Ca^{++}. Interference with these processes consequently will alter the serum levels (availability) of many hormones. Several metal cations have been shown to disrupt pituitary hormone release presumably by interfering with Ca^{++} flux (Cooper et al., 1987).

3. Altered Hormone Transport and Clearance

Hormones are transported in blood in the free or bound state. Lipid soluble hormones are transported in the blood by specialized transport (carrier) proteins synthesized in the liver. The same binding globulin, known as sex/steroid hormone-binding globulin (SHBG) or testosterone-estrogen-binding globulin (TEBG), can associate with either testosterone or estrogen. Glucocorticoids are bound to corticosteroid binding globulin (CBG) or transcortin in the circulation. Similarly, the thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4), are transported in the blood on thyroxine-binding globulin, prealbumin and albumin. Regulation of the concentration of these binding globulins in the blood is of some practical significance because there may be either increases or decreases that could affect steroid hormone availability. The levels of both TEBG and transcortin have been shown to be modified by gonadectomy and gonadal steroid hormone replacement. Salicylates and diphenylhydantoin may modify the circulating levels of T_4 because of changes in thyroxine-binding globulin. Estrogens increase the TEBG concentration in plasma, whereas androgens and pharmacologic doses of glucocorticoids decrease TEBG (Ingbar and Woeber, 1974).

The clearance of hormones is influenced by compounds that alter liver enzymes involved in hormone clearance. For example, DDT analogs are potent inducers of hepatic microsomal monooxygenase activity in vivo (Bulger et al., 1978). Induction of this activity by treatment with

DDT analogs could possibly cause a decrease in testicular androgen as a result of enhanced degradation of endogenous androgens by the monooxygenase system. Similarly, treatment with lindane (gamma-hexachlorocyclohexane) has been reported to increase the clearance of estrogen (Welch et al., 1971). However, no evidence for enhanced clearance was noted in a study by Laws et al. (1994) in which serum estradiol was measured at multiple time points after estrogen administration via subcutaneous silastic implants in doses aimed at producing physiological levels of the steroid hormone. It should be pointed out that pan-fried meat and cruciferous vegetables can induce cytochrome P4501A2 in humans (Sinha et al., 1994). Recently, a mechanistic, dosimetric model of dioxin (TCDD) effects on increasing hepatic UDP-gluconosyltransferase for removal of serum thyroid hormone (T_4) has been reported (Kohn et al., 1996).

4. Altered Hormone Receptor Recognition/Binding

Hormones elicit responses from their respective target tissues through direct interactions with either intracellular receptors or membrane-bound receptors. Specific binding of the natural ligand to its receptor is a critical step in hormone function. Intracellular (nuclear) receptors such as those for sex steroids, adrenal steroids, thyroid hormones, vitamin D, and retinoic acid regulate gene transcription in a ligand-dependent manner through their interaction with specific DNA sequences (response elements). New messenger RNAs are synthesized, processed, and translated to produce new proteins.

A number of environmental agents may alter this process by mimicking the natural ligand and acting as an agonist or by inhibiting binding and acting as an antagonist. The best known examples are methoxychlor, chlordecone (Kepone), DDT, some PCBs, and alkylphenols (e.g., nonylphenols and octylphenols), which can disrupt estrogen receptor function (Mueller and Kim, 1978; White et al., 1994). The antiandrogenic action of the dicarboximide fungicide vinclozolin (van Ravenzwaay, 1992) is the result of an affinity of this compound's metabolites for the androgen receptor (Kelce et al., 1994). Interestingly, the DDT metabolite p,p'-DDE has been found to bind also to the androgen receptor and block testosterone-induced cellular responses in vitro (Kelce, 1995a,b).

Many of the chemicals classified as environmental estrogens can actually inhibit binding to more than one type of intracellular receptor. For example, o,p-DDT and chlordecone can inhibit endogenous ligand binding to the estrogen and progesterone receptors, with each compound having IC50s that are nearly identical for the two receptors. Other compounds such as nonylphenol and the metabolite of methoxychlor, HPTE, have the ability to inhibit binding to the estrogen, progesterone, and androgen receptors with similar affinities (Laws et al., 1995).

Receptors for protein hormones are located on and in the cell membrane. When these hormones bind to their receptors, transduction of a signal across the membrane is mediated by the

activation of second messenger systems. These may include alterations in G-protein-cAMP-dependent protein kinase A (e.g., after LH stimulation of the Leydig cell), phosphatidylinositol regulation of protein kinase C and inositol triphosphate (e.g., after GnRH stimulation of gonadotrophs; thyrotropin releasing hormone stimulation of thyrotrophs), tyrosine kinase (e.g., after insulin binding to the membrane receptor), and calcium ion flux. Xenobiotics thus can disrupt signal transduction of peptide hormones if they interfere with one or more of these processes.

5. Altered Hormone Postreceptor Activation

Once the endogenous ligand or an agonist binds to its receptor, a cascade of events is initiated indicative of the appropriate cellular response. This includes the response necessary for signal transduction across the membrane or, in the case of nuclear receptors, the initiation of or alteration in transcription and protein synthesis. A variety of environmental compounds can interfere with the membrane's second messenger systems. For example, cellular responses that are dependent on the flux of calcium ions through the membrane (and the initiation of the calcium/calmodulin dependent cellular response) are altered by a variety of metal cations (i.e., lead, zinc, cadmium) (Cooper et al., 1987). Disruption of G proteins and transduction of receptor-generated signals leading to a biological response (activation of protein kinase A) occur from exposure to cholera and pertussis toxins (Gilman, 1987). Similarly, lindane, among other environmental compounds, has been demonstrated to decrease phosphatidylinositol turnover in the membrane and thus reduce protein kinase C activation. Interestingly, the well-known antiestrogen tamoxifen also inhibits protein kinase C activity (O'Brian et al., 1985). Alternatively, the phorbol esters are known to mimic diacylglycerol and enhance protein kinase C activity.

Steroid hormone receptor activation can be modified by indirect mechanisms such as a down-regulation of the receptor (temporary decreased sensitivity to ligand) as seen after TCDD exposure (including the estrogen, progesterone, and glucocorticoid receptors) (Safe et al., 1991; Safe, 1995). Consequently, because of the diverse known pathways of endocrine disruption, any assessment must consider the net result of all influences on hormone receptor function and feedback regulation.

D. RISK ASSESSMENT PARADIGM

The evaluation and analysis of reported environmental endocrine disruption phenomena should be examined from a risk assessment perspective. Generally, quantitative risk assessment includes the estimation of levels of exposure to a toxic substance that leads to specified increases in lifetime incidence rates or in the probable occurrence of an undesirable consequence (Van Ryzin, 1980). The four components of the noncancer risk assessment paradigm for human health

are hazard characterization, dose-response assessment, exposure assessment, and risk characterization (National Research Council, 1994).

The ecological risk assessment framework is conceptually similar to the approach used for human health risk assessment, with a few distinctions. Ecological risk assessment considers effects beyond individuals of a single species and may examine population, community, or ecosystem level risks. The framework consists of three major phases: (1) problem formulation, (2) analysis (which includes exposure and effects assessment), and (3) risk characterization. The endpoints for ecological risks most often considered are survival, growth, and reproduction of individuals of a few representative species and populations. While not specific to endocrine disruption effects, some limited inferences about endocrine-controlled processes may be made.

Hazard characterization focuses on the qualitative evaluation of the adverse effects of an agent on human and animal health and ecological well-being. Health endpoints of particular concern with environmental hormones are reproductive (including developmental) effects, cancer, neurological and immunologic effects.

For human health, relevant and adequate epidemiologic studies and case reports for the agent(s) are preferable. In the absence of this information, pertinent test animal toxicology studies should provide useful information. In vitro studies may provide useful data for elucidating mechanisms of toxicity but are not sufficient by themselves to characterize a hazard. Important factors to consider in the evaluation of a hazard include inherent toxicity, route of exposure, dose level, timing and duration of exposure, body burden, susceptible populations and interspecies differences, and all of the assumptions and uncertainties in the data.

Dose-response assessment is the process of characterizing the relationship between the dose of an agent and the incidence/degree of an adverse effect. Factors to consider in the dose-response assessment are the intensity or frequency of the response with increasing dose, the shape and slope of the dose-response curve, pharmacokinetics (uptake, distribution, metabolism/detoxification, elimination), and the methods used for extrapolation of data from surrogate or sentinel species to ecological endpoints or to humans.

The exposure assessment component of the paradigm attempts to measure the intensity, frequency, and duration of exposure to an agent in the environment or to estimate hypothetical exposures that might arise from the release of new chemicals. Factors to consider in the exposure assessment include the amount of the agent in the environment; reactivity; half-life; environmental fate and disposition of the agent; the magnitude, duration (acute, subchronic, lifetime), schedule (timing), and route of exposure (oral, inhalation, dermal, aquatic); the size and nature of the exposed population; and all of the uncertainties and assumptions in the estimates.

Risk characterization is the process of estimating the incidence of a health or ecological effect under various conditions of human and biotic exposure. It draws together the hazard, dose-

response, and exposure assessments. It discusses the assumptions, uncertainties and limitations of all of the data.

With respect to recent reports of hazard (i.e., endocrine disruption causing human health or ecological effects), a critical element for risk assessment is the exposure assessment component. Without a clear understanding as to the magnitude and distribution of exposure, and the potency and nature of endocrine activity, the development of a credible risk assessment for specific endocrine-disrupting agents is not feasible. Another factor to consider in the evaluation of possible risk is whether testing paradigms in past or current use are capable of identifying an agent as an environmental endocrine disruptor adequately.

It should be emphasized here that this special report is an *interim* effects and analysis document until the NAS releases its assessment report on environmental endocrine disruption. The current document focuses primarily on human health and ecological hazard effects (characterization) as found within peer-reviewed literature.

E. CONTROVERSY WITHIN THE SCIENTIFIC COMMUNITY

In the wake of media coverage dealing with possible reproductive health and cancer concerns (e.g., Raloff, 1993, 1994), a few toxicologists have questioned whether these adverse health effects can be attributed to environmental endocrine disruption (Stone, 1994; Safe, 1995; Houghton and Ritter, 1995). Arguments for a demonstrable link between hormone-disruptive environmental agents and human reproductive health effects are supported by the fact that many pesticides and other agents with estrogenic or antiandrogenic activity operate via hormone receptor mechanisms. However, in the few studies of suspected weak estrogens, like the alkylphenols, some 1,000 to 10,000 times more of the weak estrogen is required to bind 50% of the estrogen receptor than estradiol itself (White et al., 1994). In other assays, 10^6 times more of the agent may be required than for estradiol. Of course, crucial to risk assessment is the need to know how many receptors must be occupied before activation of a response can ensue. For some hormones such as human chorionic gonadotropin (hCG), as little as 0.5% to 5% receptor occupancy is required for full activation of response. For other hormones (those that require protein synthesis for expression of effect), higher levels of receptor occupancy are needed (Bolander, 1994).

In general, due to the precise yet adaptable control mechanisms and the intertwined nature of the hormonal balance, modest amounts of chemical exposure seldom compromise normal physiological functions. Fluctuations of hormone concentration and receptor activities, by design, absorb some environmental and physiological challenges to maintain homeostasis in adults. Only when the equilibrium control mechanisms are overwhelmed do deleterious effects occur. An important question is whether homeostatic mechanisms are operative in the embryo

and fetus. Alpha-fetoprotein, to which endogenous sex steroids bind avidly, is thought to exert some protective function in developing fetuses to elevated estradiol that occurs during pregnancy. However, it is known that free estradiol, under experimental conditions in female rats, may have access to brain and other target organs in the fetus and neonate (Montano et al., 1995). DES is not bound to alpha-fetoprotein (Sheehan and Young, 1979) and is not metabolized by the placenta as is estradiol (Slikker et al., 1982). Whether other xenoestrogens behave in a similar manner is not known.

The production of any hormone in the endocrine system is the result of a chain of events involving precisely choreographed interactions of many other endocrine organs. Therefore, manifestation of an endocrine disorder may be associated with multiple changes in hormone concentrations.

Some investigators (Gierthy et al., 1991; Soto et al., 1992) have proposed the use of in vitro assays to screen for estrogenic or other hormonal activity. While steroid receptors bound to their ligand act as transcription factors for gene expression in the target tissue, simple in vitro screening assays based on binding to a receptor are not sufficient in themselves for measuring hormone activity. Binding of ligand to its specific receptor must be correlated with a physiologic response. For such screening assays to be accepted as indicative of hormonal alteration, they must be thoroughly validated in a number of qualified, independent laboratories. This validation requires the correlation of receptor binding with a physiologic endpoint, for example, induction of the progesterone receptor (Laws et al., 1994), increase in uterine peroxidase (Cummings and Metcalf, 1994), or an increase in vitellogenin in the case of the estrogen receptor. Furthermore, before screening assays can be used in a "tier approach" for evaluating hormone effects, in vitro assays need to be validated in vivo (in the whole animal). In the case of estrogen-mimicking agents, uterotrophic responses, progesterone receptor induction, or gonadotrophin inhibitory responses in ovariectomized rats or mice should be undertaken for validation in the whole animal. While estrogenic effects have been cited as examples in this document, it is important to realize that any hormone has the potential of being disrupted in one way or another by an environmental agent, and similar considerations as for estrogenic effects apply.

III. SPECIFIC ENDPOINTS OF CONCERN

A. HUMAN HEALTH EFFECTS

1. Female Reproductive and Developmental Effects

a. Ovary and Reproductive Tract

With the exception of endometriosis and vaginal and breast cancer, few recent reports have attributed environmental endocrine disruptions as a causative mechanism for seriously affecting human female reproduction. The issues of endometriosis and breast cancer in humans have been raised and are discussed in separate sections below. Structural abnormalities of the uterus and oviducts, reproductive dysfunction, and nonneoplastic lesions such as parovarian cysts have been associated with prenatal exposure to the estrogenic compound DES in laboratory animals (McLachlan, 1993). Most of these same multigenerational adverse effects due to DES exposure also have been reported in women (Poskanzer and Herbst, 1977). "Estrogenism" in livestock caused by toxins associated with the fungal genus *Fusarium* has been associated with uterine hypertrophy, decreased ovarian size, abortion, fetal resorption, and premature birth (Siegmundo, 1979). These findings indicate that when hormonal balance is disturbed, the reproductive health of the mother and the developmental and reproductive soundness of the offspring, both male and female, may be in jeopardy.

In developmental toxicity testing studies, emphasis is placed on the timing of the dose of a compound such that it coincides with organogenesis and on the subsequent recording of any birth defects that might occur. However, concerns have been raised over the possibility of multigenerational effects of endocrine disrupting chemicals that persist or do not appear until after environmental exposure has ended. This hypothesis proposes that maternal animals, including humans, store endocrine-disrupting agents in their fat prior to reproduction, then mobilize these agents during periods of egg laying, pregnancy, or lactation (Colborn et al., 1993). As a result of this mobilization of stored agent(s) within maternal animals during critical windows of embryonic or fetal development and vulnerability, there may occur immediate or latent adverse effects on the offspring that are likely to be irreversible. This phenomenon is suggested in conclusions by Guillette et al. (1994) from their observations of Florida alligators. These studies hypothesize a decrease in the number of male hatchlings when maternal animals are exposed to high concentrations of the pesticide dicofol before fertilization. Of note is the fact that the manufacturing process of the dicofol spilled was such that it was contaminated with DDT and its degradates.

It should be noted that in the two-generation reproductive testing protocol, animals are exposed during several life stages and any multigenerational adverse reproductive effects due to environmental endocrine disruption should be detected with the endpoints added to the new protocol (EPA, 1995).

i. Background

The reproductive life cycle of the female mammal may be divided into phases that include fetal, prepubertal, cycling adult, pregnant, lactating, and reproductively senescent stages. Although there are a limited number of studies evaluating reproductive function in the female following toxicant exposure, it is important that each stage of the life cycle be examined thoroughly before one can assume that the female is not influenced by environmental endocrine disruptors. Traditionally the endpoints that have been used to evaluate the female's reproductive capability include the ability to become pregnant, pregnancy outcome, and offspring survival and/or development. Although reproductive organ weights may be obtained and these organs examined histologically in test species, these measures do not necessarily detect abnormalities in dynamic processes such as estrous/menstrual cyclicity or follicular atresia unless degradation is severe. Similarly, toxic effects on pubertal onset have not been examined routinely, nor have the long-term consequences of exposure to suspected toxicants on reproductive senescence.

Irreversible developmental effects are those that affect the vulnerable developing organism, frequently at the time where organ systems are beginning to be laid down. Physiological effects are those that occur anytime after development and may be reversible. Eroschenko (1981) reported that administration of Kepone to pregnant rats or mice during the main period of fetal organogenesis results in fetal toxicities and malformations. Gellert and Wilson (1979) demonstrated that the female offspring of chlordecone (Kepone)-treated dams exhibit persistent vaginal estrus and anovulation.

The consequences of disruption of the ovarian (estrous) cycle can signal exposure to a reproductive toxicant that affects endocrine function. For example, perinatal exposure to DES or methoxychlor not only induces premature vaginal opening (Gray et al., 1989), but often leads to the presence of an acyclic condition (persistent or constant estrus) in the adult (Cooper et al., 1986a). This condition is the result of the agent's ability to masculinize the developing, potentially female, brain. Such animals fail to achieve normal ovulatory LH surges, and their ovaries typically contain numerous polyfollicular or polycystic follicles and no corpora lutea. Prolonged exposure to DES or methoxychlor during adulthood also will lead to persistent or constant vaginal estrus because of direct estrogenic action on the vagina. However, in this case, the exposed adult female's ovaries become atrophied due to the suppression of gonadotropin secretion by the estrogenic compounds. It has been reported that exposure to certain chlorotriazine herbicides

(i.e., Atrazine, Simazine, or Cyanazine) also will induce a persistent estrous condition in certain strains of rats (i.e., Sprague Dawley but not Fischer 344) (Eldridge et al., 1994). In fact, it has been hypothesized that this condition is responsible for the early onset of mammary gland tumors in rats fed diets containing the chlorotriazines during the
first year of life (Stevens et al., 1994). However, in a more recent study by Cooper et al. (1995), it was shown that Atrazine did not prolong the number of days in estrus, but there is a dose-dependent increase in the number of diestrous days (in both Sprague Dawley and Long-Evans hooded rats). At higher doses, the female's ovaries were atrophied and gonadotropin levels were low. At lower doses, Atrazine appeared to induce repetitive pseudopregnancies. The reason for the apparent discrepancies between these reports is not clear. However, it is clear that Atrazine, and apparently several other chlorotriazines, can disrupt ovarian function in the adult female rat and that an endocrine mechanism is involved. The mechanism of action of the chloro-s-triazines appears to be estrogen receptor independent (Connor et al., 1996), and the alterations observed in the regulation of estrous cycling are apparently due to a disruption in hypothalamic-pituitary regulation of ovarian function (Cooper et al., 1996).

Importantly, compounds other than those that interact directly with estrogen or other steroid hormone receptors can alter the onset of puberty as well as ovarian function in adulthood. For example, it has been known for some time that administration of prolactin to female rats could advance the onset of puberty (Advis and Ojeda, 1978; Advis et al., 1981). These effects can be induced by agents that disrupt CNS regulation of prolactin secretion resulting in hyperprolactinemia. Thus, placing a dopamine receptor blocker, such as sulpiride, in the drinking water of prepubertal females advances the age of vaginal opening (Advis et al., 1981).

Chloroquine, an antimalarial agent, is reported to block calcium-calmodulin-mediated responses. It is not surprising that chloroquine exposure will lead to a disruption of estrous cyclicity (Okanlawon and Ashiru, 1992), because follicular steroidogenesis and pituitary hormone secretion are dependent, in part, on calcium-calmodulin-mediated processes.

The human ovarian follicle is vulnerable at several points in its development, and a transient toxic insult to a specific locus and time period may result in an adverse effect not only to the follicle, but also to the resulting corpus luteum. In other words, insult to the Graafian follicle and subsequent alterations in the sequence of its maturation can lead to luteal dysfunction following ovulation (McNatty, 1979). Because the corpus luteum is essential to the maintenance of early pregnancy in humans, any insult to the ovarian follicle that gives rise to the corpus luteum has the potential to adversely affect pregnancy outcomes.

Several excellent reviews have dealt with the ovarian follicle as a target for xenobiotics (e.g., Richards, 1986; Mattison and Thomford, 1989). In addition to the oocyte itself, the target may include cells of the stratum granulosum, the cumulus mass, the basal lamina, or the theca

interna and externa. Within the stratum granulosum, basal granulosa cells, parietal granulosa cells, cumulus cells, gap junctions, gonadotrophin, and other membrane or intracellular hormone receptors may serve as loci for ovarian toxicants. The adverse effects of antineoplastic agents on antral follicles and the sparing of primordial follicles have been demonstrated (Damewood and Grochow, 1986). The toxic effects of cyclophosphamide on human granulosa cell cultures have been reported (Ataya et al., 1990). A dose-related decrease in progesterone secretion by human granulosa cells occurs with increasing concentrations of the activated form of cyclophosphamide at levels used therapeutically. Human cumulus granulosa cells have been used to screen reproductive toxicants (Haney et al., 1990). Vinblastine inhibits progesterone secretion by human granulosa-luteal cells (Teaff and Savoy-Moore, 1991). Methoxychlor, a pesticide that when metabolized exhibits estrogenic activity, reduces serum progesterone and impairs implantation in rats treated during the first week of pregnancy (Cummings and Laskey, 1993). Premating treatment of female rats with the insecticide heptachlor also decreases implantations, increases resorptions, and decreases serum estrogen and progesterone (Rani and Krishnakumari, 1995).

Within the oocyte, the zona pellucida, oolemma, cortical granules, yolk, chromosomes, and spindle all serve as potential targets for exposure to toxic chemicals. The oocyte is particularly sensitive to methotrexate and cyclophosphamide (Hansmann, 1974; Jarrell et al., 1987, 1991). Greatest risk to the oocyte occurs on the days just prior to ovulation (Paul and Himmelstein, 1988). Also susceptible to chemical insult are the thecal components (interna cells with LH receptors, fibroblasts and smooth muscle cells of the externa, and elements of the vascular bed). Delayed ovulation and overripeness of ova in rat studies result in chromosomal anomalies leading to early embryonic death (Fugo and Butcher, 1966; Butcher and Fugo, 1967). If mature oocytes remain in the human Graafian follicle past mid-cycle, the incidence of oocyte abnormalities increases (Hertig, 1967).

Mattison and coworkers (1990) have called attention to the basal lamina as a permeable barrier to xenobiotics. Studies in the human female are meager, however. The anesthetic drugs thiopental and thiamylal traverse the follicular wall and have been found in follicular fluid of patients undergoing laparoscopy for oocyte retrieval (Endler et al., 1987). In another study of 47 women, oocyte recovery rates and subsequent embryo cleavage rates were inversely related to chlorinated hydrocarbon concentrations that included DDT, PCBs, and hexachlorobenzene (Trapp et al., 1984). Buserelin, a GnRH agonist employed in in vitro fertilization programs also has been found in human follicular fluid at 10% to 50% of serum concentrations (Loumaye et al., 1989). While the above observations document the potential for specific chemical insult to the ovarian follicle, many of these agents are genotoxic or cytostatic chemicals, and it remains to be demonstrated whether the mechanism of action for these and other agents is via an endocrine disruption pathway.

