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ESTROGENICITY AND ENDOCRINE DISRUPTION

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INTRODUCTION

During the past 50 years, much of the remarkable progress made in terms of agricultural production, standard of living, and length of human life has been associated with development and application of highly specialized, synthetic industrial chemicals. Production of some chemicals was, for certain diseases, initially associated with increased risks due to elevated occupational exposures. This prompted the creation of state and federal regulatory agencies, e.g., the Occupational Safety and Health Administration, to set acceptable levels for workplace exposures.

In 1962, Rachel Carson's *Silent Spring* implicated pesticides such as dichloro-diphenyl-trichloro-ethane (DDT) in the decline of some wildlife populations. Studies in the late 1960s and early 1970s discovered measurable levels of persistent organochlorine contaminants such as polychlorinated biphenyls (PCBs) and DDT and its metabolite 1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene (DDE) in almost every component of the global ecosystem, including fish, wildlife, and humans (Holden and Marsden 1967; Jensen 1966; Risebrough et al. 1968). These observations led in many countries to regulatory actions restricting or banning production and application of PCBs and DDT. Agencies such as the U.S. Environmental Protection Agency (EPA) regulated environmental release of synthetic industrial chemicals, especially lipophilic compounds such as PCBs and DDE, that both persisted and bioaccumulated in the environment. Regulatory actions have resulted in decreased levels of most organochlorines in the Great Lakes region and have brought about "dramatic improvements in reproductive success and significant increases in the populations of cormorants, gull, terns, herons, and other predatory birds in the Great Lakes basin" (Tremblay and Gilman 1995).

Colborn, Vom Saal, and Soto (1993) reviewed wildlife reproductive and developmental problems associated with exposure to synthetic organochlorine compounds such as PCBs and other 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin)-like compounds in the Great Lakes region. Many different structural classes of industrial chemicals released into the environment exhibit potential endocrine-disrupting activities. Thus, the researchers hypothesized that exposure to these compounds during critical periods of development, i.e., in utero or early postnatal, could result in irreversible damage to wildlife and possibly humans. Concern about effects of endocrine-disrupting environmental contaminants was heightened by the publication of several papers in the early 1990s. Carlsen and coworkers (1992) reported that their analysis of 61 publications on human semen-quality indicated that, between 1950 and 1990, sperm counts had

declined globally from 113 x 10^6 per milliliters (ml) to 66 x 10^6 per ml. Sharpe and Skakkebaek (1993) hypothesized that "the increasing incidence of reproductive abnormalities in the human male may be related to increased estrogen exposure *in utero*." Sharpe (1995) extended this hypothesis to include DDE, after Kelce and coworkers (1995) showed that this compound was an antiandrogen. The observation that in Lake Apopka, Florida, alligators exposed to chemicals leaking from a waste disposal site had reduced penis size (Guillette et al. 1994, 1996) further supported the human studies and led to numerous articles in the popular press and to production of the British Broadcasting Corporation television program "Assault on the Male." In 1992 and 1993, it was reported that PCB and DDE levels were higher in breast cancer patients than in controls (Falck et al. 1992; Wolff et al. 1993), a finding that led to the hypothesis that xenoestrogens were preventable causes of breast cancer in women (Davis et al. 1993).

The debate over the effects that environmental endocrine disruptors have on reproductive capacity in males and breast cancer in women has spurred research in several areas. Results often have been controversial, and consensus among investigators has been difficult to achieve. This review will present the opinions of scientists who have performed research on endocrine-disrupting chemicals (EDCs) and will provide key background information and some new data with implications regarding the effects of EDCs on human health.

Environmental Impacts of Endocrine-Disrupting Chemicals

Introduction

The scientific community and the general public have shown great interest in the possibility that wildlife has been affected by exposure to chemicals altering endocrine system functioning. Although not new, the concept that environmental chemicals influence growth, reproduction, and development in wildlife has garnered renewed attention since publication of a workshop summary entitled "Chemically Induced Alterations in Sexual Development: The Wildlife/Human Connection" (Colburn and Clement 1992) and of the book *Our Stolen Future* (Colborn, Dumanoski, and Myers 1996). Studies summarized in these and other scientific articles have highlighted numerous examples of compromised growth and reproduction, altered development, and abnormal behavior in various taxa, including invertebrates, fish, reptiles, birds, and mammals. The effects can be correlated or, in some instances, causally linked with exposure to EDCs (Fairbrother et al. 1999; Kime 1998; National Research Council 1999; Van Der Kraak 1998: Van Der Kraak et al. 1998).

This section reviews several case studies describing wildlife responses attributed to EDCs. A subsequent section discusses the underlying uncertainties associated with defining the extent to which endocrine disruption affects wildlife populations, and outlines the research needed to improve understanding of the risks posed by EDCs.

Case Studies

One of the best examples of population-level effects of EDCs comes from studies of the masculinizing actions of tributyltin (TBT) on reproduction in marine gastropods (Matthiessen and Gibbs 1998). Tributyltin originating from antifouling paints used to treat boat hulls induces a form of pseudohermaphroditism termed *imposex* in female neogastropod molluscs. This imposition of male sex organs, i.e., penis and vas deferens, can lead to sterility. A related condition termed *intersex* occurs in some gastropods and involves TBT-induced alterations of the oviduct. Tributyltin use has been associated with gastropod population declines; a ban on its use in many countries has been related to decreased imposex incidence and increased gastropod numbers.

Endocrine disruption in fish has been investigated extensively in both the field and the laboratory. Compelling evidence for the effects of environmental EDCs comes from around the globe:

1. The induction of hermaphroditism and estrogenic responses in rainbow trout and roach downstream of sewage treatment works in the United Kingdom has been attributed to synthetic estrogens from birth control pills and to chemicals with estrogenic activity (alkylphenols) present in the effluent (Matthiessen 1998; Tyler and Routledge 1998).
2. Blue sac disease and early life-stage mortality in Great Lakes salmonid populations, especially in Lake trout, has been attributed to TCDD and to related aryl hydrocarbon receptor (AhR)-ac-

tive compounds (Cook, Zabel, and Peterson 1997; Giesy and Snyder 1998).