The effects of environmental endocrine disruptors on hypothalamic-pituitary regulation of ovulation are discussed elsewhere. Finally, environmental estrogens also may interfere with fertility by disrupting implantation. In rats, Cummings and Perreault (1990) found that methoxychlor increased the speed of embryo transport through the oviduct (an estrogen-dependent process) and therefore prevented implantation because of insufficient time for uterine preparation.

ii. Toxicity Testing in Animals and Extrapolation to Humans

Recently, the Office of Pesticide Programs, EPA, reviewed multiple databases in an attempt to identify those chemicals with a clear effect on female reproduction. Records for 63 chemicals screened for noncancer health effects were evaluated. Eight chemicals were considered to be potential female reproductive toxicants because they exhibited one or more of the following: ovarian vacuolation (of unspecified attribution), ovarian stromal hyperplasia, hemorrhagic ovaries, reduced number of corpora lutea, increased uterine weights, uterine metaplasia, or cystic uteri. Data are briefly summarized below. In some of these cases, the reported adverse female reproductive effects occur at doses that exceed the lowest observed adverse effect level for other adverse, noncancer health effects. Consequently, these other endpoints of toxicity currently "drive" the risk assessment. In other words, the female reproductive effect via a potential endocrine disruption mechanism did not provide the critical effect for any of those pesticides.

In the data review for the chemical **dicofol**, ovarian vacuolation is reported in a multigenerational reproductive study in rats. However, the effect occurs at a dose level that is 10 times the dose of acceptable human exposure. Nevertheless, there is a hint that this finding in female rats may be associated with hormonal disruption because the complete database indicates that multiple endocrine target organs and multiple species are affected. It also is concluded that the reported ovarian vacuolation is associated with enhanced steroidogenic activity. The question of the purity of the chemical and its possible contamination with DDT has been raised.

Hexaconazole causes decreased numbers of corpora lutea and decreased uterine weight in mice dosed at 225 mg/kg/day (in a 29-day range-finding study, MRID# 41142801, HED Doc# 007917)[1]. In a mouse carcinogenicity study at the highest dose level tested (26.3 mg/kg/day), nominally decreased numbers of cystic glands in uteri and increased numbers of hemorrhagic ovaries are noted (MRID# 40944809, HED Doc# 007917).

Molinate causes reduced fertility and ovarian histopathology in rats at 50 ppm in the diet (2.5 mg/kg/day) with a no observed effects level (NOEL) of 0.03 mg/kg/day in a two-generation

[1] MRID# and HED Doc# refer to EPA's Office of Pesticide Programs database files.

study (MRID# 41333402, HED Doc# 008449). In the case of molinate, a carcinogenic potency factor (Q*) of 0.11 mg/kg/day^{-1} (based on ovarian hyperplasia and cancer) has been used to estimate carcinogenic risk. In this case, mutagenicity studies on molinate were both positive and negative (Caswell file# 444). In addition, mutagenicity has been suggested in an inhalation study where abnormal sperm were observed in males treated at 0.64 mg/m^3. This inhalation study demonstrated reduced implants in untreated females bred to treated males (MRID# 41589203, HED Doc# 008449). The NOEL was 0.30 mg/m^3. The reproductive effects and the ovarian histopathology and mutagenic effects may have mechanisms in common, but no hormonal disruption has been reported.

The pesticide **oxydemeton-methyl** induces cholinesterase inhibition at dose levels two orders of magnitude lower than dose levels that affect multiple reproductive organ toxicity. Increased numbers of female rats show no corpora lutea at 2.5 mg/kg/day of oxydemeton-methyl, a dose level that also causes increased epididymal vacuolation and testes weight decreases in males and severe brain, plasma, and red blood cell cholinesterase inhibition in both sexes. At these high dose levels (near lethality), neurotransmitters may have caused hormonal disruption at the pituitary level. Although documentation has been found for only one pesticide, other organophosphates may have this potential at high dose levels.

Iprodione (MRID# 42825002 and 42637801, HED Doc# 010570), **procymidone** (Caswell file# 703J), or **vinclozolin** (MRID# 43254703 and 43254704, HED Doc.# in review) administration result in ovarian stromal cell tumors, sex cord tumors, and/or luteomas (small, benign lutein cell tumors) in rats and/or mice. The lowest observable effect levels for ovarian effects in lifetime studies for these three pesticides were as follows: iprodione--600 mg/kg/day in mice, procymidone--100 mg/kg/day in rats, and vinclozolin - approximately 3.0 mg/kg/day in rats (MRID# 43254703, HED Doc# in review). Of the three pesticides, procymidone and iprodione are regulated by a Q* (because of carcinogenic concern) at even lower dose levels than the reference dose (RfD). Extensive endocrine studies indicate that vinclozolin and procymidone cause increases in LH and testosterone levels following binding to and inhibition of the androgen receptor (Kelce et al., 1994; Murakami et al., 1995; MRID# 42344104; MRID# 41824305). Iprodione causes similar effects in the ovary, testis, and accessory sex glands of rats and mice but may operate through a different mechanism. However, these data have not been fully reviewed at this time. Androgens are necessary for follicular growth and ovulation. They appear to play an important role in regulating follicular development in both the immature and mature cycling rat (Beyer et al., 1974; Kumari et al., 1978). They also induce atresia of preantral follicles (Louvet et al., 1975) and play a role in hCG-induced ovulation. Important to the discussion of antiandrogen exposure to the female, cyproterone acetate has been reported to accelerate the rate of atresia and subsequently transform the atretic preovulatory follicle into an ovarian cyst (Peluso et al., 1979).

Exposure to **pronamide**, in long-term carcinogenicity studies in rats, results in ovarian histopathology at 48.8 mg/kg/day in addition to thyroid and liver histopathology (MRID# 41714001, 41714002, 42093401, 41714001, and 41714002, HED Doc# 00899 and 009683). Testis, thyroid, and liver tumors are seen at ≥ 8.46 mg/kg/day. Pronamide does alter thyroid-stimulating hormone and thyroid hormone levels in the blood; however, an evaluation of the reproductive hormones has not been conducted.

In summary, the review of the multiple data sets available to the Office of Pesticide Programs produced a rather limited set of compounds that may be considered endocrine disruptors in the female. Studies conducted under testing guidelines currently required are not designed specifically to detect endocrine mechanisms or specifically endocrine disruption; they are designed to detect effects on endpoints of reproductive concern that may occur throughout several life stages of the animal regardless of their mechanism of action. Specific procedures for identifying better measures of potential endocrine disruption are being developed and incorporated in the more recent testing guidelines for development and reproduction (U.S. EPA, 1995) and are discussed in the new Reproductive Toxicity Risk Assessment Guidelines (U.S. EPA, 1996). Thus, future assessment of potential reproductive hazards should be facilitated. However, it should be noted that additional data may be required if results from studies conducted under the new guidelines indicate a need to further characterize the effects for regulatory purposes.

iii. Conclusions

Studies conducted under testing guidelines currently required are not designed specifically to detect endocrine disruption. Specific procedures for characterizing some endpoints of endocrine disruption are being developed and incorporated in updated testing guidelines for reproduction. With the inclusion of endocrine-sensitive endpoints in these guidelines, the effects of environmental agents on aspects of reproduction that involve endocrine disruption, particularly during development, will be better understood.

b. Endometriosis

i. Background

Endometriosis is a painful reproductive and immunologic disease of women characterized by aberrant location of uterine endometrial cells. It affects approximately 5 million women in the United States from 15 to 45 years of age (Holloway, 1994). Endometrial tissue usually occurs in or on ovaries, uterine ligaments, rectovaginal pouches, and pelvic peritoneum. Endometriosis often causes infertility, dysmenorrhea, and pelvic pain. Dysmenorrhea is caused by the sloughing of the estrogen-induced proliferation of the ectopic endometrial implant and the internal bleeding that follows. The etiology of this disease is unknown, but several hypotheses have been proposed. The regurgitation theory proposes that menstrual backflow occurs through the uterine tubes with implantation of endometrial cells in extrauterine sites. The metaplastic theory proposes endometrial differentiation from coelomic epithelium. The vascular/lymphatic dissemination theory provides a mechanism to explain extra pelvic implantation. A review of this disease has been published (Olive and Schwartz, 1993).

An association between women with endometriosis and high blood levels of PCBs has been reported (Gerhard and Runnebaum, 1992). In 1993, research showed a link between TCDD (dioxin) exposure and the development of endometriosis in rhesus monkeys (Rier et al., 1993). The severity of this lesion was dependent on the dose administered ($p<0.001$) over a 4-year period. Ten years following dosing, three of seven animals exposed to 5 ppt dioxin (43%) and five of seven animals exposed to 25 ppt dioxin (71%) had moderate to severe endometriosis. In contrast, the frequency of disease in the control group was 33%, similar to an overall prevalence of 30% in 304 rhesus monkeys housed at the Harlow Primate Center with no dioxin exposure. Pair-wise comparisons between controls and the 5 ppt group and the 25 ppt group were $p<0.05$ and $p<0.025$, respectively. This 15-year study, on a limited number of animals, suggests that latent female reproductive abnormalities may be associated with dioxin exposure in rhesus monkeys. Of course, other factors (diet, facilities) at the Harlow Primate Center may be contributing to the high background incidence in controls and the resident population. It is interesting that both dioxin and PCBs are ligands for the Ah receptor, which is known to suppress the immune system (Harper et al., 1993; Tryphonas, 1994). Recently, Arnold et al. (1996) concluded in a reproductive toxicology study in rhesus monkeys, that the incidence and severity of endometriosis lesions did not have any relationship with the ingestion of the PCB Aroclor 1254.

Boyd and coworkers (1995) have conducted a small clinical study to test the hypothesis that serum dioxin concentrations have an association with human endometriosis. Serum samples from 15 women with laparoscopically diagnosed endometriosis (5 each with the disease classified as mild, moderate, or severe) and an equal number of geographically and age-matched controls with

a history of fertility and no clinical evidence of endometriosis were analyzed for the presence of 22 of the most common dioxin, furan, and PCB congeners. No statistically significant correlations between disease status and serum levels of halogenated aromatic hydrocarbons were found. These preliminary data, admittedly on a limited population, suggest that serum dioxin concentrations *may not* be related to human endometriosis. What may be seen in monkeys, therefore, may not apply to humans.

ii. Toxicity Testing in Animals and Extrapolation to Humans

Whether current body burdens of dioxin contribute to background prevalence of endometriosis in monkeys and whether a specific chemical plays a causative role in the etiology of human endometriosis remain to be determined. An ongoing epidemiology study of victims contaminated with dioxin in the 1976 industrial accident in Seveso, Italy, and who had serum concentrations of 56 ppb should provide valuable human data on the possible role of dioxin in human endometriosis.

iii. Conclusions

The evidence for supporting the hypothesis that dioxin and PCBs are causally related to human endometriosis via an endocrine-disruption mechanism is very weak. Further epidemiologic and clinical research needs to be done to evaluate the possible role of chlorinated hydrocarbons in the etiology of endometriosis in women.

c. Breast Cancer

i. Background

This year, more than one-half of a million Americans will succumb to cancer, making it the Nation's second leading killer after cardiovascular disease. Of this number, 46,000 will die of breast cancer, the second leading cause of cancer deaths in women after lung cancer (Silverberg and Lubera, 1988). It is estimated that one in eight or nine women in this country will develop breast cancer in her lifetime. Over the past 20 years, the incidence of breast cancer has increased by 1% a year, due in part to improved diagnostic procedures (mammography) and early detection of small tumors (Feuer and Wun, 1992; Miller et al., 1994). Even with earlier detection, mortality rates have remained level over the past 50 years despite improved therapies. While considerable information on risk factors for human breast cancer etiology is available (sex, family history, age, race, age at menarche, decreased parity, unopposed estrogen therapy), the elucidation of the precise roles that chemical carcinogens, physical (radiation and electromagnetic fields) and biological agents (viruses), varied lifestyles (diet, exercise, alcohol consumption, abortion, and oral contraception), and genetic susceptibility (oncogenes and tumor suppressor genes) have to

play in the initiation, promotion, and/or progression of this disease in humans makes the task a monumental challenge.

It has been suggested that women exposed to certain persistent pesticides, such as organochlorines (e.g., DDT), PCBs, and/or polycyclic aromatic hydrocarbons (PAHs), have an increased risk of developing breast cancer in their lifetime (Morris and Seifter, 1992; Davis et al., 1993; Wolff et al., 1993; Davis and Bradlow, 1995). In general, these compounds are lipophilic and environmentally persistent. That some of these agents exhibit weak estrogenicity is the basis for an "estrogen window" hypothesis that they may be contributing to an increased risk of breast cancer. This hypothesis is based on the concept that extended, unopposed estrogen exposure during in utero development, puberty, and the perimenopausal periods increases the risk of breast cancer in susceptible women. Whether extended estrogenic exposure acts as a complete carcinogenic factor or as a promoter is not known. The estrogen-receptor complex interacts with the genome and is mitogenic in responsive tissues. Wolff et al. (1993) linked breast cancer to moderate levels of DDE, a breakdown product of the estrogenic pesticide o,p'-DDT. In a more recent nested case-control study designed to evaluate organochlorine levels in case patients long before breast cancer diagnosis, adjusting for other known risk factors for breast cancer and stratified across racial/ethnic subpopulations, Krieger and coworkers (1994) concluded that DDE and PCB exposure did not increase the risk of breast cancer in the total population, but the researchers did report that DDE levels among black case patients were higher than levels in black control women. An earlier followup retrospective cohort study of women exposed occupationally to elevated PCBs failed to demonstrate an excess risk of breast cancer mortality (Brown, 1987). A recent small, nested case-control study enrolled in a polybrominated biphenyl registry showed that women with serum PBB levels of 2-4 ppb had a higher estimated risk for breast cancer than women with less than 2 ppb (Henderson et al., 1995). It should be noted that many of these chemicals have been banned in the United States and levels of them in the environment have been declining in this country. In two recent epidemiologic reviews of the breast cancer problem and the possible role of organochlorine chemicals in its etiology, the weight of evidence for an association between organochlorines and human breast cancer was found not compelling (Houghton and Ritter, 1995; Ahlborg et al., 1995). The issue of smoking and breast cancer is controversial. Exposure to cigarette smoking during adolescence increases a woman's risk of breast cancer (Palmer and Rosenberg, 1993). In MCF-7 breast cell cultures, however, several polynuclear aromatic hydrocarbons that bind to the Ah receptor and that are constituents of cigarette smoke decrease estrogen-induced cell proliferation (Chaloupka et al., 1992).

It should be noted that while members of the organochlorine class of pesticides, which include four remaining registered pesticides (dicofol, endosulfan, lindane, and methoxychlor), may produce reproductive and developmental effects in test species, the existing data do not support

their potential for inducing mammary gland tumors. Among the organochlorines that have been banned or canceled (DDT/DDE, chlordane, heptachlor, mirex, aldrin/dieldrin), the target organs for carcinogenesis include the liver and thyroid. There are no reports in the Office of Pesticide Programs's registration database of an association between DDT/DDE exposure and rodent mammary gland tumors, thereby providing little support to the hypothesis linking these substances with human breast cancer. Of course, this assumes that rodent studies are predictive of human breast cancer.

A recent report by Brown and Lamartiniere (1995) has shown that DES, genistein, and o,p'-DDT administered to Sprague-Dawley female rats resulted in enhanced epithelial cell proliferation and differentiation of abdominal mammary glands. TCDD was inhibitory, and Aroclor 1221 and 1254 showed no significant cell proliferation increases. Other reports indicate that the herbicide Atrazine induces mammary gland tumors in Sprague-Dawley female rats (Wetzel et al., 1994; Stevens et al., 1994).

A recent publication has appeared indicating that some 75% of the current incidence of human breast cancer in the United States is attributable to past exposures to ionizing radiation (Gofman, 1995). Whether this hypothesis holds up to scientific scrutiny has yet to be determined.

ii. Toxicity Testing in Animals and Extrapolation to Humans

The study of chemically induced carcinogenesis of the mammary gland has been difficult and slow. With an increased number of women entering the workplace in recent years, the opportunity for exposure of women to potentially hazardous chemicals has increased. One explanation for the slow progress in studying risk in women is the lack of appropriate, biologically based animal models for understanding mechanisms by which toxicants interact with female reproductive target tissues and the resulting health effects that follow exposure.

A complicating factor in animal testing programs for predicting human breast cancer is the variability in susceptibility to chemical carcinogens among rodent strains. For example, Sprague-Dawley rats have high spontaneous rates of mammary tumors, while Fischer, ACI, and Copenhagen strains exhibit lower rates of mammary gland tumors (Isaacs, 1986). Independent investigators have used a wide variety of rodent strains in evaluating chemically induced carcinogenesis of the mammary gland. This fact makes interpretation of past data difficult when comparing data and extrapolating across species and strains. Pertinent is the report that one of the triazine herbicides (Atrazine) induces mammary gland tumors in Sprague-Dawley female rats but not in the Fischer 344 (Wetzel et al., 1994).

The evaluation of chemicals in laboratory rodents has been the cornerstone of the National Toxicology Program (NTP) for identifying those chemicals most likely to cause cancer in humans. The species most often used by the NTP are the inbred Fischer 344 rat and the hybrid B6C3F1

mouse (Huff et al., 1991). Recently, Dunnick et al. (1995) have reviewed NTP's chemically induced mammary gland carcinogenesis rodent studies. Out of 450 chemicals tested, 34 cause mammary gland neoplasms. Of these, 29 chemicals are positive in female rats; 4 of the 29 cause mammary gland neoplasms in both male and female rats and mice. These four chemicals are 1,2-dibromoethane, 1,2-dichloroethane, glycidol, and sulfallate, all genotoxic chemicals. The finding of mammary gland tumors in male rodents is notable because the occurrence of breast cancer in human males is a rare phenomenon. Six other chemicals (benzene, 1,3-butadiene, dichlorvos, ethylene oxide, methylene chloride, and nitrofurazone) cause mammary gland neoplasms in female mice (Huff et al., 1991). It should be kept in mind that the above 2-year cancer studies do not include pregnancy and lactation in their experimental design, which can influence expression of mammary gland carcinogenesis (Grubbs et al., 1985).

In addition to strain differences, current testing paradigms in laboratory rodents for mammary carcinogenesis may not be adequate for predicting whether a chemical agent is a human mammary gland carcinogen. Evidence for this comes from a number of studies demonstrating differences in mechanism(s) of mammary tumor development between species. For example, in high incidence strains for developing mammary gland tumors, nulliparous mice develop fewer mammary gland tumors in contrast to multiparous mice (Zwieten, 1984; Russo et al., 1989). However, in humans, full-term pregnancy followed by lactation reduces the risk of breast cancer. Furthermore, with few exceptions, spontaneous mammary adenocarcinomas in rats and mice are rare and fail to metastasize to distant organ sites (Williams et al., 1981). Whether this is due to the presence of tumor suppressor factors or some other mechanism is worthy of study. This lack of metastasis in rodents is quite different from that seen in human populations, where undifferentiated breast cancer cells can take up residence in bone, liver, brain, and lung and thereby contribute to the high mortality seen in clinical situations. In addition to differences in metastatic capability, routine chronic testing and two-generation reproductive studies in laboratory animals are done at high doses and in homogeneous animal populations. These doses usually are considerably higher than the concentrations likely to be experienced by human populations, which exhibit varied genetic heterogeneity. Furthermore, there is in vitro evidence that interspecies differences exist in metabolizing toxicants. For example, human and rat mammary gland co-cultures with V-79 cells respond differently to mutagenic PAHs (benzo[a]pyrene and 7,12-dimethyl benz[a]anthracene) (Gould et al., 1986).

iii. Conclusions

Given the sparse human epidemiologic data on the association between organochlorines, PAHs, and PCB exposures and human breast cancer, it is *not* possible to attribute to them a cause and effect at this time (Key and Reeves, 1994; Houghton and Ritter, 1995). Further

epidemiologic investigations in geographical regions with elevated breast cancer incidences (e.g., Long Island, NY) are needed as well as complementary mechanistic studies in appropriate and predictive laboratory animals.

2. Male Reproductive System Effects

a. Background

Abnormality in the expression of the genome or interference with the action of gene products, as well as acceleration of the rate of cell division, can be induced in male reproductive organs by chemicals having endocrine activity. Because the male reproductive endocrine system involves components from the hypothalamus and pituitary as well as the testes, opportunities for disruption exist at multiple levels and with a variety of types of endocrine action. Of particular importance are chemicals with the ability to affect testosterone production directly or by influencing the control of gonadotropin production. Thus, chemicals with estrogenic, antiandrogenic, or Ah receptor binding activity are primary suspects, as are chemicals that influence the synthesis or release of FSH, LH, or prolactin. Included are chemicals that interfere with hormone receptor synthesis or function. While the adult male reproductive system can be affected adversely by disruption of the endocrine balance, the development of the male reproductive system pre- and postnatally appears to be particularly susceptible and uniquely sensitive. For that reason, this discussion is focused on developmentally induced effects.

Very early embryos have the potential to develop either a female or male reproductive system. In mammals, including humans, development of the male phenotype requires activation of the SRY gene on the Y chromosome. In the absence of expression of that gene, the female phenotype develops. The mechanisms of action of the SRY gene product have not been elucidated fully, but a cascade of events is initiated. These events have been reviewed by George and Wilson (1994) and Byskov and Hoyer (1994), and their descriptions are summarized below.

The embryonic gonads are formed by migration of primordial germ cells (gonocytes) to the urogenital ridge of the mesonephric kidneys where they and other somatic cells from the urogenital ridge form a gonadal ridge. Somatic cells from the urogenital ridge include precursors of Sertoli and Leydig cells. This process begins early in week 4 of gestation in humans, and the migration is completed during week 5. During week 6, the first morphologic sign of male sexual differentiation is seen when somatic cells (primordial Sertoli cells) in the gonadal ridge form spermatogenic cords.

Before this time, the sexually undifferentiated fetus has formed two paired ducts called the Wolffian duct and the Mullerian duct. These ducts terminate in a structure called the urogenital sinus. Before 8 weeks of gestation, these internal structures as well as the external genitalia of genetic males and females are indistinguishable morphologically. During week 8 in the male, the

Mullerian ducts begin to regress due to the action of anti-Mullerian hormone (AMH) produced by the primordial Sertoli cells. Completion of this regression is essential for formation of normal phenotypic males. Following Mullerian duct regression, the Wolffian ducts form the epididymis, vas deferens, and seminal vesicles. The urogenital sinus forms the prostate gland as well as the bladder and initial urethra. Simultaneously, the external genitalia develop to form the penis, including the penile urethra, and scrotum. With the exception of Mullerian duct regression, these sexual differentiation events are under the control of testosterone that is produced by the fetal Leydig cells. Testosterone is also necessary for completion of Mullerian duct regression but is ineffective without AMH. Target cell responses to testosterone (and dihydrotestosterone) are mediated via the androgen receptor (AR).

During the latter two-thirds of human gestation, important events include development of the testes, development of the penis, and migration and descent of the testes into the scrotum. During that period and postnatally, testis development continues with proliferation of gonocytes, Sertoli cells, and Leydig cells. These processes all require testosterone and/or dihydrotestosterone (produced from testosterone) and normal AR function to proceed normally.

Thus, two hormones have been identified that are directly involved in differentiation and development of the male reproductive tract. These are AMH and testosterone. Interference with AMH expression or action would result in failure of the Mullerian ducts to regress and presence of rudimentary components of the female reproductive tract in otherwise phenotypic males, that is, a pseudohermaphrodite condition. Interference with production or action of testosterone would cause failure of or limited development of the male reproductive tract in general. Depending on the extent of that interference, the consequences would be complete or partial failure of the male reproductive system to develop. Variation in the timing of interference could cause differential effects (Silversides et al., 1995). Effects that could be seen include the following:

Incomplete development of the external and internal genitalia, including an underdeveloped penis (hypospadia or microphallus). These conditions can preclude copulation.

Failure of the testes to descend into the scrotum (cryptorchidism). Cryptorchidism in humans is associated with increased incidence of testicular cancer (Forman et al., 1994) and impaired spermatogenesis.

Incomplete proliferation or maturation of gonocytes (precursor cells of sperm) and/or Sertoli cells that would result in reduced capability to produce sperm. It has been suggested, but not proved, that the presence of fetal germ cells in postpubertal testes could be the origin of germ cell tumors that develop in young men (Skakkebaek et al., 1987).

Incomplete proliferation of Leydig cells or interference with Leydig cell function that could limit androgen production, delay or prevent onset of puberty, and affect sexual behavior in adults.

b. Influence of Hormones on the Mammalian Male Reproductive System

The action of androgen, mediated via the AR, is essential for normal development of the mammalian male reproductive system. Under normal physiological conditions, testosterone and dihydrotestosterone are the primary androgens that activate the AR. Three classes of chemicals that have been shown to influence androgen level when administered during the developmental period are of particular concern. Those are chemicals having antagonistic properties with the AR (antiandrogens), those that interact with the estrogen receptor, and those that interact with the Ah receptor.

i. Antiandrogens

Chemicals that can bind to the AR without activating it, and simultaneously prevent binding of true androgens, are called antiandrogens. Examples of antiandrogens are the pharmaceutical hydroxyflutamide, the pesticides procymidone (Hosokawa et al., 1993) and vinclozolin (Gray et al., 1994), and the DDT metabolite p,p'-DDE (Kelce et al., 1995a,b). O,p'-DDT has weak estrogenic activity. The recognition that the major metabolite is an antiandrogen introduces another mechanism for the effects of DDT. Also, in addition to their high affinity for the estrogen receptor, estradiol and DES have affinity for the AR (Kelce et al., 1995a,b). Therefore, it is possible that the mechanism by which estrogenic chemicals impair development of the male reproductive system may be via antiandrogenic properties rather than or in addition to activity related to estrogen receptor activation. Other compounds with estrogenic activity that have the ability to affect the male reproductive system adversely, e.g., chlordecone and methoxychlor (Bulger and Kupfer, 1985), have not been investigated for antiandrogenic properties.

Failure to activate the AR due to low androgen levels or antiandrogen activity would produce results similar to the less severe alterations seen in individuals with defective ARs. The range of those effects is seen clearly in human 46,XY genetic males who have defects in the AR (androgen insensitivity syndrome [AIS]). AIS in humans has been reviewed by Quigley et al. (1995). As discussed below, similar effects have been observed in genotypic males exposed prenatally to DES.

An example of an environmental chemical that has antiandrogenic properties is the fungicide vinclozolin. Gray et al. (1994) administered vinclozolin to pregnant rats from gestation day 14 to postnatal day 3. Male offspring had a variety of reproductive effects that are characteristic of interference with AR action. Effects observed included reduction of anogenital distance to that characteristic of females, impaired penis development, existence of vaginal pouches, prostate gland agenesis, delayed preputial separation, and reduced or absent sperm production as judged by seminiferous tubule atrophy.

ii. Estrogens

A series of papers and reports have appeared indicating that the human male reproductive system, as well as that of certain wildlife species, has been compromised seriously in recent decades. Reported effects, which have included reduced sperm production, improper development of the penis, cryptorchidism, and testicular tumors, are described in a report commissioned by the Danish Environmental Protection Agency (Toppari et al., 1995). It has been hypothesized that these effects are due to exposure in utero to exogenous chemicals with estrogenic activity (Sharpe, 1993; Sharpe and Skakkebaek, 1993). Sharpe et al. (1995) have produced reductions in rat testicular weight and sperm production rate with relatively high exposure levels of the estrogenic environmental chemicals octyphenol, octyphenol phenoxylate, and butyl benzyl phthalate as well as DES. Below, evidence that human male reproduction has been compromised is summarized and evaluated.

A report by Carlsen et al. (1992) described the results of a meta-analysis of human semen studies published between 1938 and 1991. Published data from a total of 61 studies were evaluated. Those studies were conducted in several different countries and examined differing and often selected populations of men. The report concluded that human sperm production had declined by approximately 50% over that period. The investigators' calculations, which were derived from combining studies, suggested that a decline in mean sperm concentration from 113 $\times 10^6$ to 66 $\times 10^6$ sperm per ml of semen was accompanied by a mean ejaculate volume decline from 3.4 to 2.75 ml over that period of approximately 50 years. The authors concluded that there was no obvious, valid reason to believe that human sperm production had not declined, but they acknowledged that no basis existed in those data to demonstrate that the downward trend was continuing.

The conclusions reached by Carlsen et al. (1992) and subsequent publications from that group have been challenged on two fronts. The first is whether an actual decline occurred and if so, whether the decline was limited to the period prior to 1970. The second is whether such an effect on sperm production might actually have been caused in humans by exogenous agents with estrogenic activity.

Issues raised regarding the conclusion that sperm production has declined include the following:

The validity of comparing results obtained from different populations of men from different geographic areas and different times (Sherins, 1995; Olsen et al., 1995). Of particular concern is the fact that the large majority of men in the different studies were from selected populations that included presumed fertile men presenting for vasectomies, male partners in infertile couples, and volunteer semen donors for artificial insemination procedures. The analysis also has been challenged on the basis of whether the criteria for inclusion in the studies might have changed due

to a change in World Health Organization criteria for judging sperm count to be inadequate for normal fertility (Bromwich et al., 1994). It is not clear that this latter criticism is valid, but the challenge has not been refuted effectively (Farrow, 1994).

The lack of control for abstinence time before provision of the semen sample (Sherins, 1995). Increasing abstinence interval results generally in increasing sperm concentration and volume of ejaculates. A systematic decrease in abstinence interval could explain much of the purported decrease in sperm concentration and semen volume.

The limitations in amount of data prior to 1970 and the use of a linear regression approach to describe the behavior of the combined data. As indicated by Olsen et al. (1995), only 12% of the total subjects in the meta-analysis were in the first 30 years. Thus, the studies from which the higher baseline of sperm count was determined do not form a robust base. Also, application of more sophisticated approaches to modeling of the data indicates that a stair-step procedure is more appropriate. Stair-step modeling with the combined data concludes that sperm count dropped between the group of studies prior to 1970 in comparison with those after 1970, but also indicates that from 1970 to 1990, sperm count held steady or possibly increased. It must be recognized that such modeling only describes the behavior of the data mathematically and does not address biological plausibility.

Evidence for a general decline in sperm production from other sources is conflicting. Auger et al. (1995) examined the sperm count and semen volume of first ejaculates provided by healthy fertile men volunteering as semen donors at the author's Paris clinic from 1973 to 1992. Declines in sperm count (89×10^6 to 60×10^6) were reported during that interval. However, the researchers did not find a decline in semen volume. Irvine et al. (1996) and Ginsburg et al. (1994) have reported similar results. On the other hand, comparison of several studies published between 1958 and 1992 (Suominen and Vierula, 1993) supports a concept that no decrease in sperm count or semen volume has occurred in Finnish men. Also, MacLeod and Wang (1979), whose laboratory in New York provided a large proportion of the men included in the Carlsen et al. (1992) meta-analysis for the pre-1970 period, concluded that sperm concentration or semen volume had not changed in an equivalent population of men 20 years later. Further, Fisch et al. (1996) and Paulsen et al. (1996) found no change in semen parameters at multiple locations in the United States, including New York. It should be recognized that all of these studies were done on selected populations. Thus, while there may be reductions in sperm production in some locations, available data do not support the concept that there has been a general reduction. Because of the limitations in virtually all of the data, the conclusions should be viewed as tenuous.

Important information on the ability of exogenous estrogenic chemicals to disrupt human male reproductive system development is available from maternal exposures to DES. Particularly important are two papers describing effects on male offspring resulting from pregnancies during

which women were treated with DES (Gill et al., 1979; Wilcox et al., 1995). Those women participated in a controlled clinical trial (the Chicago Lying-In Study) to examine effects of DES given to prevent loss of pregnancy. DES was given in daily doses that increased from 5 mg during the seventh week of pregnancy to a maximum of 150 mg by week 34. Women began receiving DES between weeks 7 and 20, and the period in gestation at which treatment was initiated was therefore not constant. Controls were given placebos. The male offspring exposed to DES in utero had increased incidence of genital malformations, including epididymal cysts (nonmalignant; 21% vs. 5% for controls) and testicular abnormalities (11% vs. 3%) that included small (hypoplastic) testes, and microphallus (Gill et al., 1979). A history of cryptorchidism was found in 17 of the 26 exposed men with hypoplastic testes as opposed to 1 of 6 placebo-exposed men with hypoplastic testes (out of 308 and 307 men, respectively). Because incidence of cryptorchidism was reported only for men with hypoplastic testes, definitive conclusions cannot be drawn about the incidence of cryptorchidism in the overall population of DES-exposed men. Overall incidence of reproductive tract abnormality (one or more major or minor abnormalities) was 32% in DES-exposed men and 8% in controls. Average sperm concentration in ejaculates from 134 of the DES-exposed men was 91×10^6 vs. 115×10^6 for 87 nonexposed controls. Most, if not all, of that significant decrease was probably attributable to the higher incidence of exposed men with hypoplastic testes. However, when the same population was recontacted at age 38 to 41, no indication was found of a decrease in fertility among these men (Wilcox et al., 1995). No report has indicated an increase in testicular cancer in this population.

In considering these results, it is important to note that DES is a potent synthetic estrogen that also has antiandrogen properties. With exposure in utero to relatively high levels of a potent exogenous estrogen, about one-third of the men who were recontacted have clinically detectable reproductive system effects. The types of effects that were observed are consistent with those that would be predicted from studies with rodents, but men appear to be less sensitive. Except as might occur from nursing, there was no postnatal DES exposure.

iii. Ah receptor agonists

A group of halogenated aromatic hydrocarbons that cause male reproductive effects have the common property that they can activate the Ah receptor (Whitlock, 1994). Where comparable, the effects on the male reproductive system are similar. The male reproductive effects of dioxin (2,3,7,8-TCDD) are presented as an example. These effects have been reviewed by Peterson et al. (1993), and their review of these effects is summarized.

Dioxin causes effects on the developing male reproductive system in rodents at lower doses than those causing effects on adult males. The effects that are induced during development appear to result from the ability of dioxin to impair testosterone synthesis, although impairment of

CNS sexual differentiation could be involved also. The low androgen level is not accompanied by increased LH levels, indicating impairment of the feedback mechanism for control of LH synthesis and release. Observed effects include decreased anogenital distance, delayed testis descent, impaired spermatogenic function, decreased accessory sex gland weights, and feminization of male sexual behavior. Recent work by Gray et al. (1995) has basically confirmed these results with dioxin and expanded them using more extended dosing during the period of organogenesis and over three generations. In the F_1 and F_2 generations, adverse effects on male fertility were seen at doses (dietary) as low as 0.01 µg/kg/day.

c. Testicular Cancer

i. Germ Cell Tumors

A substantial body of evidence has accumulated indicating that the incidence of testicular cancer in men has increased significantly. The tumors are primarily germ cell in origin. Those data have been summarized by Toppari et al. (1995). Salient features of the data include the following conclusions:

Toppari et al. (1995) have estimated that cancer incidence in men under age 50 has increased approximately 2% to 4% per annum since the 1960s in Great Britain, the Nordic and Baltic countries, Australia, New Zealand, and the United States. In Denmark, which appears to have the highest incidence, the lifetime risk of contracting testicular cancer approaches 1%.

There are marked differences in incidence levels between countries and between races. In the United States, whites appear to have a higher incidence than blacks.

Testicular cancer is the most common malignancy among men age 25 to 34, with age-specific incidence as high as approximately 25 per 10^5 in Denmark (Adami et al., 1994). Interestingly, the corresponding incidence in Finland is about 5 per 10^5. The reason for this difference is not known. Most of the tumors occurring in young men are germ cell in origin.

Cryptorchidism is associated with no more than 10% of testis cancer cases (Chilvers and Pike, 1992).

The cause of the apparent increased incidence of testicular cancer is unknown, but it has been speculated that disruption of the male endocrine system during development may be involved. That speculation is fueled by (1) the appearance of immature germ cell forms in testes of some men with testicular cancer (Skakkebaek et al., 1987); (2) a demonstrated association between cryptorchidism and testicular cancer, and (3) the predominance of testicular cancer incidence in young men. However, Gill et al. (1979) reported that none of the DES-exposed men from the Chicago Lying-In Study who were contacted approximately 25 years later had contracted testicular cancer. While Wilcox et al. (1995) did not report on the incidence of testicular cancer in those same men when recontacted at age 38 to 41, Wilcox (personal

communication) has stated that there were no cases of testicular cancer in either the exposed or unexposed men who were contacted. By age 38 to 41, that cohort was sufficiently old to have developed testicular cancer if they were at increased risk although the number of men in this study was small. These data are in accord with those of the Danish report (Toppari et al., 1995).

ii. Leydig Cell Hyperplasia and Tumors

Leydig cells are contained in the interstitial spaces between seminiferous tubules in the testis. They are responsive to LH and are the primary source of testosterone in males. A number of chemicals have been shown to increase the incidence of Leydig cell hyperplasia and adenomas in chronic toxicity studies with rodents. Although some Leydig cell tumorigens also have mutagenic properties, many do not. The demonstration of nongenotoxic bases for Leydig cell hyperplasia and adenomas in test animals and the apparently greater susceptibility of test species to these lesions has made their relevance for human risk unclear.

A workshop (Clegg et al., 1996) was convened to review the available information on Leydig cell hyperplasia and adenomas and to reach consensus about the relevance of test animal results for human risk assessment. Apparent incidence is rare and restricted primarily to white males. Comparisons with incidence in test species are tenuous because the diagnosis in test animals is from a combination of gross observation and histological examination, and in humans is from palpation in selected populations. However, available data suggest a difference in the relative susceptibility of humans to Leydig cell tumorigenesis. Because uncertainties exist about the true incidence in humans, induction of Leydig cell adenomas in test species is of concern under some conditions. The work group focused on seven hormonal modes of induction of which two, GnRH agonism and dopamine agonism, were considered not relevant to humans. AR antagonism, 5α-reductase inhibition, testosterone biosynthesis inhibition, aromatase inhibition, and estrogen agonism were considered to be relevant or potentially relevant but for which quantitative differences may exist across species. Occurrence of Leydig cell hyperplasia alone in test species was not considered to constitute a cause for concern in a risk assessment for carcinogenic potential, but early occurrence could indicate a need for additional testing. Occurrence of Leydig cell adenomas in test species was of concern as both a carcinogenic and reproductive effect if the mode of induction and potential exposures could not be ruled out as relevant for humans.

d. Conclusions

Convincing evidence exists in rodents that exposure to chemicals that have estrogenic activity, reduce androgen level, or otherwise interfere with the action of androgen during development can cause male reproductive system abnormalities that include reduced sperm production capability and reproductive tract abnormalities. The type of abnormality observed is dependent on the developmental period in which the disruption of the normal endocrine balance occurred and the extent of the disruption. Results obtained from observation of men exposed to DES in utero provide data on the potential of exogenous estrogens to disrupt the reproductive system during development in human males. These data demonstrate that male reproductive tract anomalies are produced by DES, but in a limited proportion of the men and not at a level of severity that would be predicted from studies with mice that typically might receive 100 µg/kg (Bullock et al., 1988). The data indicate that there is a decrease in sperm production that may be limited to men with other effects as well (i.e., cryptorchidism and/or hypoplastic testes). There is no evidence that fertility was reduced in that population of men. The level of estrogenic activity to which the men in the DES study were exposed was very high, but levels early in gestation were substantially lower than levels in late gestation and not all women were given DES in early gestation. Therefore, it is not possible to state with certainty that the effects observed could not have been caused by the lower levels of exposure rather than by the higher levels experienced during late gestation. Occupational exposure to chlordecone (Kepone) was reported to cause oligospermia in men, an effect that was presumed due to the estrogenic activity of that agent (Cannon et al., 1978).

Until recently, the emphasis with respect to disruption of the male endocrine system by environmental agents has been on chemicals with estrogenic activity. It has been known for some time from work with test species, and to a lesser extent with human males, that chemicals with antiandrogenic activity also can disrupt the male reproductive system. The recent revelations that agents such as estradiol and DES, as well as the DDT metabolite DDE, also have antiandrogenic activity place significantly increased importance on that mechanism of action. It is quite possible that the effects attributed to estrogenic activity are due to antiandrogenic activity instead of or in addition to estrogenic activity. Therefore, it is important that testing for endocrine-disrupting potential of environmental chemicals include the ability to detect antiandrogenic activity in addition to estrogenic activity. Testing also should be able to detect alteration in AR function as reflected in genome expression.

Controversy persists as to the allegation that human sperm production has decreased over the past 50 years. However, the firm data indicating an increase in human testicular cancer, as well as apparent occurrence of other plausibly related effects, supports the concept that adverse effects have occurred or still exist.

e. Prostate Cancer

i. Background

Carcinoma of the prostate, an androgen-dependent organ, is the second leading cause of cancer deaths in males in the Unites States and remains incurable once it has metastasized. An estimated 200,000 new cases were diagnosed in the United States during 1994, along with about 40,000 deaths (Garnick, 1994). Increased incidence of prostate cancer in recent years is due in large part to increased detection screening (digital rectal examination and serum prostate specific antigen) in men over 50 years of age (Potosky et al., 1995). Death due to prostate cancer has increased by 17% over the past 30 years despite improved diagnosis. Cancer of the prostate is a disease of men over 50, with about 1 in 10 developing the disease by age 85. There are racial differences in susceptibility, the prevalence being rare in Orientals, 20 to 30 times higher in Caucasians, and even higher in African-American males (40% higher than whites).

Little is known about the causes of prostatic cancer, but age, genetics, endocrine status, diet, and environmental risk factors have been proposed. Apparently, no causative association between smoking, alcohol, coffee, tea, or caffeine consumption with human prostate cancer has been found (Slattery and West, 1993; Moller et al., 1994; van der Gulden et al., 1994). Serum concentrations of gonadotropins (FSH and LH), testosterone, androstenedione, estradiol, and sex hormone-binding globulin are not good predictors of risk (Andersson et al., 1993). Intake of dietary fat appears to be a risk factor in some studies (Pienta and Esper, 1993; Le Marchand et al., 1994). However, a recent case-control study in Sweden has failed to find an association between diet during childhood and prostate cancer risk (Andersson et al., 1995). Controversy also exists concerning the risk of prostate cancer following vasectomy.

The possible role of chemical exposure and endocrine disruption as a contributing factor in the etiology of adenocarcinoma of the prostate must be considered. In a retrospective cohort epidemiology study of Canadian farmers linked to the Canadian National Mortality Database, a weak but statistically significant association (rate ratio = 1.19, 95% confidence interval = 0.98-1.45) between acres sprayed with herbicides and prostate cancer deaths was found (Morrison et al., 1993). In a 30-year followup study of coke oven workers, an association of coke oven emissions with significant excess mortality from cancer of the prostate has been observed (Costantino et al., 1995).

Endpoints of chemically induced carcinogenesis in animal models include incidence, tumor number, and latency (time to tumor). Shirai et al. (1992) have studied N-hydroxy-3,2'-dimethyl-4-amino biphenyl (N-OH-DMAB) induction of prostate carcinogenesis in rats. Groups of F344 rats were administered biweekly intraperitoneal injections of N-OH-DMAB at a dose of 5, 10, or 20 mg/kg body weight or of DMAB, the parent compound, at a dose of 25 mg/kg body weight,

for a total of 10 doses. Prostate carcinomas in the ventral lobe developed in an N-OH-DMAB dose-dependent manner (0, 17.6, and 66.7%, respectively) with limited tumor yields in other organs.

There is some evidence for a role of the heavy metal cadmium in prostate cancer etiology in some epidemiology and animal studies (Waalkes and Rehm, 1994).

ii. Toxicity Testing in Animals and Extrapolation to Humans

Research on the etiology of prostate cancer has been hindered by the lack of suitable animal models for study. The development and validation of animal models for testing xenobiotic chemicals that are predictive of risk for human adenocarcinoma of the prostate are essential. In contrast to its frequent occurrence in humans, prostate cancer is rare in laboratory rodents. Therefore, to make this disease more amenable for study, there is a growing effort to identify or develop a means to target carcinogenesis in the prostate gland of rodents. This goal is being approached with the use of three different methods: one method takes advantage of the unique androgenic hormone requirement for prostate growth to exaggerate the effects of carcinogens at that site, and two methods (recombinant retrovirus transduction prior to organ reconstitution and transgenic targeting) allow direct genetic manipulation of cells in the prostate gland leading to the development of prostatic malignancy (Buttyan and Slawin, 1993).

Short-term treatment of rats with chemical carcinogens produces a low incidence (5 to 15%) of prostate cancer, provided that prostatic cell proliferation is enhanced during carcinogen exposure. Chronic treatment with testosterone also induces a low prostate carcinoma incidence. A high carcinoma incidence can be produced only by chronic treatment with testosterone following administration of carcinogens such as N-methyl-N-nitrosourea (MNU) and DMAB. Testosterone markedly enhances prostate carcinogenesis even at doses that do not measurably increase circulating testosterone. Thus, testosterone is a strong tumor promoter for the rat prostate.

Transgenic mouse models for prostate cancer have been developed, inserting the mouse int-2 or the rat prostatic steroid binding protein C3(1) genes, respectively (Thompson et al., 1993; Maroulakou et al., 1994). These models offer the opportunity of studying hormone response elements in vivo and the multistage progression of adenocarcinoma of the prostate. Another promising model for human prostate cancer metastasis employs the orthotopic (but not ectopic) implantation of human prostate cells (PC-3M and LNCaP) in BALB/c nude mice (Stephenson et al., 1992). The transplantation of human prostatic carcinoma cells in nude mice is enhanced when injected in Matrigel (Passaniti et al., 1992). A review of animal models for the study of prostate carcinogenesis has been published (Bosland, 1992).

iii. Conclusions

Currently, the weight of available evidence linking herbicides or PAHs to prostate cancer is weak, and more epidemiologic and animal research is required before assigning a specific endocrine disruption (or any other) mechanism as a specific cause of human adenocarcinoma of the prostate.