3. Altered endocrine homeostasis and reproductive fitness (e.g., decreased gonad size, egg size, and fecundity; delayed sexual maturity) have been found in fish, especially in white sucker populations, exposed to pulp mill effluents (McMaster, Van Der Kraak, and Munkittrick 1996; Van Der Kraak et al. 1998).
4. Depression of plasma sex steroid hormone levels and inhibition of gonad development in marine flatfish in Puget Sound have been associated with polyaromatic hydrocarbon exposure (Fairbrother et al. 1999).

Although for certain exposures many of these responses have been recognized for more than a decade, uncertainty remains about the identity of the chemicals responsible (e.g., those in pulp and paper mill effluents) and of the mechanisms associated with their effects on reproductive and developmental endpoints.

Amphibian populations have declined worldwide; and global climate change, increased ultraviolet light levels, habitat loss, disease, pesticides, and industrial pollutants all have been implicated. Although little evidence exists to suggest that amphibians have been affected by alterations in endocrine homeostasis initiated by environmental EDCs, few studies have considered this possibility (Ankley and Giesy 1998).

American alligators in Lake Apopka, Florida exhibited decreased numbers of juveniles, decreased clutch viability, and reproductive organ abnormalities after a spill of the pesticide dicofol and sulphuric acid in 1980 (Guillette and Crain 1996). Subsequent studies showed that hatchlings of both sexes had altered gonadal structure and abnormal ratios of the sex steroids 17-beta-estradiol and testosterone; males had reduced phallus size. Linking the observed effects with a specific chemical or defining the underlying mechanism responsible for these effects has been difficult.

Numerous studies have reported that agricultural and industrial waste chemicals exert adverse effects on wild bird populations. Two major syndromes, i.e., eggshell thinning and PCB-induced teratogenesis, have been linked to chemicals functioning as endocrine disruptors (Feyk and Giesy 1998; Sheffield et al. 1998). The increased incidence of abnormal behavior in gulls inhabiting regions contaminated with DDT often is cited as evidence for certain organochlorine-induced estrogenic effects in birds (e.g., Fry 1995). The eggshell-thinning effect of DDT and its stable metabolite *p,p'*-DDE well may represent one of the most publicized incidents in wildlife toxicology, yet the underlying mechanisms mediating this response and whether this involves endocrine dysfunction have never been adequately studied. The PCB-induced syndrome of *in ovo* and chick mortality, growth retardation, subcutaneous and pericardial edema, and deformities of beaks and limbs has been attributed to chemicals such as PCBs, polychlorinated dibenzofurans, and dibenzo-p-dioxins that bind the AhR. The precise linkage between AhR binding and endocrine disruption in mediating these responses remains poorly understood.

One of the most widely cited examples of endocrine disruption in feral mammals involves mink populations near the Great Lakes. The number of wild mink in this area has decreased greatly, and feeding trials using Great Lakes fish led to adverse reproductive effects in ranched mink (Ankley and Giesy 1998). Because Great Lakes fish contain elevated levels of a number of synthetic organochlorines, including pesticides, PCBs, and heavy metals such as mercury, it has been difficult to determine which chemicals are responsible for the adverse effects or to develop the linkage between exposure, endocrine alterations, and whole-animal responses. Other studies with marine mammals, and seals in particular, have implicated organochlorine insecticides and PCBs in reproductive deficits, adrenal hyperplasia, and alterations in thyroid hormone physiology. Most of this information is rather indirect, however (National Research Council 1999; Sheffield et al. 1998). Table 1 provides examples of wildlife reproductive and developmental abnormalities attributed to EDCs.

There are numerous examples of naturally occurring phytochemicals and fungal toxins with profound effects on reproductive and endocrine function in both wild and domestic mammals eating contaminated diets (Goldman, Gray, and Cooper 1998; Kaldas and Hughes 1989). Whether synthetic environmental chemicals with similar properties may affect wildlife requires further study.

Uncertainties and Research Needs

A review of case studies listed in Table 1 dem-

Table 1. Selected examples of wildlife reproductive and developmental abnormalities attributed to EDCs (modified from Van der Kraak 1998)

Species	Site	Observation	Contaminant
Invertebrates			
Gastropods	Marine	Pseudohermaphroditism, imposex, intersex sterility, population declines	Tributyltin
Fish			
Trout, roach	English rivers	Hermaphroditism, vitellogenin in males altered testis development	Sewage effluent
Lake trout	Great Lakes	Early life stage mortality, deformities, blue sac disease	Dioxin and related AhR agonists
White sucker	Jackfish Bay, Lake Superior, MI	Reduced sex steroid levels, delayed sexual maturity, reduced gonad size	Bleached kraft pulp, mill effluent
Flatfish	Puget Sound, WA	Decreased hormone levels, reduced ovarian development, reduced egg/larvae viability	PAHs
Reptiles			
Alligator	Lake Apopka, FL	Decreased viability, abnormal gonadal development, decreased phallus size	DDE
Birds			
Waterbirds Raptors	Global	Egg shell thinning, mortality, developmental abnormalities	DDE
Waterbirds Raptors	Great Lakes	In ovo and chick mortality, growth retardation, deformities	PCBs, AhR agonists
Western gulls	California	Abnormal mating behavior, supranormal clutch size, skewed sex ratios	DDT and its metabolites
Mammals			
Mink	Great Lakes	Population decline, developmental toxicity, hormonal alterations	PCBs and dioxins

Abbreviations: AhR = aryl hydrocarbon receptor; DDE = 1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene; DDT = dichloro-diphenyl-trichloro-ethane; PAH = polyaromatic hydrocarbon; PCB = polychlorinated biphenyl.

onstrates that the variety of wildlife responses attributed to EDCs is most obvious in species inhabiting areas contaminated extensively with chemicals. But risks associated with exposure to background environmental levels of contaminants are unknown. An improved understanding of the risks posed by EDCs requires more research on the biology and physiology of sentinel species and the ability to interpret the significance of effects at the suborganismal and organismal levels in relation to population and community sustainability.

One overriding concern in all ecotoxicological investigations is that many factors besides endocrine disruption can adversely affect growth, reproduction, and survival. Wildlife is subjected to multiple stressors, so separating out the incremental risks associated with EDCs from those associated with other anthropogenic stressors is a major undertaking. Quite often, these other stressors are ignored in assessments of wildlife populations for effects of suspected EDCs. Food availability, disease, competition, and habitat loss are significant wildlife stressors, yet our understanding of how these contribute to physiological fitness often is inadequate (Munkittrick and Van Der Kraak 1999). Such factors can impinge directly on many endocrine and physiological endpoints used to evaluate the effects of EDCs. A range of other factors — including sex, age, season, reproductive status, and genetics — can contribute to variability in these biomarkers. Because sampling method and handling and capture stress can affect physiological measurements, these aspects need to be monitored closely and standardized. Another feature of wildlife studies is that comparisons often are made in relation to preselected reference locations. Given the range of nonchemical factors influencing physiological endpoints, it is difficult to identify appropriate reference populations for comparison with exposed populations. This is a concern not only for EDC studies but for environmental toxicology studies generally.

Ongoing needs are the abilities to identify substances potentially contributing to effects in wildlife

and to define their modes of delivery, biological fates, mechanisms of action, and environmentally acceptable levels. Timing and magnitude of exposure, as well as changes with time in sensitivity of target tissues, are important in defining the effects of EDCs and need to be integrated with fate and exposure data. Meeting these research needs will improve the data available for assessing risks and hazards and for developing appropriate regulatory actions.

Environmental and Natural Estrogens — Laboratory Animal Studies

Strong support for the biological plausibility of the endocrine disruptor hypothesis can be found in numerous laboratory animal studies. The well-documented reproductive tract and neurodevelopmental problems of in utero exposure to TCDD, an AhR agonist, and the developmental consequences of *p,p'*-DDE, an androgen antagonist, provide clear examples of the adverse effects of environmental chemicals possessing endocrine disruptor activity (Gray and Ostby 1987; Gray et al. 1995; Mably, Moore, and Peterson 1992; Mably, Moore, and Goy et al. 1992; Mably, Bjerke, and Moore et al. 1992). Diethylstilbestrol (DES) is perhaps the best-known example of a synthetic estrogen with adverse consequences in laboratory animals and humans (Herbst and Bern 1981; National Institutes of Health 1999; Newbold 1995).

From the late 1940s through the 1970s, physicians prescribed DES, a highly potent synthetic estrogen, to prevent miscarriage and other complications of pregnancy. Decades later, it was discovered that adolescent daughters of women who had taken the drug while pregnant developed a rare form of vaginal cancer (Herbst, Ulfelder, and Poskanzer 1971). Although the incidence of this cancer was determined to be less than 0.1% in the DES-exposed population, DES was linked to more-frequent benign reproductive tract abnormalities in an estimated 95% of DES-exposed daughters. Reproductive organ dysfunction, abnormal pregnancies, decreased fertility, and immune system disorders have been documented (National Institutes of Health 1999). Similarly, DES-exposed sons demonstrated structural, functional, and cellular abnormalities following prenatal exposure. Hypospadias, microphallus, retained testes, inflammation, and decreased fertility have been reported (National Institutes of Health 1999). Although DES no longer is used clinically to prevent miscarriage, a major concern remains that, as these DES-exposed children age, their incidence of certain cancers may be higher than the incidence in the unexposed population. A recent study (Hatch et al 1998) did not show an increased incidence in overall cancer in women exposed in utero to DES although vaginal clear cell adenocarcinomas were increased. Because the average age of these women was only 38, it will be important to continue surveillance for longer-term increases in cancer incidence.

A substantial literature base of rodent studies dating back to the early 1940s documents the detrimental effects of exogenous estrogens if a sufficient exposure occurs during critical periods of differentiation. Laboratory animal studies of DES exposure have reported similar adverse effects with other estrogenic substances. The prenatal DES-exposed mouse model has been especially useful in studying mechanisms involved in DES-induced toxicity and in replicating (and, in some instances, predicting) clinical findings in DES-exposed humans (McLachlan, Newbold, and Bullock 1975, 1980; Newbold 1995). For example, dysplastic changes in the mouse prostate are comparable to those seen in stillborn male offspring of DES-treated women. Other DES-induced abnormalities reported in both humans and mice are retained testes, decreased sperm counts, epididymal cysts, testicular tumors, anatomical feminization, microphallus, hypospadias, retained Mullerian remnants, and prostatic inflammation (Newbold 1998). In female mice, DES exposure early in development leads to subfertility/infertility, para-ovarian cysts, immune dysfunction, behavioral changes, and reproductive tract malformations and neoplasia (Newbold 1999). Comparable effects in DES-exposed humans have been reported (National Institutes of Health 1999). The possibility of second-generation effects has been reported in the DES mouse model, a fact suggesting that yet another generation may be at risk for developing problems associated with the DES treatment of their grandmothers (Newbold et al. 1998; Turusov et al. 1992; Walker 1984).