3. Hypothalamus and Pituitary

The CNS plays a major role in integrating hormonal and behavioral activity. Disturbances in these finely coordinated mechanisms can severely impair normal adaptive behavior and reproduction. During development and in adult life, the brain is a target tissue for the action of gonadal hormones. Similarly, hormones regulate many behavioral activities and vise versa (e.g., epinephrine prepares the "fight-or-flight" response; suckling releases oxytocin).

a. Mammalian Development

The developing nervous system is particularly sensitive to hormones and insult by drugs and environmental chemicals; the specific processes of sexual differentiation of the brain represent an excellent example of this sensitivity. In rodents, sexual differentiation of the CNS can be modified by experimental hormone treatments administered shortly before or shortly after birth. In contrast, differentiation of the gonads and reproductive tract occurs earlier in gestation. Before gender differentiation, the brain is inherently female or at least bipotential (Gorski, 1986). Thus, the functional and structural sex differences in the CNS are not due directly to sex differences in neuronal genomic expression, but rather are imposed or imprinted by the gonadal steroid environment during development.

In the CNS, testosterone is metabolized to both estradiol and dihydrotestosterone (DHT). In the rat, mouse and hamster, the aromatization of testosterone to estradiol is responsible for CNS sex differentiation, whereas in certain other mammals (e.g., rhesus monkey) the androgenic (DHT) pathway appears to be essential (McEwen, 1980). In humans, the role of estrogens in CNS sexual differentiation remains uncertain.

If one administers exogenous steroids (i.e., testosterone propionate) to the genotypic female rodent within the first week of postnatal life, her neuroendocrine system will differentiate phenotypically male (i.e., her brain is masculinized). Such masculinization of the female brain by the aromatization of testosterone to estrogen in the brain also is reflected in similar masculinizing effects observed with low doses of estrogen or DES, treatments without effect on the genotypic male. This "masculinized" female (1) does not ovulate, (2) has polyfollicular ovaries, (3) displays persistent vaginal estrus, (4) does not show positive feedback to gonadal hormones (i.e., an ovulatory surge of LH cannot be stimulated), and (5) exhibits sexual behavior more typical of that

observed in the genetic male. In contrast, the opposite is seen following castration in early postnatal life. Removal of the ovaries from the neonate is without major effect on sexual differentiation of the female rodent brain. However, if the testes are removed before the third postnatal day of life, this male at adulthood exhibits neuroendocrine characteristics typical of the female, including both the ability to release a cyclical surge of LH and to exhibit feminine lordotic (posture in the female of reproductive receptivity) behavior. The timing of these important developmental endocrine events responsible for sexual differentiation of the human brain remains poorly defined but appears to occur earlier in fetal development than in rodents.

A number of organochlorine pesticides, including Kepone (chlordecone) (Gellert, 1978), DDT (Bulger and Kupfer, 1985), methoxychlor (Gray et al., 1989), and the mycoestrogen zearalenone (Kumagai and Shimizu, 1982), have been shown to masculinize female rats. In contrast, purported anti-estrogens, such as tamoxifen (Döhler et al., 1984), demasculinize the male, including the size of the sexually dimorphic nucleus of the preoptic area such that it resembles that observed in the female. Exposure of newborn female rats to these xenoestrogens during the critical periods of sexual differentiation has been shown to perturb reproductive processes in later life, presumably by altering the development of the neural mechanisms regulating gonadotropin secretion. For example, the sexually dimorphic nucleus has been argued to vary with the degree of masculinization induced by phytoestrogens (Faber and Hughes, 1993). Phytoestrogens are naturally occurring nonsteroidal plant chemicals with estrogen-mimetic properties.

Investigations in the neonatal rat also indicate that analogs of DDT, i.e., 1-(o-chlorophenyl)-1(p-chlorophenyl)-2,2,2 -trichloroethane (o,p'-DDT), also may have estrogenic activity at the neuroendocrine level. Heinrichs et al. (1971) found that female rats given o,p'-DDT as neonates exhibited advanced puberty (vaginal opening), persistent vaginal estrus after a period of normal cycling, follicular cysts, and a reduction in the number of corpora lutea (anovulation). TCDD administered by gavage to pregnant female Long-Evans Hooded and Holtzman rats on gestational day 15 at 1 µg/kg causes a delay in puberty and incomplete opening of the vaginal orifice in female offspring (Gray and Ostby, 1995).

In the male rat, treatment with aromatase inhibitors such as fenarimol has been hypothesized to inhibit normal masculinization of the brain (Hirsch et al., 1987). The antiandrogen vinclozolin, which acts as an AR blocker and does not reduce the aromatization of testosterone to estrogen, was not found to alter male sexual behavior after perinatal treatment (albeit the reproductive tract was affected). Although a hormonal influence on sexual differentiation of the CNS may vary somewhat among different species, some role for gonadal hormone modulation of CNS development has been indicated in most animals studied.

In summary, sexual differentiation may be affected by a variety of environmental

compounds. Although the majority of effort has focused on those compounds reported to have steroidogenic activity, it may be premature to assume that other nonsteroidal compounds are without effect on sexual differentiation of the brain. The masculinizing effects of androgens on the female brain can be partially blocked by neuroactive drugs such as reserpine and chlorpromazine, while pentobarbital and phenobarbital provide more complete protection against testosterone (Arai and Gorski, 1968). The mechanisms through which such interactions occur remain to be elucidated. These observations suggest that other mechanisms involved in sexual differentiation of the CNS may render this process susceptible to disruption by environmental compounds that do not necessarily possess steroidogenic activity.

b. Multiple Control of Pituitary Hormones

The synthesis and release of pituitary hormones is under the feedback control of hormones (e.g., steroids) circulating in the blood as well as by releasing and inhibiting hormones or factors manufactured within specialized neurons located in the hypothalamus. The releasing hormones in turn are regulated by several types of feedback signals and by multiple nervous influences that include the classical neurotransmitters (e.g., acetylcholine, catecholamines, serotonin) and several neuropeptides (e.g., opioids, galanin, neuropeptide-Y) (Kalra and Kalra, 1983). As a result, it has been demonstrated that many pharmaceutical agents can modify pituitary hormone secretion. This may be brought about by a direct action on the pituitary by synthetic steroids (e.g., DES-induced increase in prolactin synthesis) or agents that act on pituitary receptors directly (e.g., bromocryptine inhibition of prolactin release) or through compounds that affect neurotransmitter or neuropeptide regulation of releasing factors. The effects of various therapeutic agents on reproductive function are well established. These drugs may either depress CNS activity (i.e., anesthetics, analgesics, and tranquilizers) or stimulate it (i.e., antidepressants and hallucinogens). In fact, a variety of such agents often are used to probe the central control of neuroendocrine function. Drugs of abuse also have been shown to alter the hormonal control of reproduction through a CNS mechanism.

Delta-9-tetrahydrocannabinol (delta-9-THC), the major psychoactive component of marijuana, significantly reduces LH, FSH, prolactin, and testosterone concentrations in the blood and causes decrements in sexual organ weights (Dalterio et al., 1978). In the female rat, delta-9-THC has been shown to suppress serum gonadotropin secretion, disrupt estrous cyclicity, and delay sexual development. Correspondingly, studies in the rhesus monkey have shown that a single injection of delta-9-THC produces a longstanding depression of gonadotropin levels (Smith et al., 1980). In humans, similar reports of decreased testosterone levels and significant changes in sperm count and morphology have been reported, although there is not general agreement in this regard (Smith et al., 1980). There is general consensus that the influence of delta-9-THC on

reproductive function is mediated through changes in hypothalamic control of pituitary function. Similarly, opiates also appear to exert their primary effect on the hypothalamic-pituitary axis. Such changes in central regulation of the neuroendocrine axis result in dysfunction of the gonads and sex accessory organs in both humans and laboratory animals.

A number of recent studies have examined the effect of xenobiotic exposure on the regulation of the ovulatory surge of LH in the rat. The timing of this endocrine event is critical for normal fertilization and pregnancy. Although there are differences in ovarian cycle length in rats and humans, considerable homology exists in these two spontaneously ovulating species in the CNS-pituitary mechanisms controlling LH secretion. The generation of the LH surge is under control of the pulsatile release of hypothalamic GnRH. This releasing factor is in turn regulated by hypothalamic neurotransmitters (especially norepinephrine) and opioid peptides (enkephalins) and gonadal steroids. Agents that disrupt the synthesis of norepinephrine (e.g., fusaric acid, α-methyl-para-tyrosine [Kalra and Kalra, 1983]) or agents that interfere with α-noradrenergic (α-NE) receptor stimulation (e.g., phenoxybenzamine and phentolamine [Ratner, 1971; Plant et al., 1978]) will disrupt the pattern of GnRH secretion and consequently the LH surge. Similarly, morphine exerts an inhibitory effect on LH secretion in the male and female of several mammalian species (see Cooper et al., 1986b for review). Goldman et al. (1990, 1991) have shown that a single exposure to the formamidine pesticide chlordimeform can, depending on timing, inhibit the ovulatory surge of LH and that this effect is mediated via an inhibition of hypothalamic α-NE receptors. Furthermore, Cooper et al., (1994) demonstrated that this disruption of the LH surge in the female rat can alter the outcome of the ensuing pregnancy (i.e., reduce litter size). The dithiocarbamates are known to lower CNS norepinephrine through an inhibition of the enzyme dopamine-β-hydroxylase, which synthesizes norepinephrine from dopamine. Stoker et al. (1993) have shown that thiram (tetramethylthiuram disulfide) also interferes with the generation of the LH surge, delaying ovulation and altering pregnancy outcome. This effect on female fertility does not appear to be restricted to disruption of noradrenergic neurotransmission, because methanol (Cooper et al., 1992) and sodium valproate (Cooper et al., 1994) have been found to have the same effects on the LH surge, ovulation, and pregnancy outcome. Cocaine administered subcutaneously causes a dose-dependent disruption of estrous cyclicity, reduced serum LH levels, and reduction of ovulation in female rats (King et al., 1993). Valproic acid exerts its effect on hormone secretion by binding to the γ-aminobutyric acid receptors and mimicking the effects of this neurotransmitter in both the rat and human (Jones, 1991). The mechanism by which methanol alters LH secretion remains to be determined.

Because steroid hormones have a significant role in the regulation of anterior pituitary function, it is not surprising that xenoestrogens also may modify this influence on the hypothalamus and pituitary. In the male, many of the adverse effects of exposure to

xenoestrogens on testicular function have been attributed to a direct action on the testes (e.g., see Cooper et al., 1986b for review). However, adverse effects of estrogens on male reproduction also may be mediated by a direct action on the hypothalamus and pituitary, tissues that are rich in estrogen receptors (Pfaff and Keiner, 1973). Furthermore, changes in pituitary hormone secretion were noted sooner and at lower doses of DES than those required to alter any testicular measures (Cooper et al., 1985). Doses of methoxychlor that have no detectable effect on testicular function or reproductive performance in the male rat (i.e., 25 and 50 mg/kg/day) elevate serum and pituitary prolactin levels (Goldman et al., 1986).

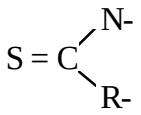
4. Thyroid Effects

a. Background

The thyroid gland consists of two lobes of endocrine tissue located just below the larynx on each side of the trachea. The function of this organ is to secrete thyroid hormones, which are critical for normal growth and differentiation and are important regulators of overall metabolism in most tissues. The functions of this gland are susceptible to insult by dietary factors, pharmacologic agents, and environmental chemicals that may interfere with thyroid hormone biosynthesis, transport, or receptor interactions.

The basic precursors of thyroid hormone biosynthesis are iodide (primarily from dietary sources) and thyroglobulin (a glycoprotein found in the thyroid follicular cells). Iodide must first be taken up from circulation, a process that can be inhibited by a number of ions such as thiocyanate and perchlorate. After the iodide is trapped in the gland, it is oxidized to hypoiodate, a reaction mediated by thyroid peroxidase. The active form of iodide is then coupled to the tyrosine residue of the thyroglobulin, resulting in the formation of monoiodotyrosyl and diiodotyrosyl residues. Coupling of monoiodotyrosyl and diiodotyrosyl residues forms triiodothyronine (T_3), or coupling of two diiodotyrosyl residues forms thyroxine (T_4). T_3 and T_4 are stored within thyroglobulin or secreted into the circulation by a proteolytic reaction. T_4 is highly bound to transport proteins, such as thyroxine-binding globulin (TGB), and transthyretin, in circulation and is converted to T_3 (the active form of the hormone that binds to the thyroid receptor) in peripheral tissues. Biosynthesis and secretion of thyroid hormones are under feedback controls of the hypothalamic (thyrotropin-releasing hormone, or TRH)-pituitary (thyroid-stimulating hormone, or TSH)-thyroid axis. Although a great many compounds disrupt the synthesis of T_3 and T_4, with few exceptions, they can be classified into three main groups according to their basic chemical structure: thionamides (e.g., propylthiouracil and mercaptoimidazole), aminoheterocyclic compounds (e.g., sulfonylureas such as tolbutamide), and substituted phenols (e.g., resorcinol and salicylamide). Derivatives of thiourea, including thiouracils, cause functional hypothyroidism and hypertrophy, hyperplasia, and

hypervascularization of the gland (Schalock et al., 1979). The thioureas, the aminothiazoles, and the mercaptoimidazoles, which inhibit thyroid hormone formation, all contain the following configuration in which R may be a sulfur, oxygen, or nitrogen atom (Ingbar and Woeber, 1974).



The serum carrier proteins TBG and transthyretin are important to the half-life and biological activity of thyroid hormones. Humans have both these proteins; however, rodents lack TBG but have transthyretin (Porterfield, 1994). The presence of the carrier proteins allows larger quantities of these fat-soluble hormones to be carried in the blood and delays excretion and metabolism of the hormone. They also may play an important role in the availability of the hormones for placental transport. Because some environmental toxicants (e.g., PCBs) can compete with thyroid hormone for binding to these carrier proteins, the toxicants can lower the availability of the hormone to the tissue (McKinny, 1989; Bastomsky et al., 1976).

Abnormalities of thyroid function are among the most common of all endocrine disorders. The two major categories of thyroid disease are hyperthyroidism and hypothyroidism. The altered thyroid state may lead to a number of physiological abnormalities, including changes in the basal metabolic rate (increased in hyperthyroidism, and decreased in hypothyroidism); lipid metabolism (lipemia, hypercholesterolemia, and fatty infiltration of the liver in hypothyroidism and a decrease in serum cholesterol in hyperthyroidism); cardiovascular functions; gastrointestinal functions, especially food intake and energy expenditure as well as alterations in gastric motility and absorption (i.e., glucose uptake); and muscle function (Hedge et al., 1987).

While thyroid hormones play key roles in the maintenance of homeostasis, they are particularly important to processes involving growth and development. The most striking effects of these hormones are observed during maturation of the brain. The absence of thyroid hormones during this period produces multiple morphologic and biochemical alterations and in humans leads to irreversible mental retardation. Conversely, a pattern of accelerated maturation is associated with hyperthyroidism, although these changes should not be viewed as beneficial as they invariably lead to neurochemical and behavioral deficits. While sparse data exist for humans, it is known that the period between the end of the first trimester of gestation and 6 months after birth is the period of active neurogenesis and most active phase of the brain growth spurt. The brain is particularly vulnerable to various insults during this period. Specific receptors for T_3 exist both in the cerebrum and cerebellum, are present at a higher concentration at an early age, and are preferentially found in neuronal cells with regional differences in their distribution. Most of the biochemical effects of hypothyroidism become irreversible if replacement therapy is delayed after

the critical period of development, which in rats usually spans the first 10 to 14 days after birth (Dussault and Ruel, 1987). Experimental perinatal hypothyroidism, in which circulating T_4 was virtually eliminated by drug treatment (e.g., propylthiouracil [PTU], methimazole) or surgery, is associated with overall growth retardation, delayed morphologic and neurochemical development of the brain with attendant deficits in neurobehavioral maturation, malformations of the organ of Corti and auditory dysfunction (Deol, 1973; Uziel et al., 1980, 1981), alterations of peripheral nervous system, and developmental delays in eye opening and weaning (Porterfield, 1994).

Numerous environmental agents have been reported to alter thyroid hormone levels in humans, wildlife animals, and laboratory animal models. Typically, hypothyroidism is the consequence of exposure to environmental chemicals (PCB, TCDD, methoxychlor, thiocarbamide, and sulfonamide-based pesticides, to name a few), as indicated by reduction of thyroid hormones in circulation, TSH elevation, and thyroid follicular neoplasia. A partial list of these compounds from EPA's Office of Pesticide Programs' Health Effects Division's database may be found in table 1 (appendix). The putative mechanisms of thyrotoxicity may vary and include specific damages to the endocrine gland (e.g. PCB), alterations of hypothalamic-pituitary-thyroid axis (e.g., methoxychlor), interferences of hormone transport, and receptor interactions (e.g., PCB). Curran and DeGroot (1991) have called attention to the effect of hepatic enzyme-inducing drugs that metabolize and clear thyroid hormones from the circulation and thus alter hormone control mechanisms (increasing TSH), which could lead to thyroid hyperplasia and tumors. Recently, a mechanistic model of carcinogenic effects of TCDD on thyroid follicular tissue in the rat has been demonstrated (Kohn et al., 1996). Consequently, it should be noted that environmental agents that produce hypothyroidism can have potential physiological and developmental adverse impacts on an organism.

Perhaps the most studied examples of environmental agents that alter thyroid function are the polyhalogenated biphenyls (including the polybrominated biphenyls and PCBs) and the family of chlorinated dibenzo-p-dioxins (TCDD). Both groups of compounds are present in the environment, and some PCB contamination is seen virtually everywhere in the United States. There are multiple forms of these compounds, and their actions on the thyroid depend both on the specific form studied and the dosage of toxin used. Some forms of these toxicants are quite stable, and because they are fat soluble, they are accumulated in the adipose tissue. They can be bioconcentrated in the environment, and fish from contaminated waters can contain relatively high amounts. The toxicants cross the placenta and are also concentrated in milk so that the fetus and newborn can be exposed by a contaminated mother both through the placenta and through her milk (Takagi et al., 1976; Collins and Capen, 1980). PCBs, dioxins, and the active thyroid hormones T_4 and T_3 show similar structural properties that appear to be important in molecular recognition in biochemical systems (McKinny, 1989).

In laboratory animals, manifestations of thyrotoxicity induced by environmental agents resemble those produced by drugs or surgery. For instance, development of the CNS cholinergic neurons are exquisitely sensitive to the thyroid status. In rats, perinatal exposure to some PCBs (specific congeners or mixtures such as Aroclor 1254) has been shown to lower serum T_4 and reduce choline acetyl transferase (ChAT) activity (Juarez et al., 1994) in the hippocampus and basal forebrain. ChAT is an enzyme involved in the synthesis of acetylcholine, a neurotransmitter considered important to learning and memory. T_4 replacement was capable of reversing the PCB-induced deficits in ChAT. The particular susceptibility of the developing peripheral auditory system to thyroid hormone deprivation is well known. The onset of evoked cochlear electrical activity (which is postnatal in the rat) is delayed by hypothyroidism and is returned to normal by thyroid hormone administration (Meyeroff, 1979; Uziel et al., 1980). Consistent with the hypothyroidal effects of PCBs, Aroclor 1254 was found to produce permanent auditory deficiencies following perinatal exposure (gestational day 6 to postnatal day 21), in a manner similar to those elicited by the goitrogenic drug PTU (Goldey et al., 1995a,b).

In humans, hypothyroidism has been linked to occupational exposure to PBBs (Bahn et al., 1980) and PCBs (Murai et al., 1987). Many of the symptoms of PCB poisoning such as epidermal abnormalities, fatigue, mental apathy, and memory deficits are similar to those resulting from non-PCB-induced hypothyroidism. Accidental exposure to PCBs by pregnant women in Yu-Chen, Taiwan, led to a host of delays in physical and mental development of their offspring not dissimilar to those associated with hypothyroidism (Hsu et al., 1985; Chen et al., 1992). These included weight and size deficits at birth that persisted as the children matured (a hallmark of hypothyroid effect in animal models) and IQ deficits. In addition, children born to women who ate more PCB-contaminated fish had lower IQ and exhibited behavioral problems (Jacobson et al., 1990). Recent clinical studies further demonstrated hypothyroid status in the infants whose mothers have been exposed to PCB, dioxin, and dibenzofurans (Koopman-Esseboom et al., 1994), while high levels of these environmental contaminants in the breast milk have been related to reduced neonatal neurological capacity and high incidence of hypotonia (Huisman et al., 1995). Indeed, perinatal exposure to PCB and TCDD are of particular concerns to the risk assessment for human health. Maternal ingestion of these contaminants results in its transfer to human neonates through the placenta and by breastfeeding (Nishimura et al., 1977; Masuda et al., 1978). Children's exposure to these lipophilic chemicals can be 10 to 40 times greater than the daily exposure of an adult (World Health Organization, 1989).

Although the actions of thyroid hormone in higher organisms are critical to normal growth, differentiation, and metabolic regulation, there is an increasing body of data suggesting a critical involvement of thyroid hormones in the carcinogenic process. There are data demonstrating that the thyroid status of experimental animal models and humans dramatically affects tumor

formation, growth, and metastasis (Guernsey and Fisher, 1990). Relevant to the issue of endocrine disruptors are the findings that thyroid hormones dramatically stimulate the proliferation kinetics of MCF-7 mammary cancer cells in culture and that antiestrogens prevent the stimulatory effects of T_3 on MCF-7 proliferation (Zhou-Li et al., 1992). It also has been reported that estrogen stimulates postconfluent cell accumulation and foci formation of human MCF-7 breast cancer cells (Gierthy et al., 1991) while dioxin (TCDD), a potent inducer of differentiation and an antiestrogenic substance, inhibits this process (Gierthy and Lincoln, 1988).

5. Endocrine Disruptors and Immunotoxicology

The interrelationship of endocrine and immune systems is complex, but research into this area is progressing rapidly (Weigent and Blalock, 1987; Fuchs and Sanders, 1994; Weeks et al., 1992). The elucidation of this interaction between endocrine and immune systems is made more challenging with the addition of species diversity (e.g., shellfish, fish, birds). However, where evaluated, endocrine and immune functions are somewhat similar to those found in mammals (Colborn and Clement, 1992; Dickerson et al., 1994; Hontela et al., 1995; Ross et al., 1995).

It is beyond the scope of this document to assess the relationship between endocrine and immune systems. A recent review on the interactions of the immune, neural, and endocrine systems has been published (Besedovsky and Del Rey, 1996). Instead, the present discussion briefly summarizes a few of the key immunotoxicology issues in the context of endocrine function.

Immune systems in most vertebrate animals typically consist of a diffuse and complex set of lymphoid structures and of innate and inducible immune functions (such as phagocytosis, antibody formation, and cell-mediated immunity). The purpose of these immune systems is most often to protect such organisms from various forms of foreign invaders. The deleterious effects of chemicals on the immune system of animals has been briefly reviewed by De Guise et al. (1995). Three classes of undesirable effects have been identified that may occur when the immune system is perturbed by exposure to chemicals in the environment: (1) immunodeficiency or immunosuppression; (2) alterations of natural, genetically controlled host defense mechanisms; and/or (3) hypersensitivity or allergy. The alteration of (mammalian) immune responses is often reflected by changes in an organism's susceptibility to disease agents, parasites, latent viral infections, and even tumor formation (Dean et al., 1994; Luster et al., 1992, 1993; Office of Technology Assessment [OTA], 1991).

Several xenobiotics, such as therapeutic drugs, pesticides, metals, and/or other persistent environmental contaminants (dioxins, PCBs, PAHs, etc.), are either already known to or are suspected of directly and adversely impacting immune structures and functions in humans, laboratory and field mammals, avian species, fish, and even invertebrates (Anderson, 1992; Anderson and Zeeman, 1995; Dickerson et al., 1994; Fairbrother, 1994; Gleichmann et al., 1989;

Luster et al., 1988; Luster et al., 1992, 1993; Ross et al., 1995; Zeeman and Brindley, 1981; Zeeman, 1996). Dioxin (2,3,7,8-TCDD) is just one contaminant that has been demonstrated to impact a wide variety of immune parameters, for example, thymic atrophy, antibody responses, and impaired disease resistance (Dean et al., 1994; Gleichmann et al., 1989; Luster et al., 1992; OTA, 1991; Smialowicz et al., 1994). Besides TCDD, several other xenobiotics have been implicated as possibly affecting immune structures and functions. Recent reviews on immunotoxicity and the possible impacts of heavy metals (Bernier et al., 1995), pesticides (Thomas, 1995), and PCBs (Tryphonas, 1995) in relation to human health and the Great Lakes have been published.

Similarly, it is known that immune systems are also regularly being directly and indirectly affected by normal endocrine functioning in animals (Fuchs and Sanders, 1994; Hall and Goldstein, 1986; Weigent and Blalock, 1987). For example, the human immune system can be orchestrated by the normal circadian rhythms found in the release and action of glucocorticoid hormones, such as cortisol (Hrushesky, 1994).

There is a normal daily rhythm in the levels of cortisol found circulating in the blood of humans (and in other vertebrate animals). In humans, the highest cortisol levels are found in the morning and the lowest in the evening. An inverse relationship also is found between the blood cortisol levels and the immune parameters of inflammatory responses and numbers of circulating leukocytes. This rhythmic endocrine presence probably is a dominant feature influencing the fact that the number of leukocytes in the blood fluctuates regularly with a variation of as much as 50% in a day (Hrushesky, 1994).

This effect is not surprising because cortisol is one of the stress (flight or fight) hormones known to adversely impact the number of white blood cells found in the blood. The adverse impacts of glucocorticoids, especially cortisol and cortisone, on the immune systems of other vertebrates in the environment also seem somewhat consistent with the experiences found in mammals (Anderson and Zeeman, 1995; Fowles et al., 1993; Weeks et al., 1992; Zeeman and Brindley, 1981).

As is amply demonstrated in other parts of this review, endocrine system functioning can be adversely impacted by a wide variety of xenobiotics (Kubiak et al., 1989; Colborn and Clement, 1992; White et al., 1994; Fry, 1995; LeBlanc, 1995). In fact, humans have intentionally developed and already widely used such chemicals as pesticides in the environment (e.g., insect growth regulators). Therefore, it should not be surprising to find that the adverse impacts of xenobiotics on endocrine functioning can thereby (directly and indirectly) also significantly influence the structures and functions of the immune system and its normal protective responses against foreign bacteria, viruses, parasites, and so on.

This is an important area for consideration. However, as should be evident from the brief

presentation above, these relationships are complex and actively evolving. Despite the best of intentions, it probably will not be easy to tease out the complex relationships between the impacts of numerous diverse xenobiotics on (1) endocrine functions, (2) immunologic functions, and (3) the diverse types of species in which these chemicals could have an impact on both these systems and their interactions.

B. EFFECTS ON AQUATIC LIFE AND WILDLIFE

1. Background

There is increasing evidence that a number of chemicals in the environment may disrupt the endocrine systems of aquatic life and wildlife. This includes both manmade chemicals (xenobiotics) and chemicals that occur naturally in plants such as phytoestrogens.

a. Synthetic Chemicals (Xenobiotics)

Many synthetic chemicals have been labeled as suspected environmental endocrine disruptors and are addressed briefly below. These include alkylphenols, bisphenol-A, 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzo-furan (TCDF), PCBs, and some pesticides.

Some of the chemicals thought to be environmental endocrine disruptors are in commerce today in the United States; however, many other xenobiotics have been prohibited previously from use in the United States because of their adverse effects on human health and the environment. Some of these xenobiotic chemicals not in use today in North America persist in the environment. They are transported and deposited via atmospheric transport from other parts of the world that still use them or from previous environmental contamination (Geisy et al., 1994). Environmental residues of some xenobiotic compounds have decreased after these chemicals were banned or canceled, but many others have leveled off because of physical properties that cause them to accumulate in sediments, be re-released to the aquatic environment, and accumulate in the tissues of organisms.

Purdom et al. (1994) suggested that alkylphenol-polyethoxylates (APE), originating from the biodegradation of surfactants and detergents during sewage treatment, and ethynylestradiol, originating from pharmaceutical use, are the two most likely sources of the estrogenic substances present in sewage effluent. Alkylphenols, such as nonylphenol, are commonly used as antioxidants and also are degradates of the biodegradation of a family of nonionic surfactants (such as APE) during sewage treatment (Jobling and Sumpter, 1993).

Nonylphenol and other alkylphenols have been reported to leach from plastics used in food processing and packaging, such as food grade polyvinyl chloride (Junk et al., 1974; Brotons et al., 1995). In the development of a screening assay for estrogenic compounds, nonylphenol was

discovered to leach from polystyrene laboratory ware (Soto et al., 1991) and bisphenol-A was released from plasticware during autoclaving (Krishnan et al., 1993).

TCDD and TCDF also are suspected of being environmental endocrine disruptors. They are byproducts of the paper, wood, and herbicide industries and are formed in the incineration of some chlorinated organic compounds (Schmidt, 1992).

PCBs are a class of compounds that have approximately 113 congeners present in the environment. PCBs, which disrupt hormone pathways involved in, for example, male fertility (Sager, 1983), were banned from further production in the United States in 1976 under the Toxic Substances Control Act, but these agents were used widely between 1930 and 1970 as additives in products such as paints, plastics, rubber, adhesives, printing ink, and insecticides (Peakall and Lincer, 1970). While 31% of total PCBs manufactured are currently estimated to be present in the global environment, only 4% of cumulative world production can be accounted for as degraded or incinerated. Many PCBs are still in use in older electrical equipment (e.g., transformers), in containment storage, or in dumps or landfills. Releases from these sources can result in continuing PCB pollution for years to come (Tanabe, 1988).

Evidence also exists that pesticides such as alachlor, DDT, dicofol, methoxychlor, chlordane, and many others can disrupt the endocrine systems of fish and feral species. Various pesticides with suspected endocrine disruption capabilities are listed in table 2 (appendix).

b. Phytoestrogens

Phytoestrogens, which are hormone-mimicking substances naturally present in plants, are suspected of interfering with the endocrine systems of grazing animals (see review by Hughes, 1988). Specific compounds that have been identified as phytoestrogens include coumestrol, formononetin, daidzein, biochanin A, and genistein. In all, more than 300 species of plants in more than 16 families are known to contain estrogenic substances (Hughes, 1988). Some examples of plants that contain phytoestrogens include beets, soybeans, rye grass, wheat, alfalfa, clover, apples, and cherries. These agents are responsible for the depression of fertility observed in sheep grazing on clover pastures, decreasing serum progesterone or pituitary LH. Plant sterols in paper pulp mill effluent also may be responsible for the masculinizing effect observed in fish downstream of pulp mills (Davis and Bartone, 1992). It should be noted that some phytoestrogens (e.g., naringenin) can be both estrogenic and antiestrogenic (Ruh et al., 1995).

2. Endocrine-Related Effects

We know that certain chemicals can affect normal endocrine function and that certain endocrine-disrupting chemicals can substantially reduce some animal populations. We also know that there can be extreme differences in the susceptibility between species to these chemicals.

These differences are exploited specifically by chemists in the development of pesticides designed to disrupt insect endocrine systems through an array of compounds, which are collectively referred to as insect growth regulators. Thus, the endocrine systems of insects have been intentionally targeted for insecticidal activity. These chemicals include juvenile hormone mimics (e.g., methoprene), antijuvenile hormone analogs (e.g., precocene), chitin synthesis inhibitors (e.g., diflubenzuron), ecdysone analogs (e.g., tebufenozide), and molting disruptants (e.g., fenoxycarb). These insect growth regulators were developed to be not only efficient pesticides, but also to be highly specific to insects without risk to other nontarget animals, especially vertebrates. Although these compounds can be active against some insect species and not others, studies have documented the sensitivity of certain nontarget arthropods, especially crustaceans, to these compounds (Christiansen et al., 1977a,b, 1979; Cunningham, 1976; Forward and Costlow, 1978; Landau and Rao, 1980; Nimmo et al., 1980; Touart and Rao, 1987). Besides the insect growth regulators, the well-known case of DDT and its effects on avian eggshell thinning has been linked to endocrine pathways (Jefferies, 1975). Evidence is accumulating that many chemicals released into the environment can disrupt normal endocrine function in a variety of fish and wildlife.

Some of the deleterious effects observed in aquatic life and wildlife that may be caused by endocrine-disrupting mechanisms, as summarized by Colborn et al. (1993), include the following:

- Abnormal thyroid function in birds and fish (Moccia et al., 1981, 1986; Leatherland, 1992)
- Decreased fertility in birds, fish, shellfish, and mammals (Shugart, 1980; Leatherland, 1992; Gibbs et al., 1988; Reijnders, 1986)
- Decreased hatching success in fish, birds, and reptiles (Mac et al., 1988; Kubiak et al., 1989; Bishop et al., 1991)
- Demasculinization and feminization of fish, birds, reptiles, and mammals (Munkittrick et al., 1991; Beland, 1989; Guillette et al., 1994; Fry and Toone, 1981)
- Defeminization and masculinization of fish and gastropods (Davis and Bartone, 1992; Ellis and Pattisina, 1990)
- Alteration of immune function in birds and mammals (Erdman, 1988; Martineau et al., 1988).

3. Representative Examples

a. Invertebrates

In field studies, Reijnders and Brasseur (1992) report that female marine snails with male genitalia, including a penis and vas deferens, are now common. The cause of this phenomenon is exposure to tributyltin (TBT) compounds, which are used as marine antifouling paints on ships.

TBT is an extremely toxic chemical that, at sublethal levels, also appears to have significant hormonal effects, leading to what appears to be an irreversible induction of male sex characteristics on females (imposex) (Gibbs and Bryan, 1986).

Bryan et al. (1988) found that populations of the dog-whelk snail (*Nucella lapillus*) were disappearing or diminishing in many locations along the United Kingdom coast due to the effects of TBT. Gibbs et al. (1988) found that there was a direct dose-response relationship between exposure of the snails to TBT and the degree of imposex induced. This effect can be seen at levels (expressed as elemental tin concentrations) below 0.5 ng/l (wt/vol) equal to parts per trillion (ppt), although reproduction appears unaffected at these low levels (Gibbs et al., 1988). At slightly higher levels, 1 to 2 ppt, the penis becomes larger, and in some animals, the vas deferens tissue grows over the genital papilla and the organism becomes effectively sterilized. As concentrations increase, practically all of the animals become sterile. Finally, at levels of 10 ppt or higher, oogenesis is suppressed and spermatogenesis is initiated.

In additional studies, Bryan et al. (1988) specifically tested the ability of six tin compounds to induce imposex on female dog-whelks that were already slightly affected by this condition. Because of the widespread use of antifouling paints, the authors report that in England and Wales it is impossible to find unaffected populations. The six compounds were tested both by dissolving them in seawater over a 14-day exposure period and, in separate experiments, by a single injection to compensate for lack of absorption from water of some of the compounds. TBT was the most effective at inducing imposex. Neither di- nor monobutyltin had an effect on the snails. A fourth compound, triphenyltin, was also ineffective in inducing imposex, even though its toxicity is comparable to that of TBT for some organisms and has pesticidal and antifouling uses similar to TBT. A fifth chemical, tripropyltin, was accumulated from solution by the snails to a higher concentration than TBT and induced imposex, but it was far less effective than TBT. Tetrabutyltin was reported to cause a marginal increase in female penis size, but again, it was much less effective than TBT. Given TBT's strong effect, the authors concluded that the presence of imposex in dog-whelks may have utility as a biomarker for TBT. This is borne out by additional studies.

Bright and Ellis (1990) surveyed marine snails in northeast Pacific neogastropods for signs of imposex. They examined eight different species of marine snails in areas that contained differing amounts of TBT pollution, including four species from the genus *Nucella* (but not *Nucella lapillus*, the species of snail studied by, e.g., Gibbs and Bryan, 1986, which does not occur in the northeast Pacific). Imposex could be confirmed in all but one species of snail (*Amphissa columbiana*, Dall). One species, *Nucella emarginata*, showed the clearest positive relationship between degree of imposex and TBT concentrations due to its relatively short lifespan and much earlier age of maturity relative to the other species. Sterility due to imposex

(and consequent blockage of the genital pore) could be detected in only two of the eight species examined: *N. lamellosa* and *Neptunae phoenecia*. Evidence of a negative effect due to TBT pollution on a population of snails *(N. lamellosa*) was seen in a sampling of the Victoria Harbour breakwater, the most polluted of three sites examined. Juveniles of this species were underrepresented, and many adult females retained their egg capsules due to blockage of the genital pore. Bright and Ellis (1990) note that the selective loss of reproductive potential observed for *N. lamellosa* could potentially result in an alteration of the competitive interactions between sympatric species of *Nucella* (different species of *Nucella* often co-occur within the intertidal zone of British Columbia).

Ellis and Pattisina (1990) further report on imposex observed in neogastropod molluscs from Singapore, Malaysia, and Indonesia, again with positive association with boat and ship traffic (and implied, although not measured, TBT contamination). The authors note that imposex has been widely observed (at least 45 species studied), and available studies suggest that TBT pollution may be a worldwide phenomenon. Because other molluscs are also sensitive to the effects of TBT (e.g., oysters and other bivalve molluscs), TBT pollution has both commercial and ecological impact. Furthermore, because of TBT's ability to bioaccumulate, it also raises concerns about the possibility of having a reproductive toxicant in the human food supply (Ellis and Pattisina, 1990). TBT has been found in bivalve molluscs and fish species eaten by man, although levels of these residues in edible tissues (e.g., 0.08 to 0.9 mg/kg in salmon in the United States, and < 10 to 5600 µg/kg in Chesapeake Bay oysters) are considered to be "safe" levels (Fent, 1996). Cooking does not degrade or remove the TBT. Whether TBT causes the above reproductive effects through an endocrine disruption mechanism awaits further study.

Field and laboratory observations after implementation of chemical controls indicate that TBT does have reproductive effects and that these effects, at least on marine snail populations, can be mitigated. Matthiessen et al. (1995) found that periwinkle (*Littorina littorea*) in two British estuaries showed steady population increases as TBT residues in water and sediments declined as the result of a partial ban on TBT use in 1987 by the United Kingdom. Unlike the dog-whelk (*Nucella lapillus*), the periwinkle does not undergo imposex in response to TBT exposure, which results in decreased egg production due to blockage of the genital pore (Bryan et al., 1988; Bauer et al., 1995; Matthiessen et al., 1995). Nonetheless, a slightly different masculinizing phenomenon has been observed in periwinkles, intersex, which is correlated with TBT exposures (Bauer et al., 1995). The intersex phenomenon differs from imposex in that there is no superimposition of male organs (penis or vas deferens) on the female but instead, there is a malformation of the pallial oviduct whereby it takes on a progressively more masculine form, with five distinguishable stages identified by Bauer et al. (1995). Based on field observations, Bauer et al. (1995) postulate that the threshold concentration for intersex development is about 15 ng TBT

as Sn/liter and that the degree of intersex noted in environmental populations may be potentially useful as a biomonitor for TBT, especially in areas where populations of *Nucella* are not present.

In terms of actual reproductive effects, Matthiessen et al. (1995), in laboratory studies, showed that exposures to TBT resulted in decreased egg production by the periwinkle. None of the test concentrations used--0, 10, 100, 330, and 1,000 ng/liter (nominal)--affected snail growth rate compared with the controls, nor was imposex (examined for) seen, nor the intersex phenomenon described by Bauer et al. (1995) noted. Egg production was measured beginning 2 months after treatment began. Egg production was more or less reduced on a seasonal basis and reductions became more evident at progressively lower exposure concentrations with increasing exposure time. At the end of the 12 months of exposure, egg production was significantly depressed at exposure concentrations in the range of 20.5 to 107.6 ng/liter (measured), concentrations that often had been exceeded in the study estuaries before implementation of the ban on TBT use. In experiments looking at egg development and hatching on freshly collected eggs from a relatively uncontaminated site, Matthiessen et al. (1995) found lower rates of hatching compared with controls but at levels much higher than those depressing egg production (e.g., the lowest concentration tested, 560 ng/liter, caused only a slightly lower hatching rate than the control level) and therefore concluded that this aspect of TBT toxicity was less important than egg production. However, the authors also noted that experiments looking for potential longer-term effects on the veliger should be conducted before concluding that egg production depression is the most sensitive or important effect.

Moore and Stevenson (1994) reported intersexuality in the harpacticoid copepods, *Paramphiascella hyberborea, Halectinosoma similidistinctum, H.* sp., and *Stenhelia gibba*. These benthic invertebrates were taken in the vicinity of a sewage outfall near Edinburgh, Scotland. However, the investigators did not find a correlation between intersex frequency and proximity to the discharge.

b. Fish

Purdom et al. (1994) reported that there were both public and scientific concerns about the effects of synthetic estrogens (from birth control pills) entering rivers in the United Kingdom as early as 1985. This concern was heightened when British anglers reported catching fish with both male and female characteristics; these hermaphroditic fish were caught in lagoons below sewage treatment plants (Purdom et al., 1994). The particular fish species is known as a roach, *Rutilus rutilus* (Harries et al., 1995). Purdom et al. (1994) hypothesized that the widespread use of contraceptive pills and the subsequent release of ethynylestradiol (via sewage treatment plants) might account for the occurrence of these hermaphroditic fish. To determine how widespread estrogens might be in the ambient waters of Great Britain, investigators used a biomarker

approach where male rainbow trout (*Onchorhynchus mykiss*) were placed downstream from sewage treatment works and periodically sampled for the presence of vitellogenin in the blood serum.

Vitellogenin is a phospholipoprotein that is synthesized in the liver of female oviparous vertebrates. The induction of vitellogenin is naturally induced in females in response to an estrogen, typically estradiol-17ß (Nimrod and Benson, 1996; Copeland et al., 1986). Vitellogenin leaves the liver and enters the bloodstream where it is utilized by the ovary. In the ovary, vitellogenin is transformed into two major types of yolk proteins, lipovitellins and phosvitins (Ng and Idler, 1983).

Purdom et al. (1994) reported the results of placing the caged rainbow trout in the effluents of sewage treatment plants throughout Great Britain. Five series of field trials began in 1986 and continued through 1989. Overall, the results of the 4-year survey indicated that effluents from sewage treatment plants contained an estrogenlike substance(s) as measured by the vitellogenin assay (Sumpter, 1985). A survey of six rivers and tributaries of the United Kingdom has now been completed (Harries et al., 1995). Estrogenic activity as measured by the method of Purdom et al. (1994) has shown there is estrogenic activity in three of them. In one river, the Aire, the vitellogenin concentration in male fish was similar to gravid female fish in unexposed sites; retardation of testicular growth was also observed. Nonylphenol, a breakdown product of nonylphenol ethoxylate surfactants used in wool scouring plants near the Aire, is speculated to be the causative agent. Laboratory experiments with adult male trout showed that nonylphenol induced both vitellogenin formation and testicular inhibition (Harries et al., 1995; Jobling et al., 1996). However, in other rivers, there has been no correlation between a specific chemical (e.g., nonylphenol) and vitellogenin formation. Of particular importance are the studies by Harries et al. (1995) that indicate related alkylphenols, for example, and various unrelated estrogenic chemicals (e.g., o,p′ DDT, arochlor, bisphenol A) can act in an additive fashion in vitro. Thus, individual chemicals could be present in the environment at concentrations below what is needed to elicit an estrogenic effect, but collectively they could induce some estrogenic activity.

Pelissero et al. (1993) improved the vitellogenin assay by developing a procedure to isolate rainbow trout hepatocytes, treat the cells with a suspected estrogen, and then measure the vitellogenin that is secreted into the culture medium. Jobling and Sumpter (1993) utilized this in vitro bioassay to evaluate the estrogenic activities of alkylphenol ethoxylates and their breakdown products. The results are summarized in table 4 (appendix).

The results indicate that the vitellogenin assay can be a useful biomarker for detecting exposure to estrogens in the environment. Ability to expand field studies has been limited by the availability of vitellogenin antibodies. Polyclonal antisera have been raised against purified vitellogenin from a wide variety of species; however, these antisera have been extremely species

specific. Recently, there has been significant research to develop "universal" antibodies that will recognize all fish, if not all vertebrate vitellogenins (Folmar et al., 1995; Heppell et al., in press; Denslow et al., in press; Palmer and Palmer, 1995). In question is the biological significance of vitellogenin formation in male fish. Nimrod and Benson (1996) cited a case where male rainbow trout died from kidney failure, possibly due to the formation of excessive amounts of vitellogenin. Experiments by Jobling et al. (1996) indicated that high levels of vitellogenin formation in male rainbow trout was accompanied by a decrease in testis growth as measured by the weight of the testes compared with total body weight (gonodasomatic index). Spermatogenesis also was affected.

An example of the masculinization of a fish species is given by Howell et al. (1980), who reported that 4 miles downstream from pulp and paper mills in Florida, mosquito fish females were masculinized and developed the male sex organ called the "gonopodium." These masculinized females sometimes attempted to mate with normal females, or when placed together, with each other. Furthermore, males were found to be hypermasculinized, displaying normal but hyperaggressive mating behavior. When placed in a tank with a normal male and three normal females, the hypermasculinized male established dominance and was free to court the females without competition (Howell et al., 1980). Chemicals in the effluent were not identified. Howell et al. (1980) noted, however, that this masculinizing effect was not likely to be due to natural conditions and paralleled laboratory experiments using known androgens, which induce the precocious appearance of male secondary sexual characteristics in males and masculinization of females. Commenting on this work, Davis and Bartone (1992) noted that kraft mill effluents contain phytosterols (e.g., tall [pine] oil contains 25% to 35% phytosterols), which can be converted microbially to C-19 steroids, which may exert the observed androgenic effects. The authors noted that bleached kraft mill effluents also contain other substances, for example, chlorinated organic substances, including dioxins and furans, which may have endocrine-disrupting effects.