Thus, studies on developmental exposure to the estrogenic compound DES have provided an important model for delineating problems associated with exposure to estrogenic compounds in both animals and humans. Diethylstilbestrol-induced effects on male and female reproductive tracts strongly support the endocrine disruptor hypothesis, especially for potent estrogens given at sufficient levels during develop-

ment.

Laboratory studies by Vom Saal and coworkers (1997) showed that early developmental exposure to low doses of bisphenol A increased prostate weight in offspring whereas higher doses decreased prostate weight. Because the low-dose "inverted-U" dose-response curve observed for bisphenol A but not for alkylphenols is controversial and could not be replicated in another study (Cagen, Waechter, and Dimond, et al. 1999), the phenomenon requires further research and validation.

Whereas environmental contamination with synthetic estrogenic chemicals has engendered concern, naturally occurring substances are well-known to have estrogenic activity. The phytoestrogen genistein, found in soy products, is just one example. Experimental animal studies with genistein have shown altered volumes of the sexually dimorphic nucleus in the preoptic area of the hypothalamus, abnormal reproductive tract development, altered mammary development, and changed pituitary responsiveness to gonadotropin-releasing hormone (Faber and Hughes 1993; Levy et al. 1995). Other studies reported that long-term effects of developmental exposure to genistein included increased incidence of neoplasia in reproductive tract tissues (Newbold et al. submitted). Another soy constituent, coumestrol, was reported to have mixed estrogen agonist and antagonist activities in laboratory animal studies, and the pattern of estrogenic responses induced by coumestrol were somewhat unique. In some studies, coumestrol acted additively with estradiol to increase uterine weight and to decrease estrogen receptor (ER) binding; in other studies, coumestrol was reported to dampen the effects of endogenous estrogens on other endpoints. These variations point to the complexity of the mechanisms responsible for differences.

Because dietary intakes of synthetic estrogenic compounds are much lower than those of naturally occurring phytoestrogens, it has been suggested that effects of synthetic chemicals are negligible. But because many synthetic compounds bioaccumulate, their effects may be enhanced. Moreover, mechanistic studies show differences among phytoestrogens, estradiol, and synthetic estrogens in binding to ER subtypes and patterns of ER-associated gene responses (Kuiper et al. 1997). Differences occur in responses to synthetic and to natural estrogenic compounds.

Laboratory animal studies of chemicals disrupting the endocrine system show that the most important time of exposure is early in life, e.g., prenatal exposure. If exposure occurs during critical periods of development, effects may be different from those stemming from adult exposure and may last throughout the animal's life. These effects can change the course of development and the reproductive potential of offspring, with outcomes depending on the specific developmental period(s) of exposure. It is difficult to identify specific endocrine disrupting chemicals because effects may not be expressed until offspring reach maturity, or later, even though the critical exposure period occurred during the early embryonic, fetal, or neonatal stage.

Endocrine Disruptors and Human Health

Industry, environmental groups, toxicologists, and regulators are becoming increasingly concerned about the potential human reproductive and developmental effects of EDCs. These agents have been linked to a variety of adverse reproductive and developmental human-health outcomes including but not limited to breast, ovarian, and endometrial cancers; endometriosis; infertility; prolonged time to pregnancy; increased spontaneous abortion rates; decreased male:female birth ratios; increased testicular cancer in young men; increased prostate cancer; decreased semen quality; increased frequency of testicular maldescent; increased hypospadias prevalence; and precocious puberty. Epidemiological studies have demonstrated that incidences of some adverse reproductive/developmental outcomes are increasing although it is difficult to link these changes with specific exposures to synthetic chemicals in general or to EDCs specifically. Furthermore, whether there is a true change in prevalence for outcomes such as infertility and prostate cancer is unclear. The reported changes may be an artifact of statistical reporting, improved diagnostic procedures, medical management changes, or heightened awareness by the community in general and the medical profession in particular. In other instances, meta-analyses of breast, ovarian, and endometrial cancers have failed to identify a statistical association between the adverse outcome of concern and exposure to synthetic chemicals (Adami et al. 1995; Houghton and Ritter 1995; National Research Council 1999).

The endocrine disruptor hypothesis — which is both intriguing and controversial — requires further research. Based on evidence from wildlife studies and

on reports of occupational exposure and animal experiments, the hypothesis certainly is plausible. The role of synthetic chemicals and endocrine pathways associated with male reproductive-tract problems has not been demonstrated, however. Explanations alternative to synthetic chemicals must be identified and investigated with equal vigor and determination. This research is especially important in light of the potential medical, legal, economic, social, and ethical implications associated with EDC-induced effects in the human population.

The absence of evidence for synthetic chemical effects on reproductive outcomes in the general population may be due to several factors. First, there may be no effect or the studies may lack sufficient power to detect a response. Also, there is the difficulty of exposure determination or estimation. Few studies of adverse effects provide such direct measurement of exposure as was provided for the semen-quality studies. Exposure often is inferred from unrelated studies of human-tissue residue levels, surrogate exposure-markers (e.g., umbilical cord blood), maternal serum, or proximity of residence to a suspected point source. Accurate exposure estimates are complicated further by the long latency periods between exposure and effect of interest (e.g., male reproductive-tract developmental abnormalities, breast cancer, semen quality), rarity of the event being studied (e.g., testicular cancer, endometriosis, male reproductive-tract developmental abnormalities), inability to measure exposure directly (e.g., fetal exposure during organogenesis), and absence of a chemically naive population to serve as a control group.

Sample-size limitations (e.g., of endometriosis effects) and dependence on retrospective studies (e.g., of breast cancer or semen quality) complicate attempts to determine whether effects are present and to identify potential causative factors. The sample-size problem was illustrated by Mayani and colleagues (1997), who reported that, to detect a twofold increase in endometriosis — assuming a prevalence of 10%, significance level of 0.05, and 90% power — 286 women with endometriosis and 286 age-matched controls would be required. But no studies have employed sample sizes anywhere near this large; thus, it has not been possible to conclude that exogenous chemicals are or are not involved in the pathobiology of this disease. Finally, large normal variations in outcome; poor predictive values of outcome measures; sample bias; and regional differences (for instance, in semen quality), despite similar chemical exposures, further complicate efforts to identify causative factors.