Endocrine disruption affecting development and fertility also was noted in several other fish species exposed to bleached kraft mill effluent, with greater or lesser effects noted depending on the fish species studied. As in the study by Howell et al. (1980), the agent or agents actually causing the observed effects were not determined. Munkittrick et al. (1991) reported that near a bleached kraft mill on Lake Superior, white suckers had lower than normal levels of steroid sex hormones in their blood, took longer to mature, developed smaller gonads, and had fewer eggs at maturity. McMaster et al. (1991), in a followup to this study, found similar results--both male and female fish reached maturity at an older age, the females contained fewer eggs at maturity, the males had reduced development of secondary sexual characteristics (i.e., nuptial tubercles), and there were reduced plasma steroid levels throughout the year, including testosterone and 17α,

20β-dihydroxyprogesterone in both sexes, as well as 11-ketotestosterone in males and estradiol-17β in females. Van Der Kraak et al. (1992), in an additional study on this population of white suckers, determined that the endocrine effects of bleached kraft mill effluent (including reduced gonadotropin secretion by the pituitary, depressed steroidogenic capacity of the ovarian follicles, and altered peripheral metabolism of steroids) were caused by the effluent's acting at multiple sites in the pituitary-gonadal axis. Eggs failed to increase in size with age at the bleached kraft mill exposure (BKME) site, as opposed to the (nonexposed) reference site where there was an age-related increase in egg size (McMaster et al., 1991). Nonetheless, although eggs were smaller at the BKME site, and although male fish at the BKME site exhibited sperm having reduced motility (but not significantly different milt volume, spermatocrit level, or seminal plasma constituents), this had no effect on egg fertilization or hatchability, initial larval size, or larval survival (McMaster et al., 1991; Van Der Kraak et al., 1992). Furthermore, although prespawning BKME females were older than those in the reference site, there was no difference between sites in mean fecundity (note: this is a negative result for the BKME population; one would expect the population with the higher percentage of older fish to have a higher mean fecundity). While the observed changes in the BKME white suckers can be described as unhealthy, and, indeed, Van Der Kraak et al. (1992) noted that it is remarkable that fish having such aberrant gonadotropin and steroid levels are able to successfully spawn at all, the consequences of these changes to the exposed population are difficult to predict and would require additional (population dynamics) studies.

Munkittrick et al. (1992) further reported that these hormone-related changes were not improved after 1 year with the addition of secondary treatment of the mill effluent or with a 2-week shutdown of mill activities. The authors noted that the lower levels of circulating steroids were due to an inability, or reduced ability, of the hypothalamic-pituitary-gonadal axis to respond to alterations in steroid levels and a reduced ability to synthesize steroids. The authors further concluded that one cannot tell if the persistence of these steroid abnormalities in the BKME site after secondary treatment is due to food chain contamination from past pollution or whether secondary treatment has not removed the responsible chemicals. Other exposed fish studied include lake whitefish (*Coregonus clupeaformis*), which experience similar changes to white suckers in terms of reduced gonad size, reduced egg size, and increased age to maturity. However, while the white suckers are capable of producing viable eggs, Munkittrick et al. (1992) reported that the lake whitefish appeared to be experiencing reproductive problems. In contrast, the long-nose sucker (*Catastomus catastomus*) that was also examined showed much less effect than either species, but even here there was an altered age distribution of the spawning population (with older fish, on average), characteristic of the BKME population (Munkittrick et al., 1992). Although not explored by Munkittrick et al. (1992), an issue that immediately suggests itself as

needing study is how the differential sensitivity of coexisting populations of fish species to endocrine disruptors alters the ecological balance between the species. In cases where species are competitive with each other, even a subtle difference in effects could shift what was a delicate balance of populations and cause one species to greatly decrease in numbers, or even go locally extinct.

More subtle effects of endocrine disruptors on fish species also have been observed. Thomas (1990) reported preliminary studies wherein he exposed adult female Atlantic croakers (*Micropogonias undulatus*) to sublethal concentrations of lead, cadmium, benzo[a]pyrene, and PCBs. For all of these chemicals, he found significant decreases or increases in plasma steroid levels, ovarian steroid secretion, and ovarian growth in these fish. In more detailed studies, he exposed croakers collected at the beginning of the reproductive season to a mixture of Aroclor 1254 in the diet (0.5 mg/100 g body wt/day) for 17 days or to 1 ppm cadmium dissolved in 30% salinity seawater for 40 days. Significant, but opposite effects, on the reproductive system were seen with these exposures, and the results in both cases suggested that the hypothalamic-pituitary complex was the major site of toxic action (Thomas, 1990). With PCBs, there was suppression of ovarian growth and a decrease in plasma estradiol concentrations. There were also decreases in plasma vitellogenin levels and hepatic estrogen receptor concentrations. The author concluded that the effects seen with PCBs implied an impairment of gonadotropin secretion by the pituitary. On the other hand, with exposure to cadmium, both ovarian growth and plasma estradiol were increased, as was plasma gonadotropin secretion. For cadmium, a direct stimulating effect on the pituitary appeared to be the case, as was further indicated by in vitro studies (Thomas, 1990). Either treatment, the author judged, could inhibit the reproductive success of this fish species by causing oocytes to mature outside of the normal (optimum) spawning period.

In a case of a widespread effect, exposure to endocrine-disrupting chemicals is suspected of affecting thyroid function and impacting fertility and embryo survival and development in Great Lakes salmon (Leatherland, 1992). In one study, Moccia et al. (1981) found that in salmon from British Columbia (a relatively pristine population), the thyroid morphology was typical of a normal, nonpathological gland. In contrast, thyroid tissue collected from Great Lakes salmon was invariably altered and abnormal in appearance (Moccia et al., 1981). Even in Great Lakes salmon where no *overt* goiters were apparent, there was extensive follicular hyperplasia, with the follicles assuming abnormal, nonspherical shapes. In other fish, the histopathology was even more abnormal, revealing loss of follicular organization and, in some fish, large masses of aggregated epithelial cells that were difficult to distinguish from neoplasms (Moccia et al., 1981). Leatherland (1992), in continuing studies, noted that in every one of the Great Lakes, thyroid hyperplasia and hypertrophy have been found in 100% of the salmon stocks analyzed in the past two decades. (It should be emphasized that grossly visible lesions, e.g., thyroid hyperplasia and

reproductive effects, have been observed in "clusters" and in some lakes have actually declined, e.g., in Lake Ontario coho salmon, where different genetic stocks were introduced beginning in the 1970s. Nonetheless, while the incidence of gross lesions has changed in some areas, "the prevalence of thyroid hyperplasia has been consistently 100% for the last 18 years, regardless of salmon species, lake of origin, or gender" [Leatherland, 1992].) Leatherland (1992) concluded that a 100% prevalence of abnormal thyroid histology provides the most convincing evidence of a biologically active environmental factor affecting the function of the endocrine system in Great Lakes fish. Salmon are not the only affected species. Herring gulls throughout the Great Lakes have been found with enlarged thyroids (Peakall and Fox, 1987).

The agent causing these thyroid and reproductive effects has not been determined. Leatherland (1992) believes that feeding experiments that he and others have conducted point to an agent that affects the endocrine system, is readily metabolized or eliminated, and is not bioaccumulated; however, even this hypothesis is tentative. A common problem that arises from abnormal thyroid function is goiter, a condition characterized by an enlarged thyroid. The follicular cells produce colloid, and if they are unable to iodinate it, the follicles become congested with colloid, and do not make functional thyroid hormones. Without the feedback inhibition by thyroid hormones, TSH from the pituitary is elevated and stimulates the thyroid, which enlarges in an attempt to meet demand (Marieb, 1989). Goiter can be caused by a lack of iodine in the diet or by chemicals in the environment that act at multiple steps in the process from synthesis of thyroid hormone to postreceptor activation as discussed earlier. In the case of the Great Lakes salmon, lack of iodine also has been postulated as the cause of the observed thyroid effects in salmon, and this cause of the observed thyroid effects either in whole or in part cannot be completely ruled out at this time. However, Leatherland (1992) argues strongly from physiological and ecological observations that iodine deficiency is not the likely or even primary cause of the observed thyroid effects. It should be emphasized, however, that there is no firm evidence linking thyroid hyperplasia observed in Great Lakes salmon with any specific chemical contamination (LC Folmar, personal communication).

Furthermore, the epidemiologic observations for the goitrogenic effects seen in salmon have not been mentioned for either indigenous or other, more "purely freshwater" introduced fish species, which would strengthen a linkage of goitrogenic effects to a possible toxic chemical etiology.

Sonstegard and Leatherland (1976) noted that the particular significance of the observed effects on salmon is that, if goitrogenic substances are involved in the etiology of the observed thyroid effects in fish, such substances could potentially affect human health because fish are eaten and the substances are passed on to human consumers. The effects of these substances on fish populations or other wildlife populations also deserve more study (Sonstegard and Leatherland,

1976). As in mammals, with some differences in the particulars, the thyroid gland and the hormones it produces are involved in such things as metabolism, particularly carbohydrate metabolism, and growth, having, as in mammals, a permissive rather than a directly controlling role (Gorbman, 1969). In teleosts (fish having a bony skeleton, as opposed to cartilaginous species such as sharks), growth of the skeletal elements is particularly sensitive to the state of the thyroid gland. Thyroid hormones also appear to have a role, through feedback with the CNS, on teleost behavior, including general orientation, motor behavior and activity, and perhaps migratory behavior as well (Gorbman, 1969).

c. Amphibians

Many populations of frogs, toads, and salamanders are in decline in North America and worldwide (Blaustein and Wake, 1995). Several reasons have been put forth for the declines, including habitat loss, disease, ultraviolet radiation (UV), and pollution. The role of endocrine-disrupting chemicals to these declines, if any, is unknown. Hypotheses that a disrupted endocrine process could weaken an immune system response and make an individual amphibian more susceptible to a bacterial pathogen or less resistant to a UV stress have not been fully explored. Because monitoring efforts for these populations also have been very limited, a concerted effort would be needed to confirm or rule out an endocrine-disrupting chemical etiology for any of the population losses. Because anurans (frogs and toads) have both aquatic and terrestrial life histories and are subject to varied and multiple exposures (oral, dermal, and inhalation) at different stages in their life cycle, this class of vertebrate might represent a unique sentinel animal model for laboratory and field exposure studies.

d. Reptiles

Perhaps the best known example of putative environmental disruption is that from Florida's fourth largest lake, Lake Apopka. In 1980, a chemical spill from nearby Tower Chemical Company contaminated the lake. Guillette et al. (1994) reported this spill as a mixture of dicofol, DDT, and DDE. The spill was characterized as being primarily dicofol. More specifically, Tower Chemical Company was a manufacturer of generic chlorbenzilate, which was produced from DDT feedstock. Dicofol is closely related to chlorbenzilate and is a byproduct of its manufacturing process. Dicofol and chlorbenzilate both principally degrade to dichlorobenzophenone. The relative proportions of DDT, DDE, other DDT-related materials, dicofol, chlorbenzilate, and dichlorobenzophenone in the spill are not definite, but certainly all of these compounds were represented.

A variety of endocrine-related abnormalities have been reported as a consequence of this spill. The majority of male alligators from this lake appear to have been demasculinized, with

their phalluses one-half to one-fourth the normal size. Histologically, their seminiferous tubules show abnormal development and are marked by the presence of cell types and cell structures not seen in male alligators from (relatively unpolluted) Lake Woodruff (Guillette et al., 1994). Lake Apopka male alligators were further characterized by having extremely low serum levels of *both* testosterone and estrogen, but comparatively more estrogen (Guillette et al., 1994). This diminished hormone level and altered ratio was evident in the eggs, hatchlings, and juvenile animals (Guillette et al., 1994, 1995a,b, 1996). For example, male Lake Apopka hatchlings had a ratio of estradiol to testosterone of 2 versus the 0.5 ratio seen in normal animals (Guillette et al., 1994). The female alligators, on the other hand, were "super-feminized" having an estradiol to testosterone ratio twice as high as normal. Histologically, the ovaries of Lake Apopka females were marked by the presence of numerous polyovular follicles and polynuclear oocytes, which were never observed in alligators from Lake Woodruff (Guillette et al., 1994). A population of juvenile male alligators from Lake Apopka exhibited smaller penis size and plasma testosterone was much reduced compared with similar-sized animals from Lake Woodruff (Guillette et al., 1996). It should be mentioned that the hypothesis that these abnormalities in male sexual development heretofore attributed to xenoestrogenic activity of DDT and its metabolites may be mediated through inhibition of the AR (Kelce et al., 1995a,b).

Red-eared turtles in Lake Apopka also are being demasculinized. Amniotic fluid concentrations of estradiol and testosterone indicate that no turtle hatchling has a normal androgen synthesis pattern. Histopathologically, the hatchlings have either normal appearing ovaries or else are intersex, having ovotestes, with no normal males observed (Guillette, 1994).

The effects of this spill in Lake Apopka apparently include not only the developmental effects noted above, but also effects on hatching success and population growth. For example, in Lake Apopka, only 5% to 20% of alligator eggs hatched in each nest examined, compared with a normal hatching rate of 65% to 80% (Woodward et al., 1993). Furthermore, the mortality rate of Lake Apopka hatchlings was close to 50% in the first 2 weeks, a rate that is 10 times higher than that in nests from unaffected areas. Woodward et al. (1993) noted that juvenile alligator densities on Lake Apopka declined by 90% during 1980 to 1987. They attributed this decline to acute reproductive failure, perhaps due to exposure to DDD and DDE as demonstrated by the association of decreasing egg viability and the 1980 spill (Woodward et al., 1993). Alligator eggs from Lake Apopka were found to have p,p'-DDE at levels 5.6 ppm (wet weight) (Heinz et al., 1991), roughly twice that known to adversely affect the eggs and embryos of bald eagles. However, in an earlier study, Heinz et al. (1991) looked at hatching success in 1985 of artificially incubated eggs from Lake Apopka that contained significantly higher levels of organochlorine pesticides compared with Lake Griffin (where eggs were relatively clean). Of the analytes, p,p'-DDE was present at the highest concentration in Lake Apopka eggs with a geometric mean

concentration of 3.5 ppm wet weight (vs. 0.58 ppm in Lake Griffin.) The levels of heavy metals were similar in both lakes and did not appear to be present at harmful levels. While hatching success was lower for Lake Apopka eggs compared with Lake Griffin, within Lake Apopka there was no clear association between pesticide levels in eggs and hatching success. Given this lack of association, Heinz et al. (1991) concluded that the observed depression in egg viability could not be readily attributed to the organochlorine or metal compounds (toxaphene, dieldrin, DDT and its metabolites, nonachlor, chlordane and oxychlordane, and 16 metals) analyzed for and detected. Hexachlorobenzene, hexachlorocyclohexane, heptachlor epoxide, PCBs, endrin, mirex, and dicofol and its metabolites were analyzed also, but not detected in 1985.

The example of Lake Apopka demonstrates the difficulty of determining the exact causative agent in cases where a mixture of chemicals and heavy metals is involved and points up the need for coordinating both laboratory and field studies in these cases. It also points up the need to focus not only on direct mortality, but also on the far more common, but less easily measured, sublethal effects of endocrine disruption which may have detrimental consequences to populations in the long-term (and especially as these disruptions occur to embryos, adversely affecting the organization of the reproductive, immune, or nervous systems) (Guillette et al., 1995a,b).

As another case in point, Bishop et al. (1991) collected snapping turtle eggs from five locations in the Great Lakes region and assayed them for a variety of organochlorine contaminants, including hexachlorobenzene, o-chlordane, t-nonachlor, p,p'-DDE, mirex, dieldrin, heptachlor epoxide, pentachlorobiphenyls, dibenzo-p-dioxins, and dibenzofurans. Based on analyses of the eggs, two of the sites could be classified as relatively highly contaminated (total PCBs 1,500-3,000 ppb, DDE 500-900 ppb, other [total] organochlorine pesticides 250-500 ppb, and [total] dioxins/furans 0.06-0.15 ng/g or [ppb] wet weight); two others as moderately contaminated (total PCBs 300-500 ppb, DDE 40-80 ppb, other [total] organochlorine pesticides 100 ppb, and [total] dioxins and furans 0.01-0.02 ng/g or [ppb] wet weight); and the fifth site as relatively clean (total PCBs 30 ppb, DDE 8 ppb, other [total] organochlorine pesticides 5 ng/g or [ppb] wet weight, and dioxins and furans [not detectable]). (Data have been rounded off and combined in this paper for comparative purposes; see Bishop et al., 1991, for exact figures.)

There was a strong statistical association between the presence of these chemicals (especially the PCB congener 2,3,3',4,4'-pentachlorobiphenyl) and decreased hatching success and increased developmental abnormalities. However, the study could not conclusively demonstrate that any particular organochlorine chemical analyzed was the responsible agent. Interaction analyses of the variables examined indicated that site effects were more strongly correlated with developmental abnormalities than individual contaminant levels in eggs. That is, although there was a strong correlation between the presence of these chemicals individually and adverse effects, there was a stronger relationship between adverse effects and areas of high contamination in

general. The authors judged that no single chemical substance could be conclusively implicated as the causative agent for the observed developmental effects. They concluded that controlled reproductive effects studies of polychlorinated chemicals on this species of turtle would make the results of this study more convincing.

A further complication that must be considered is the way in which sexual development is normally regulated in vertebrates. Among mammals, the development of the male reproductive tract and sexual characteristics are regulated by androgens (including testosterone) and anti-Mullerian hormone (as discussed earlier). However, in many poikilotherm (cold-blooded) vertebrates (fish and reptiles), individuals lack sex chromosomes and have evolved other mechanisms of sexual differentiation. The determining factor may be the temperature at which embryos develop; in others, it may be the social surroundings that control sex determination. Finally, some individuals may reproduce asexually by a process of parthenogenesis. Pertinent to this discussion is the fact that alligators, many turtles, and some lizards establish their gender during embryonic development coincident with differentiation of the gonads. Temperature regulation of sexual differentiation takes place in an all-or-nothing fashion. Temperature acts by modulating enzymes and sex steroid receptors. Depending on the species, the embryos develop into males predominantly at low, intermediate, or high temperatures; females develop at different temperatures (Crews, 1994). Reptiles with temperature-dependent sexual determination (TSD) should be good indicators of estrogenic response (personal communication, David Crews, University of Texas at Austin, 1996; Bergeron et al., 1994).

Gross and Guillette, reproductive endocrinologists at the University of Florida, completed a laboratory study taking advantage of the TSD (Gross and Guillette, 1994). They wanted to determine if the abnormalities seen in Lake Apopka's alligators could be induced with normal eggs treated with DDE. They took eggs from Lake Woodruff, a relatively clean lake, and painted estradiol on some and DDE on others. They then incubated the eggs at a temperature that, in a clean environment, would produce mostly male hatchlings. For the eggs treated with DDE (or estradiol), there was observed, when measured at hatching, a decrease in allantoic testosterone concentrations that mimicked the estrogen-testosterone ratios seen in the eggs collected from Lake Apopka. Estradiol, but not DDE, also increased allantoic estradiol levels. These observed hormone ratios indicate a strong demasculinizing effect due to exposure to these chemicals. In a followup interview concerning this work, Hileman (1994) reported that 80% of the eggs painted with estradiol produced females. Those eggs painted with DDE produced 20% female, 40% intersex, and 40% male hatchlings.

In another experiment using a TSD species, Bergeron et al. (1994) dosed the eggs of the red-eared slider turtle, *Trachemys scripta*, with various combinations and concentrations of 11 PCB compounds. The test substances were dissolved in 95% ethanol and applied to the outside

of the shell of the eggs. Two compounds, both hydroxylated forms of PCBs, 2',4',6'-trichloro-4-biphenylol and 2',3',4',5'-tetrachloro-4-biphenylol, resulted in a significant percentage of turtles hatching as females at temperatures that normally produced males. In the case of 2',4',6'-trichloro-4-biphenylol, there was 100% sex reversal at the high dose (100 µg or approximately 9 ppm). Both of these compounds when tested in mouse tissue also showed marked estrogen receptor affinity (McKinney et al., 1990). Although no other PCBs (whether hydroxylated or nonhydroxylated) showed sex reversal, Bergeron et al. (1994) postulated that the two active hydroxybiphenyls could exist in steady-state concentrations in the aquatic environment as metabolites of other PCBs. Furthermore, when these two compounds were combined, they had a synergistic effect. There was a significant increase in ovarian development at a dose of 10 µg (about 0.9 ppm), a dose tenfold less than the effect observed when the chemicals were tested singly. Estradiol-17β, the positive control chemical, gave similar results when applied at a dose of 0.5 µg (0.04 ppm). Bergeron et al. (1994) noted that the PCB concentrations showing estrogenic effects and disruption of normal gonadal differentiation in their turtle experiments are similar to average concentrations of PCBs found in human breast milk.

As with fish, vitellogenin induction is thought to have some utility as an estrogenic biomarker of exposure to environmental endocrine disruptors for amphibia and reptiles. To test this, Palmer and Palmer (1995) injected 1 µg/g estradiol-17β (E_2), 1 µg/g DES, 250 µg/g o,p'-DDT, or 1 µg/g o,p'-DDT (ip, dissolved in corn oil) into adult male red-eared turtles (*Trachemys scripta*) and adult male African clawed frogs (*Xenopus laevis*). Single injections of test substance were given daily for 7 days, and plasma was collected on day 14 for analysis. Both the DES and estradiol treatments induced relatively high concentrations of vitellogenin. DDT induced smaller amounts, in a dose-dependant manner, and corn-oil only (control) animals showed no extractable vitellogenin in their plasma. On the basis of the results of these laboratory studies, Palmer and Palmer (1995) concluded that the vitellogenin assay may be a useful biomarker of xenobiotic estrogen activity in reptiles and amphibians in wild populations as well. Palmer and Palmer (1995) also noted that in the case of lipophilic compounds, like o,p'-DDT, which have estrogenic activity and which also bioaccumulate, there may be negative impacts on fertilizability of the egg and development of the embryo as these lipophilic contaminants are mobilized and transferred to sensitive tissues during the reproductive and developmental processes.

e. Birds

Hatching success of birds also has been suspected of being affected by environmental hormones. DDT and DDE continue to be a problem in the Great Lakes due to these chemicals' persistence and ability to bioaccumulate (Colborn, 1991). Reproductive success of the fish-eating Forster's tern was dramatically impaired on organochlorine-contaminated Green Bay, Lake

Michigan (Kubiak et al., 1989). Compared with the Wisconsin control eggs from Lake Poygan, eggs from Green Bay had an order of magnitude higher residues of TCDD, PCDD, and PCBs (201 pg/g vs. 2175 pg/g). Hatching success of eggs at Green Bay was 75% lower than that of those at Lake Poygan (Kubiak et al., 1989). In the 1983 nesting season, hatchability of Forster's tern eggs taken from other nests and artificially incubated was about 50% lower for Green Bay than for Lake Poygan (Kubiak et al., 1989).

The insecticide chlordecone (Kepone) reportedly also has an estrogenic effect, as observed in Japanese quail fed diets contaminated with 10, 40, 80, or 160 ppm chlordecone for 6 to 26 days. Effects were observed in a dose-dependent fashion for all the doses after 26 days of exposure, and very rapid changes were noted at the highest dose, with effects approaching those of estradiol-17β, the positive control (Eroschenko, 1981). In these experiments, Kepone was found to stimulate the female reproductive system of immature quail, but decrease follicular development, induce ovarian regression, and inhibit ovulation and egg-laying in adults (Eroschenko, 1981). With chronic exposure, eggs laid by treated birds were significantly weaker and thinner shelled than control birds. Additional studies by Palmiter and Mulvihill (1978) and Eroschenko and Palmiter (1980) indicate that Kepone competes for, and binds to, estrogen-sensitive cells in the reproductive system. Also, messenger RNAs for conalbumin and ovalbumin were induced. Such induction of egg white protein synthesis is also typical of estradiol. Kepone also affects male birds, causing highly dilated seminiferous tubules, a reduction in germinal epithelium and reduced numbers of sperm (Eroschenko and Wilson, 1975; Eroschenko, 1981).

Fry et al. (1987) noted that gulls are relatively resistant to the eggshell thinning effects of organochlorine compounds such as DDT; however, gulls appear to be much more sensitive to the teratogenic (specifically, the feminizing) effects of chemicals identified as having estrogenic properties (e.g., DDT and methoxychlor). Indeed, gulls appear to be 10 to 50 times more sensitive to chemicals inducing feminization than chickens, Japanese quail, or finches, other species that have been tested using estrogenic teratogens (Fry et al., 1987). These pollutant effects may be the cause of locally observed population declines and skewed sex-ratios of breeding populations of Western gulls in California and Herring gulls in the Great Lakes in the 1960s and 1970s (Fry et al., 1987). In examination of this hypothesis, Fry et al. (1987) injected the eggs of Western and California gulls with estrogenic compounds (o,p'-DDT, p,p'-DDT, and methoxychlor) at concentrations (2, 5, 20, 50, and 100 µg/g [ppm] fresh egg wt) that would simulate levels that have been observed in eggs in the environment. The positive control compound, estradiol, injected even at the lowest concentration (0.5 ppm) caused complete feminization of male embryos, such that male embryos could only be distinguished histologically by the presence of seminiferous tubules in the left ovotestis. O,p'-DDT at 5 ppm and higher and methoxychlor at high concentrations (20, 50, and 100 ppm) also caused extensive feminization

(e.g., persistence of right oviducts in female embryos, left or left and right oviducts present in males, and right testes of feminized males either normal or reduced in size). A 4:1 mixture of p,p'-DDE plus p,p'-DDT also resulted in feminization of male and female embryos at the high dose of 50 ppm. Embryos from eggs injected with p,p'-DDT or p,p'-DDE alone were not noticeably affected at the doses tested. It should be mentioned again that the above studies treated p,p'-DDE as an estrogen when it has recently been shown to be a potent AR antagonist (Kelce et al., 1995a,b).

In addition, Fry et al. (1987) examined several colonies of Glaucus-winged gulls (*Larus glaucescens*) breeding in localized polluted areas of Puget Sound, Washington. Average eggshell thinning was 8, 9, and 10%, respectively, in the three target sites of Seattle, Tacoma, and Shelton. This is a remarkable amount of thinning for a gull species and, as Fry et al. (1987) noted, is comparable to thinning caused by high levels of DDT in Lake Michigan in the 1960s. A significant percentage (50, 86, and 100%, respectively) of birds from these three sites also had persistent right oviducts, evidence of exposure to an estrogenic substance, and also a high frequency of supernormal clutches of eggs. Interestingly, Puget Sound, historically, has not been characterized by extensive amounts of pollution by DDT, unlike other areas where the above-observed effects have been noted. However, high levels of PCBs and PAHs--both classes of compounds that also are considered to be environmental endocrine disruptors (this paper)--are characteristic pollutants in the Sound, and birds from urban areas of Puget Sound have been found with comparatively high levels of these compounds in their tissues (Fry et al., 1987). Fry et al. (1987) concluded that because only very low levels of DDE have ever been found in Puget Sound, the specific cause of the observed eggshell thinning and feminization of Glaucus-winged gulls in this area is unknown.

Moccia et al. (1986) did histologic examinations of 213 herring gulls collected from nine colonies in the Great Lakes basin between 1974 and 1983 and also of birds from a single colony in the Bay of Fundy (a coastal marine population) between 1977 and 1982. Abnormal thyroid histology was the rule for gulls from the Great Lakes area, versus those from the Bay of Fundy, which demonstrated normal thyroid structure. Epithelial hyperplasia, microfollicular organization of the thyroid tissue, and enlarged thyroids (goiter) were prevalent in gulls from the Great Lakes but not from the Bay of Fundy. Moccia et al. (1986) noted that the Great Lakes region is deficient in concentrations of iodine in both soil and water, and iodine deficiency can cause goiter. Indeed, iodized salt is legislated for use by the human population in this area. Nonetheless, the spatial and temporal differences in thyroid pathology seen in the gulls, compared with the interlake differences in iodine content, does not, according to Moccia et al. (1986), support a hypothesis of iodine deficiency being the sole cause of the observed thyroid abnormalities in the gull populations sampled.

Moccia et al. (1986) also noted that a number of substances present in the Great Lakes food chain, including PCBs, PBBs, DDT, DDD, DDE, dieldrin, and mirex, reportedly affect thyroid activity in birds. In this study, the authors found that those colonies of gulls with the highest prevalence of epithelial hyperplasia were from those sites that were most contaminated with PCBs and polyhalogenated aromatic hydrocarbons. Furthermore there has been a temporal decline in the incidence and severity of abnormal thyroid histopathology, corresponding to a temporal decrease in contaminant levels in the gulls. A similar decrease also has been observed in salmon populations in the Lakes. Given that the herring gull diet consists in large part of fish, and that Great Lakes fish (Coho salmon) have been found to accumulate substances found to be goitrogenic in rats, Moccia et al. (1986) hypothesized that the agents responsible for the goiter and thyrotoxic effects observed in Great Lakes herring gulls are probably some fishborne polyhalogenated hydrocarbons, but probably not PCBs (which produce an effect that is histologically different from that observed in the Great Lakes gulls). Specific identification of these substances remains to be determined.

f. Mammals

Laboratory evidence of the effects of estrogenic environmental hormones on sexual differentiation was demonstrated in a study by Gray (1982). Female hamsters treated neonatally with 0.25, 0.5, or 1 mg/pup of Kepone (chlordecone) or 20 µg/kg of estradiol benzoate were masculinized but not defeminized. They had normal estrous cycles but displayed abnormal sexual behavior by mounting receptive females (Gray, 1982).

The linkage of observed effects on wild mammalian species to environmental endocrine disruptors is somewhat tenuous, with perhaps certain populations of marine mammals providing the most likely examples of such an association. As in the example of herring gulls along the Great Lakes, the common theme appears to be a diet of fish contaminated by chemicals that have demonstrated or suspected influence on endocrine systems affecting reproduction and immunocompetence (e.g., PCBs, DDT, DDE, mirex, mercury). Reijnders (1986) reported on the collapse of a population of common seals (*Phoca vitulina*) in the western most part of the Wadden Sea, The Netherlands. In 25 years, between 1950 and 1975, the seal population in this area plummeted from 3,000 to fewer than 500 animals. The western (Dutch) area of the Wadden Sea is heavily polluted due to pollutants carried to this portion of the sea by the Rhine River. A comparative analysis of organochlorine chemicals and heavy metals in the tissues of seals from the western and northern portions of the Wadden Sea revealed that only PCB levels were significantly higher in the western seal population. PCBs are the chemical agents thought to be the cause of the poor reproduction observed in the western population.

To investigate this hypothesis, Reijnders (1986) fed two groups of 12 female common seals fish taken from different areas. Group 1 received fish species caught in the western part of the Wadden Sea; Group 2 received those fish caught in the northeast Atlantic. Analysis of the fish for chemical residues (aldrin, dieldrin, endrin, heptachlor, hepox, α,β,γ-hexachlorocyclohexane, pentachlorobenzene, hexachlorobenzene, p,p'-DDE, o,p'-dichlorodiphenyl-dichloroethane, p,p'-dichlorodiphenyl-dichloroethane, and PCBs) showed PCBs and p,p'-DDE to be significantly higher in fish taken from the western portion of the Wadden Sea versus levels in fish taken from the northeast Atlantic. The seals were fed their respective diets for approximately 2 years, during which time the average daily intake of Group 1 seals was 1.5 mg PCBs and 0.4 mg p,p'-DDE and of Group 2 seals, 0.22 mg PCBs and 0.13 mg p,p'-DDE. Reproductive success was significantly lower in Group 1 versus Group 2 seals. Profiles of hormones from the two seal groups showed no significant differences in circulating blood levels of progesterone or estradiol-17β between the two groups on a circumannual basis. However, the rise in estradiol levels of nonpregnant seals in Group 2, which indicates follicle growth, was not seen in nonpregnant seals in Group 1 (although too few seals [two] were nonpregnant in Group 2 to test the significance of this result statistically). Also, the level of elevated estradiol in the combined Group 1 seals was statistically lower than that of Group 2 seals but apparently was still high enough to result in reproductive success in some of the animals.

In additional experiments, Reijnders (1986) fed American mink (*Mustela vison*) livers of fish from the Wadden Sea or mink chow dosed with pure PCBs (Clopen A-60 or A-30). Mink were affected equally under both regimens, with reproductive effects evidenced even at very low doses (25 µg/day). Reijnders (1986) concluded that available evidence indicated that PCBs were the likely cause of the reproductive failure observed in the western Wadden Sea seals. Reijnders (1986) further concluded that effects occur postovulation and perhaps especially during the period around implantation. However, whether the cause of reproductive failure is a result of impaired steroid binding capacity by PCBs and a disruption of the steroid synthetic pathways (endocrine disruption), a dominant-lethal action, or an embryo lethal effect could not be determined at the time.

In addition to possible steroidal effects, Brouwer et al. (1989) found that the seals fed the diet of Wadden Sea fish (same experimental group as Reijnders, 1986) had greatly reduced levels of plasma retinol concentrations (e.g., 55%, and 30% to 40% reductions in June 1983 and September 1983, respectively--two time periods selected for sampling and analysis during the pregnancy period) compared with seals fed the northeast Atlantic fish diet. Plasma triiodothyronine levels were also significantly reduced in the high vs. the low PCB diet in the June 1983 sampling. There were also lesser reductions in plasma total and free thyroxine at that point. Unlike the observations on plasma retinol, this relative diminution in thyroid hormone levels

apparently did not persist throughout pregnancy. The September 1983 sampling showed comparable thyroid hormone levels in both treatment groups. Brouwer et al. (1989) postulated that PCBs interfere with thyroid hormone and, especially, vitamin A metabolism in these seals, which could, over time, lead to a persistent vitamin A deficiency, resulting in retarded growth, adverse reproductive effects, skin and eye disorders, and increased susceptibility to microbial infections--effects observed in wild marine mammal populations in the Baltic, North, and Wadden Seas.

De Guise et al. (1995) similarly found that a local population of beluga whales (*Delphinapterus leucas*) in the St. Lawrence estuary, Quebec, Canada, suffered a population decline from 5,000 animals at the turn of the century to approximately 500 animals currently. Like the Wadden Sea seals studied by Reijnders (1986), this population of whales lives in a highly polluted area and does not appear to reproduce at a normal rate. Abnormalities observed in the ovaries during the reproductive cycle, the presence of relatively few pregnant animals, and the unusual occurrence of an adult hermaphroditic beluga (with two ovaries, two testes, complete male genital tract, and partial female genital tract) also were considered indicative of endocrine disrupting effects with a possible chemical etiology. Thyroid lesions (abscesses, and, in one animal, adenomas) and adrenal cortex lesions (hyperplastic nodules and serous cysts) have also been observed in this population of whales. De Guise et al. (1995) also postulated that exposure to environmental contaminants (such as PCBs, dieldrin, and 2,3,7,8,-TCDD) may be compromising the immune system of the St. Lawrence beluga whales as evidenced by a relatively high prevalence of neoplasms and observed frequent infections of mildly pathogenic bacteria in this population.

Lahvis et al. (1995) reported on the massive stranding and die-offs of bottlenose dolphins (*Tursiops truncatus*) that have occurred in the late 1980s and early 1990s. One such incident cited by Lahvis et al. (1995) involved more than 740 dolphins from New Jersey to central Florida, representing as much as 53% of the coastal migratory stock of this species (Scott et al., 1988). Gulf of Mexico dolphins experienced similar episodes of high or unusual mortality in the early 1990s, as did striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea. Lahvis et al. (1995) reported that in each of these cases the dolphins were marked by skin and organ lesions believed to be caused by (in many cases opportunistic) infections of common bacteria, viruses, and fungi. Several hypotheses have been proposed concerning the cause of the observed mortalities. In the case of the dolphin deaths in the Atlantic, the presence of a "red tide" just prior to the observed mortalities was noted. In a red tide, produced by the toxic dinoflagellate alga *Ptychodiscus brevis*, the animals would have been exposed to a neurotoxicant, brevetoxin, produced by the algae. Brevetoxin, it was suggested, could induce immunosuppression in exposed dolphins, making them susceptible to the observed opportunistic infections. Another

hypothesis was that the Atlantic dolphin developed a morbilli virus infection, which can lead also to immunosuppression and additional (opportunistic) infections. Neither of these two hypotheses are totally persuading. Lahvis et al. (1995) noted that not all of the dead dolphins contained brevetoxin, and morbilli virus infection could perhaps be secondary to some primary immunosuppressive event, as appeared to be the case in the incident involving mortalities of Mediterranean striped dolphin.

The hypothesis that these animals' immune systems were suppressed due to chronic exposure to immunosuppressive pollutants such as PCBs, p,p'-DDT, p,p'-DDE, or 2,3,7,8,-TCDD should be considered. High levels of these toxicants have been found in the stranded animals. Lahvis et al. (1995) took blood samples from 15 male bottlenose dolphins from a resident population near Sarasota, Florida, in an attempt to see if a relationship between toxicant load and immunosuppression could be determined. Immunosuppression was measured for each blood sample using lymphocyte proliferation assays. Blood samples also were assayed for concentrations of polychlorinated dibenzo-p-dioxins, polychlorinated dibenzo-furans, PCBs, pesticides, and other chlorinated compounds. Only 5 of the 15 animals were selected for analysis, with samples from the high and low ends being analyzed. This small, and necessarily biased, sample plus the lack of uncontaminated control dolphins make the conclusions of the analysis tentative. Nonetheless, the investigators found that immunosuppression as measured by this assay was positively correlated with increasing levels of pollutants, especially o,p'-DDE, p,p'-DDE, o,p'-DDT, and the PCB congeners assayed. Additional work would be necessary to confirm and further define the significance of these results as they relate to dolphin mortalities.

In Florida panthers (*Felis concolor* coryi), cryptorchidism (one or both testes retained within the body cavity) is present in 90% of the male population (Facemire et al., 1995). Furthermore, sperm abnormalities for this population are the highest reported for any feline, and sterility has been observed in at least four animals examined from this population between 1978 and 1990 (Facemire et al., 1995). While the cause of cryptorchidism is unknown, the exposure of developing embryos to endocrine disruptors is suspected. Another explanation, lack of genetic diversity in this isolated population, also has been proposed.

Facemire et al. (1995), however, while not discounting that possibility, think that exposure to environmental endocrine disruptors may also account for some, and perhaps even the larger part, of the observed reproductive abnormalities. They base their conclusions on several observations. First, the genetic diversity of Florida panthers, when compared with other species of large cats (e.g., Asian and African lions), was slightly higher or slightly lower than average, depending on the specific population being compared, and was only slightly lower to roughly equivalent to most other subpopulations of panthers (e.g., those in Texas, although a Latin American panther population had markedly higher genetic diversity than the Florida population).

Second, cryptorchidism is rare in captive panthers and has never been reported in any other species of wild feline, regardless of the degree of inbreeding. Third, several animals that have been found dead for unknown reasons or after failing health have been found to have what appear to be toxic levels of mercury in their tissues. Mercury and other contaminants, when present in the environment, can be accumulated from the aquatic food chain with the upper end of that chain represented by, in this case, the raccoon, which feeds on aquatic fish, molluscs, and crustaceans. Panthers whose diet consists of large numbers of raccoons will likewise accumulate high doses of lipophilic compounds, such as mercury, and also p,p'-DDE and PCBs, which are suspected endocrine disruptors, and which also have been found in Florida panthers and raccoons. Endocrine disruptors could possibly cause cryptorchidism by influencing the synthesis of anti-Mullerian hormone or the synthesis of androgens--for example, through an antiandrogenic effect of DDE. Fourth, Facemire et al. (1995) examined estradiol and testosterone levels from whole blood samples from 19 male (6 normal and 13 cryptorchid) and 5 female Florida panthers and found that there was no significant difference in estradiol levels between these three groups, although testosterone levels were generally greater for males and increased as the males aged. However, there were also several males whose estradiol/testosterone (E/T) ratio was relatively high, greater than 1 or near 1, and also a female panther whose E/T ratio was relatively low, 0.77, indicating possible feminization of the male and masculinization of the female animals. There were no significant differences between the hormone levels of normal versus cryptorchid males. Facemire et al. (1995) concluded that additional studies (e.g., to determine normal seasonal hormone levels of panthers, to examine other possible causes of the observed abnormalities such as vitamin A deficiency, which has been associated with a raccoon diet) should be conducted to further elucidate the observed reproductive failure of this population of panthers.

In an unusual report of endocrine disruption in mammals, possibly deserving additional followup studies, Cattet (1988) reported that 4 of 15 female black bears and 1 of 4 female brown bears in Alberta, Canada, had male sex organs, formed to a greater or lesser extent. Upon gross dissection, the bears' reproductive tract was completely female, but externally, some degree of masculinization of the genitalia was evident. This ranged from "a small piece of cartilage embedded in a muscular process attached to the ventral wall of the vaginal canal to a nearly full-sized penis-like structure with a urethra and baculum" (Cattet, 1988). The author reported no evidence for what might be the cause of this observed masculinization but suggested that these effects might be due to exposure of the developing fetus to androgen-mimetic chemicals. Another possibility suggested by Cattet (1988) for the observed pseudohermaphroditism was a freemartin type phenomenon (an intersexual female calf twin born with a male), as is seen in cattle when the blood supply of male and female twin calves are commingled. However, a freemartin

phenomenon was considered less likely because the bears examined had evidence of prior reproduction (placental scars, lactation, and cubs) whereas freemartins are usually sterile.

4. Test Methods

Ecological effects observed and suspected of being caused by environmental endocrine disruptors are listed in table 3 (appendix). For these effects and others discussed in the above paragraphs, even though an endocrine-disrupting etiology seems clear in several of these, it can still be disputed to some degree for all. What is not disputed is that a true cause-and-effect relationship is difficult to establish.

A variety of test methods are available, but it is not known which one(s) is the best to determine the effects of endocrine-disrupting chemicals on fish and wildlife. While it is beyond the scope of this document to list and discuss various tests for each hormone and process, consider just one class of hormones--estrogens, for example. Several in vitro bioassays have been developed for assessing the estrogenicity of chemicals using human breast estrogen-sensitive MCF-7-cells (Gierthy and Lincoln, 1988; Gierthy et al., 1991; Soto et al., 1992). The assays compare the cell yield after 6 days of culture in medium plus 10% charcoal-dextran stripped human serum with and without estradiol and chemicals suspected of being environmental estrogenic agents.

Many tests have been conducted to determine the endocrine action and potency of environmental chemicals by using developmental or physiologic effects as endpoints. Developmental effects are those that affect the developing organism and may result in irreversible changes. Physiological effects are those that occur any time after development and may be reversible. For example, Gellert and Wilson (1979) have demonstrated that the offspring of chlordecone (Kepone)-treated dams exhibit persistent vaginal estrus and anovulation. Eroschenko (1981) also reported that administration of Kepone to pregnant rats or mice during the main period of fetal organogenesis results in fetal toxicities and malformations in the offspring. As another example, a study by Gray et al. (1989) measured reproductive alterations in rats by age at vaginal opening, first estrus, and preputial separation in males being dosed with methoxychlor at 25, 50, 100, or 200 mg/kg/day from weaning through puberty, gestation to postnatal day 15. Methoxychlor accelerates the age at vaginal opening and first estrus. In the highest dosed group, females go from constant estrus into pseudopregnancy following mating, but do not implant. In males, methoxychlor treatment reduces growth, seminal vesicle weight, caudal epididymal weight, caudal sperm count, and pituitary weight.

Vitellogenin, whose relevance in fish has already been discussed, provides an example of a biomarker that may be determined very useful in assessing endocrine, especially estrogenic or other feminization, effects. A vitellogenin assay is available that Pelissero et al. (1993) improved

by developing a procedure to isolate rainbow trout hepatocytes, treat the cells with a suspected estrogen, and then measure the vitellogenin that is secreted into the culture medium. Jobling and Sumpter (1993) utilized this in vitro bioassay to evaluate the estrogenic activities of alkylphenol ethoxylates and their breakdown products. Their results are summarized in table 4 (appendix).

The vitellogenin assay and the MCF-7 cell assay (Soto et al., 1992) are methods that can screen for estrogenic activity. The results of these assays have actual implications for animals. For instance, nonylphenol has been shown to reduce testicular development in fish and also had a positive response in both assays. Likewise, octylphenol and its ethoxylates and benzyl butyl phthalate were estrogenic in the vitellogenin assay and both were found to reduce testicular size and sperm production in the offspring of female rats exposed to the substances via drinking water (Sharpe et al., 1995). Screening assays are not limited to breast cell cultures or hepatocytes. Routledge and Sumpter (1996) have developed an estrogen assay using the yeast *Saccharomyces cerevisae* to screen for estrogens, and this assay has been used to assess rivers in the United Kingdom for the presence of estrogenic compounds. The next challenging step will be to modify existing test methods or develop new ones to further evaluate the results of bioassays or other screening methods. For practical and cost reasons, tests will have to be developed in a tiered fashion. A consensus-building approach will be needed, and this area will be the subject of intense activity for some years to come. Furthermore, other endocrine disruption effects, in addition to estrogen or androgen mimics, will have to be evaluated as more information becomes available.

Development and use of tests targeting endocrine function could assist the risk assessor in the determination of whether a particular agent is an endocrine disruptor and of what toxicological significance. Tests may vary as the creative minds of their developers and be as numerous as there are hormones and hormone-controlled processes. Of immediate need, however, is an array of test methods utilizing in vitro, whole animal, and field-level approaches for identifying, quantifying, and elucidating endocrine-related toxicological effects. A framework establishing the more useful of available methods and for linking or "tiering" these for a coordinated assessment of potential endocrine effects is also essential for prudent regulatory intervention. The Agency is establishing a Federal advisory working group called the Endocrine Disruptor Screening and Testing Advisory Subcommittee to develop a screening and testing strategy for new and existing chemicals that may act as endocrine disruptors. This subcommittee will be composed of representatives from environmental groups, industry, academia, and Government.

IV. ANALYSIS, DISCUSSION AND RECOMMENDATIONS

A. HUMAN HEALTH ISSUES

With few exceptions (e.g., DES, dioxin, DDT/DDE), a causal relationship between exposure to a specific environmental agent and an adverse effect on human health operating via an endocrine disruption mechanism has not been established.