The semen-quality issue is presented as a case study because it has received the greatest attention in both lay and scientific presses, is highly controversial, and clearly demonstrates a number of the previously identified limitations of current studies. Attention was focused on a purported change in human semen-quality by the publication of the Carlsen and coworkers study (1992) in which concentration of human sperm was reported to have declined by approximately 2% per year for the preceding 50 years. Since this report, numerous studies have appeared in the literature. Some also have demonstrated a decrease in sperm concentration (Adamopoulos et al. 1996; Auger et al. 1995; Baker et al. 1996; Irvine et al. 1996; Menchini-Fabris et al. 1996); some have found no change (Bujan et al. 1996; Fisch et al. 1996; Paulsen, Berman, and Wang 1996); and others have discovered an increase (Benshushan et al. 1997; Vierula et al. 1996) in semen quality. These studies, regardless of their outcomes, suffer from sampling bias in that subjects either were in fertility programs or undergoing vasectomies. Consequently, one cannot generalize from these studies to the whole population. Furthermore, none of these studies reported tissue levels of any synthetic chemical or EDC. It also is perplexing to discover that reports of semen quality diverge so widely for seemingly closely related geographic regions, e.g., Finland and Denmark. Moreover, semen quality varies greatly within an individual with time and is sensitive to factors such as illness, medication use, alcohol exposure, cigarette smoke, diet, caffeine, and physical and emotional stress. Factors other than exposure to environmental chemicals therefore could account for the observed changes if they are, indeed, real.

Notwithstanding, semen quality may serve as a useful biomarker of change in male fecundity within a community. Regional differences within Great Britain, France, and Canada have been reported (Federation Francais des CECOS 1997; Ginsburg et al. 1994; Younglai, Collins, and Foster 1998). Although the reasons for these differences have not yet been explained in the literature, it seems that regional differences may reflect locally important exposures to contaminants or dietary or lifestyle factors requiring investigation and should prove useful to the general understanding of endocrine disruption.

Many reports of trends in reproductive outcomes suggest that human reproduction and development are undergoing changes potentially reflecting as yet unidentified pressures from the environment or responses

to dietary changes. For example, an increased prevalence of cryptorchidism and hypospadias has been reported in a number of countries (Kallen et al. 1986). Although no direct residue measurements were made, cryptorchidism incidence was reported to be greater in boys born to mothers living near pesticide-manufacturing plants (Garcia-Rodriguez et al. 1996). Animal studies show that EDCs may induce cryptorchidism (McMahon, Kramer, and Husmann 1995). A recent study on international trends (Paulozzi 1999) showed that incidence of hypospadias and cryptorchidism differed between regions/countries, with some increases observed before 1985. Since the mid-1980s, however, the incidence of hypospadias has not changed and cryptorchidism has declined in most areas. A decrease in the male:female birth ratio has been reported in the United States, Canada, and elsewhere. This decrease has led to speculation that estrogenic compounds may be the culprit although no plausible mechanistic explanation has been advanced. Finally, a decrease in family size has been shown for the Hutterites (Nonaka, Miura, and Peter 1994; Sato et al. 1994), a North America subpopulation unlikely to have changed its family planning practices or lifestyles throughout the preceding century. These reports, taken together, raise concerns about potentially adverse effects of environmental contaminants and possibly dietary factors on the reproductive tract and its functioning.

Most research has focused on the estrogenic activity of synthetic EDCs. More attention should be paid, however, to (1) dietary factors such as phytoestrogens, which possess similar or greater potency than synthetic EDCs, (2) mixed hormone-like activity and nonendocrine activities of EDCs and dietary factors, (3) potent anti-estrogens such as dioxins and dietary carbinols, (4) anti-androgens such as *p,p*'-DDE and vinclozolin, and (5) effects of mixtures. Dietary factors such as phytoestrogens act as aromatase inhibitors, inhibit protein tyrosine phosphorylation, and possess variable affinities for ER subtypes. Consumption of phytoestrogens is increasing as awareness grows regarding their potential health benefits. These compounds recently have been identified in amniotic fluid during the second trimester (Foster et al. in preparation) and in the urine of children, both periods of hormone-directed cellular and tissue differentiation. Exposure to these agents and other persistent contaminants at key developmental stages raises concerns for human health that have yet to be explored.

In summary, it remains to be determined whether synthetic chemicals have induced adverse changes in reproduction and development within the general human population. Some studies suggest changes in reproductive physiology and development, but the mechanisms underlying and driving these changes remain undetermined. That these changes may simply be normal responses to subtle changes in our environment — including changes in diet and lifestyle — requires further investigation.

Problems for Risk Assessment Associated with Exposures to the Estrogenic Compounds

Introduction

Hormonally active substances are of three types: natural endogenous, natural exogenous, and synthetic. Natural endogenous compounds include hormones such as estradiol or estrone, which serve as the body's natural chemical messengers. These have obvious biological purposes but also may pose risks of adverse effects. Research on fish in the United Kingdom demonstrated that estradiol and other natural hormones in oral contraceptives appeared responsible for the feminization of male fish living downstream of sewage treatment facilities (Purdom et al. 1994).

Natural exogenous substances are found in products such as fruits and vegetables (Verdeal and Ryan 1979). These phytoestrogens or fungal estrogens have significant hormonal activity. For example, soy isoflavones decrease the risk of certain adverse health effects but may, through a hormonal mechanism, cause other adverse health effects.