An important consideration in the evaluation of endocrine-disrupting mechanisms is the concept of negative feedback control of hormone concentrations. Endogenous secretion and elimination of hormones are highly regulated, and mechanisms for controlling modest fluctuations of hormones are in place. Therefore, minor increases of exogenous hormones following dietary absorption and hepatic detoxification of these xenobiotics may be inconsequential in disrupting endocrine homeostasis in the adult. Whether the fetus and the young are capable of regulating minor changes to the endocrine milieu is uncertain.

An essential question in the analysis and discussion of the issue of environmental hormone disruption for risk assessment is whether the exposure and endocrine potency levels of the agents are sufficient to adversely affect human populations. If endocrine disruption is operating through a hormone receptor mechanism, low ambient concentrations along with low affinity binding of purported xenobiotics are probably insufficient to activate an adverse response. For example, exposure concentrations of weak estrogenic alkylphenols are on the order of ppm to ppb. White et al. (1994) reported effluent concentrations from sewage discharge plants in the United Kingdom at 0.1 ppm. Approximately 1/100 of the total (bound plus free) serum estradiol available is free to activate a physiologic response in female rats (Montano et al., 1995). According to White et al. (1994), of the alkylphenols tested, it requires some 1,000 to 10,000 times more of the weak estrogen to bind 50% of the estrogen receptor than estradiol. If these data are correct, it means that 100,000 to 1,000,000 times more of the agent is needed to activate a physiological response. In other words, there would have to be 100 to 1,000 times more in the water to activate an estrogenic response. Clearly, the normal human female is able to regulate ppb concentrations of estradiol without difficulty. In addition, Safe (1995) points out that dietary exposure to xenoestrogens derived from industrial chemicals is minimal compared with estrogen equivalents from naturally occurring bioflavonoids. Furthermore, in the case of environmental estrogens as endocrine disruptors, it is known that competition for binding sites by anti-estrogens and down-regulation of estrogen receptors via Ah receptor-mediated chemicals in the environment may mitigate estrogenic effects of some chemicals (Safe et al., 1991). Taken together, the Technical Panel concludes, based on the available evidence, that exposure to a single xenoestrogenic chemical, at current environmental concentrations, is probably insufficient to evoke an adverse effect in adults. More information is needed to determine whether this holds for

the human fetus and the neonate. Also, whether additional chemicals may overcome a body burden or operate at nonestrogenic receptor sites to stimulate or inhibit estrogenic or other responses needs to be determined.

Another unknown of relevancy is whether a mixture of chemicals with endocrine-disrupting potential (via additivity [Harries et al., 1995; Soto et al., 1994] or synergy [Arnold et al., 1996]) is sufficient to elicit a response and whether antagonists within the same mixture are sufficient to negate the response (Harris et al., 1990). These uncertainties will require considerable exploration.

Another issue is whether existing guidelines and testing protocols are adequate to detect endocrine-mediated effects of a disruptor in the general population as well as in sensitive individuals (the fetus, children, the infirm, and elderly). Clearly, there are age-dependent differences in susceptibility to endocrine disruptors. In adult ovariectomized C57BL/Tw mice, three daily doses of 100 µg of clomiphene, tamoxifen, or nafoxidine or 1 µg of estradiol but not keoxifene increases uterine and vaginal weight, DNA, and protein (Chou et al., 1992). In contrast, neonatal mice given five daily doses of the antiestrogen keoxifene exhibit decreased uterine and vaginal weights at 60 days of age. Similarly, while 2,3,7,8-TCDD can inhibit certain estrogenic effects in adults, weanling Sprague-Dawley female rats are apparently insensitive to the antiestrogenic effects of TCDD (White et al., 1995). No test guidelines/protocols exist to specifically evaluate endocrine disruption effects.

For human health risk assessment two-generation reproduction studies, the new EPA harmonized reproductive and developmental toxicity testing guidelines and the 2-year cancer bioassay should be able to detect many adverse effects. However, these were not designed to identify mechanisms of action of endocrine disruption, subtle functional deficits, or "transplacental carcinogenesis" that might result following exposures at critical stages of development not currently included in testing protocols. Current tests also are inadequate to evaluate endocrine-mediated effects of mixtures. Some attempt has been made to expand on this issue under specific endpoints discussed above. Of course, it should be remembered that first-tier toxicity testing protocols are designed not to determine specific endpoints or mechanism of action, but are apical in design. As such, they employ a paradigm intended to detect a broad spectrum of endpoints and adverse effects in the overall reproductive process.

With respect to risk assessment, it should be kept in mind that all of the data should be considered in the evaluation. For example, in the case of evaluating estrogen-mimetic, natural, and synthetic chemical influences in the development of hypothalamic centers and sex differentiation of the fetus, what is the role of natural products such as the phytoestrogens in the diet of mothers? Are the adverse effects observed the result of additive, synergistic, or antagonistic mechanisms of action? In adults, do the phytoestrogens have any protective role in

regulating/restricting estrogen influences in breast cancer development? For industrial chemicals and pesticides (including inert ingredients) that are used in the workplace and home, there is a need to accurately assess the exposure posed by their uses. Basic questions such as what are the exposure potentials due to leaching from containers, dermal contact, and inhalation need to be addressed. To obtain answers to these questions, a concerted effort will be needed from industry and the Agency to compile accurate information on how these chemicals are used.

B. ECOLOGICAL ISSUES

Evidence has been presented that a number of environmental agents (both synthetic and natural) have the potential of disrupting endocrine systems in aquatic life and wildlife. The problem is characterized by varied adverse effects on the endocrine systems of a wide range of species. Effects observed include abnormal thyroid function, sex alteration, poor hatching success, decreased fertility, and reduced growth.

The evidence that has accumulated in the scientific literature is compelling that the endocrine systems of certain fish and wildlife have indeed been disturbed by chemicals that contaminate their habitats. At present, it is not clear whether the adverse effects seen at various sites are confined to isolated areas or are representative of more widespread conditions. In many cases, the chemicals identified are ones that already have been identified as problem substances due to their toxicity and persistence (DDT, PCBs, heavy metals, etc.) and therefore are heavily regulated or banned from commercial use in the United States, or the chemicals are complex mixtures (pulp mill effluents, Superfund site drainage, etc.) that are known to be hazardous and to have deleterious effects in highly exposed populations. For many of the cases, however, the evidence lacks specific cause-and-effect data, and alternative explanations for the observed effects cannot be completely ruled out. For instance, goiter in Great Lakes fish has no specific chemical or mixture of chemicals identified or specific exposure level quantified that produces the anomaly. It seems likely that there is a chemical etiology for the phenomenon, besides low iodine levels in the Great Lakes, but much more research is needed in this case and for many others as well.

It is significant that these chemicals that affect fish and wildlife in their natural habitat have been shown to cause similar adverse effects in laboratory test animals. In addition, specific chemicals have been detected in fish and wildlife coincident with the onset of adverse reproductive effects.

For virtually all toxic chemicals, the toxic action or stress imparted on an organism will likely be moderated by endocrine and immune processes that exist to maintain homeostasis. Because of this, it is difficult to elucidate whether a toxic action is directed specifically at an endocrine function or whether an endocrine process disruption is an indirect consequence of some

other stress to the immune, nervous, and/or reproductive system. This fact may provide an explanation as to why many compounds have been postulated as endocrine disruptors.

While great attention has been focused mainly on environmental estrogens (xenoestrogens) and their possible adverse effects to human and other animal well-being, it should be kept in mind that these and other environmental agents may act at several target sites promoting, directly or indirectly, endocrine disruption, disease, and adverse population effects. Furthermore, it should be kept in mind that certain pesticidal agents have been synthesized to function intentionally as hormone/growth regulators to control pest populations. Although it is clear that exogenous chemicals can interfere with hormonally mediated processes, the extent to which exposure to these environmental chemicals occurs at levels that may cause endocrine disruption is uncertain. Until additional laboratory animal, wildlife, and some human studies provide sufficient evidence for an environmental endocrine disruption phenomenon, it seems reasonable to call the endocrine disruption issue a working hypothesis.

In summary, while the majority of the effects listed above are of concern, whether these observations represent widespread or isolated phenomena and whether these effects can be attributed to a specific endocrine disruptor will require additional research.

C. DATA GAPS AND RECOMMENDED RESEARCH NEEDS

The data gaps and research needs on potential endocrine disruptors summarized below under specific human health and ecological research needs support and complement those presented in much greater detail in two recent workshops and addressed in the following documents: (1) *Research Needs for Risk Assessment of the Health and Environmental Effects of Endocrine Disruptors: A Report of the US EPA-Sponsored Workshop*, Raleigh, NC, April 10-13, 1995 (Kavlock et al., 1996) and (2) *Development of Research Strategy for Assessing the Ecological Risk of Endocrine Disruptors*, Duluth, MN, June 13-16, 1995 (Ankley et al., 1996). These latter two documents, along with ORD's research strategy proposal, present needs for research information that will be useful to the Agency in responding appropriately to potential effects of endocrine-disrupting chemicals on health and the environment.

In view of the current interest and concern in environmental endocrine disruption for human health and ecological well-being, additional epidemiologic, laboratory testing, and field studies can be undertaken to better define the nature and scope of the potential problem. Epidemiologic studies of populations environmentally or occupationally exposed may provide an insight into the actual risks posed by chemicals. Both in vitro and short-term in vivo tests could be developed and validated in independent laboratories in an effort to elucidate mechanisms. Biomarkers of exposure could be defined and their concentrations related to degree of insult (i.e., dose/response assessment). Pharmacokinetics studies will be helpful for improving risk assessments by allowing

extrapolation between species and assessing other routes of exposure. Because of the interrelationship of the endocrine glands, the potential disruption of either one could have detrimental effects elsewhere. For example, the active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3, a hormone, causes a hypercalcemia with resulting disturbance of the estrous cycle, corpus luteum dysfunction, reduced serum progesterone, and uterine function (Horii et al., 1992). In other words, disruption of one endocrine gland function may influence other endocrine glands. Additionally, the endocrine system is related to nervous and immune systems, whereby disruption of one component may affect others. Consequently, these interrelationships could be fertile grounds for research exploration of environmental endocrine disruption.

1. Female Reproductive and Developmental Research

a. Ovary and Reproductive Tract

Updated reproductive and developmental testing guidelines have been proposed recently that should improve the Agency's ability to indirectly assess hormonal disruption and the effects on laboratory test animals, but there may be a need for additional tests to specifically evaluate chemicals perceived to be endocrine disruptors.

The inclusion in the new guidelines of estrous cycle evaluation, vaginal opening, and anogenital distance measurements when appropriate may provide information on whether estrogen and androgen receptors have been affected by a given compound. Specific inclusion of ovarian and uterine weights and the histology on these reproductive organs also may help in evaluating potential endocrine active chemicals. Although all changes occurring in these organs are not necessarily specific to endocrine effects, all changes in these endocrine-sensitive organs should help indicate when further testing may be desirable. The measurement of serum hormone levels in laboratory animals at appropriate times, if incorporated into testing guidelines, should provide useful information as to whether an endocrine disruption mechanism may be operating.

Validation of certain experimental testing assays (both in vitro and in vivo), developed and used in some research laboratories for use as estrogen assays, would be a valuable first step in the development of more efficient approaches to determine whether the potential exists for agents to cause hormonal disruption. However, these studies should not be used as a sole determinant of whether a compound is an endocrine disruptor, and special in vivo studies would be necessary to support the information obtained from in vitro screening tests or computer models. Finally, research is needed to determine the feasibility of such a tier approach, the type of studies that are needed, and the impact that a battery of tests for endocrine disruption will have on the risk assessment process.

b. Endometriosis

There is a need to develop and validate laboratory animal endometriosis models for testing chemicals and xenobiotics with other than rhesus monkeys. A rat model for endometriosis has been reported (Cummings and Metcalf, 1995). The use of human endometrial transplants in nude (immunologically compromised) rodents might provide an appropriate animal model for the testing potential causative agents of endometriosis.

c. Breast Cancer

There are a number of data gaps in our understanding of mechanisms of mammary gland carcinogenesis. Traditionally, safety and scaling factors and "mathematical models" have been employed to estimate the risk to humans based on study results in test animals. Such procedures are based on assumptions that may not be realistic for predicting human hazard/risk or mechanisms. Therefore, there is a need to develop and validate biologically based dose-response test animal to human extrapolation models for studying mechanisms of toxicity and chemical carcinogenesis, thus improving human risk assessment.

Because environmental estrogenlike chemicals have been implicated as possible contributing factors in the etiology of human breast cancer, these agents could be tested in various appropriate animal models.

2. Male Reproductive Research

Testing for reproductive toxicity should include evaluation of both the quantity and quality of sperm produced. Such measures are emphasized in both the Draft EPA Guidelines for Reproductive Toxicity Risk Assessment and the Draft Two-Generation Reproductive Toxicity Test Guidelines. The recent revelations that agents such as estradiol and DES, as well as the DDT metabolite DDE, also have antiandrogenic activity place significantly increased importance on that mechanism of action. It is quite possible that the effects attributed to estrogenic activity are due to antiandrogenic activity instead of or in addition to estrogenic activity. Therefore, it is important that testing for endocrine-disrupting potential of environmental chemicals include the ability to detect antiandrogenic activity in addition to estrogenic activity. Testing also should be able to detect alteration in androgen receptor function as reflected in genome expression.

Further intense research on the population exposed to DES might allow stratification of adverse effects by timing and level of exposure. Additionally, because retrospective examinations of existing data are likely to yield ambiguous results, it is important that prospective studies of human male sperm production be conducted. Such studies should include examination of trends in testicular cancer and sperm production over time and attempt to relate results to body and target tissue burdens of chemicals known to have antiandrogenic and/or estrogenic effects. The

need for information relatively quickly dictates that existing populations of men be studied. For the long term, ideally, a study would begin with the pregnancies from which the male study population was derived. Under those conditions, evaluation of the other known or developmentally induced reproductive system effects could be done also.

Whether herbicide exposure contributes to the increasing incidence of human adenocarcinoma of the prostate and, if so, whether the mechanism is by way of an endocrine disruption have yet to be confirmed. If additional epidemiology studies support the above finding, then the next question is to identify which specific herbicide is the causative agent and what is the mechanism by which the carcinogen acts. Because the association between prostate cancer and herbicide spraying has been suggested, there is need to determine the most likely route (oral, inhalation, and/or dermal) of human exposure. If a dietary risk factor (increased fat intake) is confirmed, perhaps an oral route of exposure is most likely. Is a genotoxic effect operational, or is there an epigenetic mechanism working? Pertinent to this discussion, what is the evidence that a hormonal mechanism is contributing to the increased incidence of this disease? Are androgen-mimetic chemicals likely candidates? These and other questions require further research.

3. Hypothalamus, Pituitary, and Thyroid Research

Future efforts should concentrate on developing improved tests to identify environmental agents that alter endocrine function through their action on the CNS and pituitary. Such tests are needed to identify any adverse neuroendocrine changes that occur in response to exposure during development and/or in adulthood. These tests might include direct measures of the gonadotropins and prolactin, as well as an assessment of the functional reproductive endpoints that are regulated by the pituitary hormones. Further information is needed to better evaluate the extent to which the normal sex differences in the neuroendocrine control of gonadal function may contribute to gender differences in response to reproductive toxicants. Because the CNS may develop tolerance to exposure to environmental agents, further studies are needed to evaluate the impact of tolerance on neuroendocrine/reproductive toxicity and to determine whether or not the current tests will identify this phenomenon.

Clearly, there is a need for protocols and multiple tests to identify chemicals that have the potential of disrupting thyroid hormone function. In rat studies, propylthiouracil treatment during development impairs CNS function (i.e., hearing) in adulthood (Davenport and Dorcey, 1972). Information on effects of chemicals in both sexes and the effects of exposure to the fetus, children, and adults are necessary. Once these apical tests are developed and validated, then additional tests to ascertain mechanisms of action seem appropriate. In an effort to extrapolate test animal to human equivalence, reasonable dose-response data are needed, along with pharmacokinetics studies.

4. Ecological Research

Many questions must be addressed before the overall magnitude, extent, and specific causes of this environmental concern can be resolved. Information is needed on what chemicals or class of chemicals can be considered to be genuine endocrine disruptors. The quantity (dose) of a chemical that is necessary to cause an adverse effect is important. Next, there is a need to know whether chemicals that are suspected of being endocrine disruptors act in an additive, synergistic, or antagonistic manner. While there are several available tests that are capable of evaluating a chemical for possible unique endocrine system disruption in some animal species, it is unclear which one or ones are the most useful. Apparently, there are no avian reproductive tests to evaluate specific estrogenic effects in birds. Therefore, it is important to determine how well current screening assays predict an adverse ecological effect due to endocrine disruption. Methods need to be developed and validated to test for a cause-and-effect and a dose-response relationship to allow for sound risk assessment and regulatory decisions to be made. Additional research is needed to (1) determine whether a chemical or its metabolites have hormonal activity, and if so, what the mechanism of action is; (2) prioritize chemicals in relative potency terms of toxicity; (3) determine whether organisms are exposed to the chemical in the environment; (4) ascertain whether there are sensitive species and individuals, and (5) predict effects in the environment, including the effects on organisms, populations, communities, and ecosystems. Specifically, test methods are needed to identify potential endocrine disruptors, quantify the potency of such action, and demonstrate any adverse outcome.

"Sentinel" species (organisms used to detect effects of hazardous exposures) have been used to identify environmental contaminants. Therefore, there is a need to determine whether current sentinel species are adequate surrogates for identifying endocrine disruptors in wild and aquatic life or if other sentinel species should be identified and validated for assessing the state of ecosystems. Perhaps the development, validation, and use of amphibian and/or reptilian models would be appropriate in view of their widespread distribution and lack of information on these classes of vertebrates. Evaluations of ecological effect generally do not consider factors such as disease resistance (immune system dysfunction), behavior (mating disruption), or reproductive viability of offspring (transgenerational effects). Consequently, there is a need to determine whether existing ecological effects/endpoints are adequate for assessing endocrine system perturbation. If not, then additional effects/endpoints are needed.

Finally, there is a need to know what effects that occur at the earliest response threshold are relevant for further risk characterization and what are the population, community, or ecosystem consequences of the effects observed in fish and wildlife.

V. APPENDIX

Table 1. Selected Chemicals With Thyroid Activity: Potential to Induce Thyroid Tumors

Chemical	Thyroid Tumors	Dose Level
Alachlor	+	126 mg/kg (rats) [a]
Amitrole	+	1.04 mg/kg (rats)
Chlorpropham	-	1000 mg/kg (rats) [b]
Clofentezine	+	20 mg/kg (rats)
Ethiozin	+	80 mg/kg (rats)
Ethylene Thiourea	+	4.15 mg/kg (rats)
Maneb	*	
Mancozeb	+	30.90 mg/kg (rats)
Metiram	+	varies with species
Metribuzin	-	42.2 mg/kg (rats) [b]
Oryzalin	+	135 mg/kg (rats) [a]
PCNB	+	150 mg/kg (rats)
Pendimethalin	+	213 mg/kg (rats)
Pronamide	+	42.59 mg/kg (rats)
Zineb	*	

+ = Positive for thyroid tumors.

- = Negative for thyroid tumors.

*** = Presumed positive because of ETU; complete data not available.**

[a] Exceeds MTD (Maximum Tolerated Dose).

[b] Highest dose tested.

Table 2. Attributed Endocrine Disruption Effects in Wildlife for Some Pesticides

Pesticide	Reported effect (OPP files)
HERBICIDES	
Trifluralin	Fish vertebral anomalies
FUNGICIDES	
Benomyl	Fish growth impaired, reduced embryo survival; mysid reproduction impaired
Iprodione	Altered bird behavior, reduced egg production, reduced hatchlIng weight; mysid reproduction impaired
Mancozeb	Avian reproduction impaired, delay In egg layIng
Metiram	Avian reproduction impaired, reduced egg production, reduced fertility, Embryonic deaths
Tributyltin oxide	Imposex In snails; oyster growth anomalies
Vinclozolin	Avian reproduction impaired, reduced egg production, reduced fertility, impaired testicular development
INSECTICIDES	
Azadirachtin	Arthropod molt Inhibition
Carbaryl	Avian reproduction impaired; fish reproduction impaired
Dicofol	Avian reproduction impaired
Dieldrin/Aldrin	Avian reproduction impaired
Diflubenzuron	Reduced testosterone In birds; arthropod cuticle deposition disruption
DDT	Avian reproduction impaired, eggshell thInnIng
Endosulfan	Avian reproduction impaired, reduced egg production
Fenoxycarb	Arthropod molt Inhibition
Malathion	Fish growth reduced
Methomyl	Avian reproduction impaired
Methoxychlor	Avian reproduction impaired; fish growth reduced, impaired hatchIng success
Parathion	Avian reproduction impaired, reduced egg production, reduced adult body weight; fish reproduction impaired, vertebral anomalies; mysid growth reduced
Synthetic pyrethroids (various)	Avian reproduction impaired, eggshell thInnIng; fish reproduction impaired
Toxaphene	Avian adult growth reduced, shortened egg-layIng period, reduced hatchability; fish growth reduced, vertebral anomalies

Table 3.Organisms, Possible Chemical(s) Exposure, and Types of Effects

Organism	Chemical(s)[a]	Type of Effect
Salmon	PCBs, dioxins, organochlorine pesticides	Abnormal thyroid function
Herring gulls	PCBs, dioxins, organochlorine pesticides	Abnormal thyroid function
Western gulls	DDT and DDE	Feminization
Marine snails	TBT	Masculinization
Mosquito fish	Pulp mill effluent	Masculinization
Grizzly and black bears	Unknown[b]	Masculinization
RaInbow trout	Sewage effluent	Feminization
Alligators	Organochlorine pesticides	Demasculinization
Panther	Mercury, DDE, PCBs	Demasculinization
Suckers	Pulp mill effluent	Defeminization and decreased fertility
Atlantic croaker	Lead, cadmium, benzo[a]pyrene, and PCBs	Defeminization
Bald eagle	DDT and DDE	Decreased hatchability
Forster's tern	TCDD, PCDD, and PCBs	Decreased hatchability
Wood duck	TCDD and TCDF	Decreased hatchability
Cardinals, mockIngbirds, and thrashers	Various pesticides	Decreased hatchability
Snapping turtle	PCBs, dioxins, and furans	Decreased hatchability
Sheep	Phytoestrogens	Decreased fertility

[a] Chemical(s) to which organisms were exposed.

[b] Chemical(s) were not mentioned In the literature cited.

Table 4. Relative Estrogenic Potencies of Alkylphenol Ethoxylates and Breakdown Products [a]

Compound	Relative potency [b]
Estradiol-17ß	1
Nonylphenol ethoxylate (E0=9)	0.0000002[c]
Nonylphenol ethoxylate (EO=2)	0.0000060
Nonylphenol carboxylate	0.0000063
P-nonylphenol	0.0000090
P-octylphenol	0.00003700
P-tert butylphenol	0.0001600

[a] Source: Jobling and Sumpter, 1993.

[b] Relative potency compared to estradiol.

[c] In the MCF-7 assay p-nonylphenol had a relative potency of 0.000003 compared to estradiol (Soto et al., 1992).

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