Synthetic chemicals may have hormonal activity. For example, the insecticide Kepone has weak estrogenic activity (McFarland and Lacy 1969); the same is true for *o,p*-DDT (Welch, Levin, and Conney 1969) and the alkyl phenols (Soto et al. 1991).

In consideration of the risk associated with the hormonal activity of any substance, natural or synthetic, the toxicity and the exposure to that substance must be known. Is the critical toxicity related to the hormonal activity? Hormonal activity is not the same as toxicity: toxicity can come from many types of chemical activities. All substances exhibit some toxicity whereas hormonal activity not only may lead to adverse effects but also is essential to good health (Adlercreutz 1990).

Fundamentals of Risk Assessment

Nor is toxicity the same as risk. Risk depends on exposure to a hazard as well as timing and toxicant potency. Risk assessment of hormonally active substances is the same as for other chemicals and depends on toxicity, dose response, and exposure. With increased interest in EDCs has come the tendency to label as "endocrine disruptors" those substances with hormonal activity. This practice has been especially pronounced for estrogens. Endocrine disruption, however, implies that the hormonal activity will result in an adverse effect. Many synthetic compounds referred to as EDCs (Colborn, Vom Saal, and Soto 1993) have hormone-like activity, i.e., aid in receptor binding, but their adverse hormone receptor-mediated responses are unknown. The endocrine-dependent and endocrine-independent toxicities of these chemicals should be determined.

The potential toxicity of any chemical in an animal depends on fundamental biological processes such as absorption, disposition, and metabolism. These processes have the potential to eliminate completely a response seen in vitro or to magnify that response. In vitro testing systems often allow the substance to reach the target cell unchanged. This same substance may be changed dramatically to a more toxic or less toxic form by the metabolic system. In vivo animal studies often are used to determine the potential toxicities of substances over a range of doses, to establish the level causing an adverse effect, and to estimate the no observed adverse effect level (NOAEL). Researchers compare the NOAEL with exposure data to obtain an estimate of risk. The NOAEL divided by the exposure is the margin of exposure (MOE). Regulators use the MOE to determine whether exposure is acceptable given its predicted toxicity.

A thorough assessment of toxicity will assess acute, subchronic, and chronic toxicities. Studies of developmental and reproductive toxicities also are required routinely. A full toxicological database for a pesticide requires studies in at least four species, including rat, rabbit, dog, and mouse. *Acute toxicity* studies involve a single-day exposure and assessment of fairly general signs of a toxic response. *Subchronic toxicity* studies are conducted with a 13-week exposure and measure additional toxic endpoints. *Chronic toxicity* studies detect effects after one to two years of exposure and are used to predict carcinogenic potential. Rodent and nonrodent species such as rabbits are exposed during pregnancy, and offspring are assessed to predict *developmental toxicity*. *Reproductive toxicity* tests, which expose animals such as rats through two generations, are used to predict effects on fertility and reproduction. Other specialized toxicity studies — mutagenicity, neurotoxicity, immunotoxicity, or metabolism studies — can be performed.

Several assumptions are made when animal data are extrapolated to humans. First, it is assumed that effects observed in laboratory animals reasonably predict the type of toxic effects that would occur in humans. Second, it is assumed that the dose-response relationship observed in animal tests reflects the human dose-response relationship accurately. Third, it is assumed that high-dose experiments in laboratory animals can be used to predict, both qualitatively and quantitatively, low-dose responses in humans. Extrapolation from animals to humans is necessary because human data rarely are available or sufficient for risk assessment.

Special Issues for Hormonally Active Substances

Hormonally active substances must also be studied for their toxic effects. If thought to relate to a particular hormonal activity, developmental or reproductive landmarks may be incorporated into toxicity tests. For example, the potent estrogenic drug DES affects development of the reproductive system. Therefore, if there is concern about exposure to an estrogen, endpoints specific to estrogenic activity can be incorporated. Evidently, certain adverse effects of DES result from its estrogenic activity. This link between estrogenicity and toxicity has led to a program to screen substances for hormonal activity (U.S. Environmental Protection Agency 1998). The EPA's endocrine disruptors screening program (EDSP) is a large, two-step program that first screens substances for hormonal activity, and then tests them for toxicity. The tier 1 screening program focuses on estrogenic, androgenic, and thyroid hormone activity. This program is not designed for risk assessment purposes but to help the EPA set priorities for further toxicity testing.

Additional testing to be conducted in tier 2 (U.S. Environmental Protection Agency 1998) will include toxicity studies of compounds such as pesticides, e.g., two-generation rat reproduction studies. Some additional, hormonally-related endpoints, e.g., preputial separation, vaginal opening, and estrous cycle, will be incorporated into the existing two-generation reproduction study protocol. A major concern about risks of hormonally active agents is that developmental

exposure can result in a permanent change in an offspring that may have been a transient or inconsequential effect in an adult. The tier 2 testing is designed to identify such adverse effects in offspring. Although hormonally responsive endpoints have been included in tier 2 testing, no attempts have been made to demonstrate cause and effect between the hormonal mechanism and its presumably adverse "effect." Therefore, while the EDSP will drive additional testing based on hormonal activity, the testing will yield results on adverse effects whether or not a hormonal mechanism is responsible.

Data on adverse effects will be used to predict risk. The dose-response relationship is important for the adverse effect. For true EDCs, it is assumed that the dose-response relationship for the hormonal response will parallel that for the adverse effect. The relative potencies of these hormones therefore may be important in predictions of their relative potencies as endocrine disruptors. Comparisons of estrogenic potencies have shown that estradiol and DES are substantially more potent than most other hormonally active substances. Diethylstilbestrol is about 1,000 times more potent than most phytoestrogens, about 20,000 times more potent than bisphenol A, and about 100,000 times more potent than *o,p*-DDT (National Research Council 1999).

Exposure to hormonally active substances differs widely across populations. For example, synthetic hormones such as those used in contraceptives or in hormone replacement therapy are given at doses ranging from 20 to 200 micrograms (μg)/day (d). A typical dose of ethinyl estradiol, an oral contraceptive with estrogenic potency similar to estradiol or DES, is 20 μg/d whereas doses of synthetic estrogens for treatment of postmenopausal women are 50 to 200 μg/d. Exposures of pregnant women to DES were even higher, ranging from 5,000 to 150,000 μg/d and resulting in significant adverse effects to the offspring. Phytoestrogen exposures can be as high as one million μg/d. By contrast, bisphenol A exposures range from less than 0.5 to 2 μg/d.

Exposure assessment is complicated, for exposures can vary from day to day and from source to source. Chemical exposures can be found in the water, diet, home, or workplace. Totaling these exposures can be difficult but may be accomplished by using sophisticated statistical models such as probabilistic exposure assessments. Another issue is whether to add exposures of different substances. Basic research on hormonally active substances indicates that summing substances with differing potencies does not necessarily result in an additive response. Early studies indicating that some binary mixtures of weak xenoestrogens gave synergistic activity (Arnold et al. 1996) have not been replicated (Ramamoorthy et al. 1997). More research on EDC mixtures is required to determine their additive or nonadditive interactions.

In an appropriate toxicological model, risk assessment of hormonally active agents and EDCs depends on dose-response data. Test compounds should be administered during critical developmental periods. Data describing toxicant-induced mechanisms of action for an adverse response are important but not sufficient for risk assessment purposes. Fundamental principles of risk assessment and toxicology apply to hormonally active chemicals, and dose determines poison for both toxicants and EDCs. Potency, timing, and amount of exposure all contribute to risk estimates. Development of screening and testing procedures by the EPA should be based on the best science available.

Conclusions

In response to public and scientific concern regarding potential adverse health effects associated with EDC exposure, several government agencies and the U.S. Congress asked the National Research Council (NRC) to evaluate the scientific data and to provide advice based on them. The NRC, an agency of the National Academy of Sciences, has been used extensively to provide opinions and recommendations regarding controversial scientific issues. The NRC report *Hormonally Active Agents in the Environment* (National Research Council 1999) took more than four years to complete and was not without controversy. (Note: Two of the coauthors of this paper, James Lamb and Stephen Safe, were members of that NRC committee.) The NRC committee's evaluation of the sperm count issue and the role of EDCs on male reproductive capacity concluded as follows:

> When the data from large regions are combined and analyzed, some data sets indicate a statistically significant trend consistent with declining sperm concentrations. However, aggregation of data from larger geographic regions might not be an appropriate spatial scale for this analysis, given the significant geographic heterogeneity.

The current data are inadequate to assess the possibility of trends within more appropriately defined small regions. Acquiring data at smaller regional scales is critical to assessing the significant geographic variation in sperm concentration.

The role of organochlorine compounds in breast cancer was another early concern because two studies reported that levels of PCBs and DDE were higher in breast cancer patients than in controls (Falck et al. 1992; Wolff et al. 1993). Subsequent reports on larger patient groups from Europe, North and South America, and Mexico did not observe differences in DDE/PCB levels in breast cancer patients (Guttes et al. 1998; Hunter et al. 1997; Krieger et al. 1994; López-Carrillo et al. 1997; Moysich et al. 1998; Van't Veer et al. 1997). The NRC report concluded that "an evaluation of the available studies conducted to date does not support an association between adult exposure to DDT, DDE, TCDD, and PCBs and cancer of the breast."

These NRC conclusions suggest that current scientific evidence does not link EDCs with decreased male reproductive capacity or breast cancer in women. It should also be noted, however, that the NRC committee did not dismiss a potential role for EDCs as causative agents for adverse human health effects. Rather, it affirmed their effects on some reproductive and developmental problems in wildlife populations.

An important lesson learned from studying EDCs is that these compounds provide a unique challenge to toxicologists. Like most toxic compounds, EDCs cause dose-dependent toxic effects for most responses. The inverted U-shape curve reported by Nagel and coworkers (1997) for hazard and risk assessment of estrogenic compounds and other EDCs requires further study. The toxicity of EDCs (and some other toxicants) is influenced strongly by the animal's age and by critical windows of exposure. The unique feature of EDCs is that in utero or early postnatal exposure to these compounds can lead to permanent or irreversible effects in adults. The challenges for determining the absence or presence of an EDC-induced response in an adult population are considerable because levels of in utero or early postnatal exposure in the study group, and other dietary (lifestyle) and genetic factors potentially influencing hormonal responses are critical.

Symbols, Acronyms, and Abbreviations

AhR	aryl hydrocarbon receptor
d	day
DDE	1,1-dichloro-2,2-bis (p-chlorophenyl ethylene)
DDT	dichloro-diphenyl-trichloro-ethane (chlorophenothane)
DES	diethylstilbestrol
EDC	endocrine-disrupting chemical
EDSP	Endocrine Disruptors Screening Program
EPA	U.S. Environmental Protection Agency
ER	endocrine receptor
µg	microgram
ml	milliliter
MOE	margin of exposure
NOAEL	no observed adverse effect level
NRC	National Research Council
PCB	polychlorinated biphenyl
TBT	tributyltin
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin

Glossary

Adrenal hyperplasia. A condition of diffuse enlargement of the adrenal glands.

Agonist (see also *Antagonist*). A drug having affinity for and stimulating physiologic activity at cell receptors normally stimulated by naturally occurring substances, thus triggering a biochemical response.

Alkylphenols. A group of endocrine-disrupting chemicals used in industrial detergents, in the form of alkylphenol ethoxylates.

Androgen. General term for any male sex hormone in vertebrates.

Antagonist (see also *Agonist*). A substance tending to nullify the action of another, e.g., a drug binding to a cell receptor without eliciting a biological response.

Aromatase. An enzyme converting androgens to estrogens by desaturating a steroid ring.

Biomarker. A specific biochemical having a specific molecular feature making it useful for measuring the progress of disease or the effects of treatment.

Bisphenol A. An industrial chemical used to make polycarbonate plastic resins, epoxy resins, and other products. A common use is in the manufacture of reusable bottles and food and drink containers.

Carbinol. An alcohol derived from methanol.

c-fos. A protein involved in intracellular signal transduction.

Coumestrol. A constituent of soy reported to have mixed estrogen agonist and antagonist activities in laboratory animal studies.

Cryptorchidism. Failure of one or both testicles to descend into the scrotum.

Dose-response relationship (drug). The relationship between the dose of an administered drug and the response of the organism to the drug.

Endogenous (see also *Exogenous*). Developed or originating inside the organism. Natural endogenous compounds include hormones such as estradiol or estrone that serve as the body's natural chemical messengers.

Endocrine-disrupting chemicals. A group of chemicals able to imitate or modify the action of natural hormones. Includes industrial chemicals such as alkylphenols, bisphenol A, polychlorinated biphenyls (PCBs), and phthalates and pesticides such as DDT and dioxin. Also known as *hormone disruptors* or *xenoestrogens*.

Endocrine system. The system of glands releasing their secretions (hormones) directly into the circulatory system.

Endometriosis. A condition in which tissue more or less resembling the uterine mucous membrane (i.e., the *endometrium*) and containing typical endometrial granular and stromal elements occurs aberrantly in various locations in the pelvic cavity.

Estradiol. A hormone synthesized mainly in the ovary but also in the placenta, testis, and possibly adrenal cortex. A potent estrogen.

Estrogen. A generic term for estrous-producing steroid compounds, the female sex hormones. In humans, it has various functions in both sexes. It is responsible for the development of female secondary sex characteristics and, during the menstrual cycle, acts on the female genitalia to produce an environment suitable for fertilization, implantation, and nutrition of the early embryo.

Estrone. An estradiol metabolite possessing less biological activity.

Exogenous (see also *Endogenous*). Developed or originating outside the organism. Natural exogenous estrogenic substances can be found in products such as fruits and vegetables. These phytoestrogens or fungal estrogens have significant hormonal activity.

Gastropod. A member of the class *Gastropoda* in the phylum *Mollusca*, with a distinct head with tentacles and a long "foot" used for locomotion. Eighty percent of the 40,000 mollusc species are gastropods. Slugs and snails are common gastropods.

Genistein. A phytoestrogen found in soy products.

Hermaphroditism (see also *Pseudohermaphroditism*). The union of the two sexes in the same individual or the combination of some of their characteristics or organs in one individual; implies a discrepancy between genotype and external genitalia. Both ovarian and testicular tissues are present, either in the same or in opposite gonads.

Hutterites. A religious group originating in Switzerland during the Reformation (ca. A.D. 1530) who lives communally in rural North America. Followers of this movement are known as the *Anabaptists*, or *rebaptisers*.

Hypospadias. A congenital defect in which there is an abnormal urethral opening.

Immunotoxicity. The effect of chemicals on general body defense mechanisms.

Imposex (see also *Intersex*). A form of pseudohermaphroditism in female neogastropod molluscs. This imposition of male sex organs (i.e., penis, vas deferens) can lead to sterility.

Intersex (see also *Imposex*). A form of pseudohermaphroditism in female neogastropod mulluscs that involves tributyltin (TBT)-induced alterations of the oviduct.

"**Inverted U-shaped**" hypothesis. A dose-response curve showing adverse effects at low doses but a different response at higher doses.

Isoflavone. A class of phytochemical bioflavinoids.

Lipophilic. Having an affinity for lipids (as fats). For example, PCBs and DDE are lipophilic compounds bioaccumulating in the body's fat stores.

Littorinid. A marine snail of the family *Littorinidae*, which includes the periwinkles.

Microphallus. A congenital underdevelopment of the penis.

Mutagenicity test. Range of tests using biological systems to see whether compounds can cause genetic mutations.

Neurotoxic. Poisonous or destructive to nerve tissue (both brain and peripheral nerves).

Organochlorine. Of, relating to, or belonging to the chlorinated hydrocarbon pesticides (e.g., aldrin, DDT, dieldrin)

Organogenesis. The process of formation of specific organs in a plant or animal involving morphogenesis and differentiation.

Phytochemicals. A broad term for hundreds of differ-

ent compounds found only in plants.

Prepuce. A covering fold of skin, often used alone to designate the preputium penis.

Pseudohermaphroditism (see also *Hermaphroditism*). In females, the genotype is XX, the gonads are ovaries, but the external genitalia are virilized. In males, the genotype is XY but the external genitalia are incompletely virilized, ambiguous, or completely female.

Salmonid species. Fish commonly known as salmon and trout (members of the genera *Oncorhynchus, Salmo, Salvelinus,* and *Hucho*).

Sentinel species. Species used in assays to evaluate and to monitor ecological and human health hazards from chemical contaminants.

Teratogenesis. Production of developmental malformations.

Toxicity studies. *Acute* toxicity studies involve a single-day exposure and the assessment of fairly general signs of a toxic response. *Subchronic* studies are conducted with a 13-week exposure and measure additional toxic endpoints. *Chronic* studies detect effects after one to two years of exposure and are used to predict the substance's carcinogenic potential.

Vitellogenin. A protein, precursor of several yolk proteins, especially phosvitin and lipovitellin in the eggs of various vertebrates, synthesized after estrogen stimulation.

Xenoestrogen. Alternative term for *endocrine-disrupting chemicals*.

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