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Environmental Signaling: What Embryos and Evolution Teach Us About Endocrine Disrupting Chemicals

JOHN A. McLACHLAN

Department of Pharmacology, Tulane University Health Sciences Center, Environmental Endocrinology Laboratory, Center for Bioenvironmental Research, Tulane and Xavier Universities, New Orleans, Louisiana 70112–2699

ABSTRACT

The term "endocrine disrupting chemicals" is commonly used to describe environmental agents that alter the endocrine system. Laboratories working in this emerging field—environmental endocrine research—have looked at chemicals that mimic or block endogenous vertebrate steroid hormones by interacting with the hormone's receptor.

Environmental chemicals known to do this do so most often with receptors derived from the steroid/thyroid/retinoid gene family. They include ubiquitous and persistent organochlorines, as well as plasticizers, pharmaceuticals, and natural hormones. These chemicals function as estrogens, antiestrogens, and antiandrogens but have few, if any, structural similarities. Therefore, receptor-based or functional assays have the best chance of detecting putative biological activity of environmental chemicals. Three nuclear estrogen receptor forms—α, β, and γ—as well as multiple membrane forms and a possible mitochondrial form have been reported, suggesting a previously unknown diversity of signaling pathways available to estrogenic chemicals.

Examples of environmental or ambient estrogenization occur in laboratory experiments, zoo animals, domestic animals, wildlife, and humans. Environmentally estrogenized phenotypes may differ depending upon the time of exposure—*i.e.*, whether the exposure occurred at a developmental (organizational and irreversible) or postdevelopmental (activational and reversible) stage. The term "estrogen" must be defined in each case, since steroidal estrogens differ among themselves and from synthetic or plant-derived chemicals.

An "estrogen-like function" seems to be an evolutionarily ancient signal that has been retained in a number of chemicals, some of which are vertebrate hormones. Signaling, required for symbiosis between plants and bacteria, may be viewed, therefore, as an early example of hormone cross-talk.

Developmental feminization at the structural or functional level is an emerging theme in species exposed, during embryonic or fetal life, to estrogenic compounds. Human experience as well as studies in experimental animals with the potent estrogen diethylstilbestrol provide informative models. Advances in the molecular genetics of sex differentiation in vertebrates facilitate mechanistic understanding. Experiments addressing the concept of gene imprinting or induction of epigenetic memory by estrogen or other hormones suggest a link to persistent, heritable phenotypic changes seen after developmental estrogenization, independent of mutagenesis.

Environmental endocrine science provides a new context in which to examine the informational content of ecosystem-wide communication networks. As common features come to light, this research may allow us to predict environmentally induced alterations in internal signaling systems of vertebrates and some invertebrates and eventually to explicate environmental contributions to human reproductive and developmental health. (*Endocrine Reviews* **22:** 319–341, 2001)

Address reprint requests to: John A. McLachlan, Ph.D., Weatherhead Distinguished Professor and Director, Center for Bioenvironmental Research, Tulane University, 1430 Tulane Avenue, New Orleans, Louisiana 70112. E-mail: john.mclachlan@tulane.edu

I. Introduction

IN 1958, Dr. Roy Hertz described the "steroid cycle," anticipating what we now call endocrine disrupter research, as follows: ". . . we have to consider that the introduction of . . . [hormones into cattle feed lots] leads to the exposure . . . of individuals who might otherwise not ever in their lives come in contact with such materials . . . . This is not a theoretical consideration because we . . . now have encountered two families, each with two children, who presented with simultaneously developing gynecomastia attributable to the accidental contamination of vitamin capsules by estrogens during manufacture. If such estrogens can, by stray handling, get into such pharmaceutical preparations, can they not very readily get where they are not wanted on the farm?

There is one additional consideration in this regard . . . . The fecal excretion of these materials . . . will be dropped on the soil and . . . over generations there will be constant replenishment of the soil surface with steroidal substances of this kind. This in turn has its effect potentially on surface water-supply contamination and also potentially on the veg-

etable content of steroids in crops raised on such soil . . . . I think that we are now actually setting up a steroid cycle in our environment, and we have to give very serious consideration to its implications for our subsequent development and growth and possibly reproductive functions" (taken from the discussion following Ref. 1).

The "reproductive functions" mentioned by Hertz are, in most invertebrates and all vertebrates, under the control of an integrated network of chemical signals—the endocrine system. This finely tuned communication system relies on messenger molecules, hormones of great sensitivity and specificity, to maintain the complex information flow required for normal health. Chemicals in the environment that mimic or block endogenous hormones might upset this fine balance in ways that, while unexpected, are at least predictable based on the known biology of the endocrine system. The potential implications for human health as well as the health of numerous wildlife species are self-evident.

The emerging field of scientific inquiry commonly referred to as "endocrine disruption" is thus of growing public health and environmental concern. The concerns arise from the real and perceived deleterious effects of environmental chemicals on the development or function of the reproductive system in species as diverse as snails, alligators, and humans.

Hormonal Chaos, a recent book by Sheldon Krimsky (2), is an excellent introduction to the politics, sociology, science, history, and philosophy that pertain to endocrine disruption. Krimsky, a Professor in Urban and Environmental Policy at Tufts University, states what he terms ". . . the environmental endocrine hypothesis, [which] asserts that a diverse group of industrial and agricultural chemicals in contact with humans and wildlife have the capacity to mimic or obstruct hormone function—not simply disrupting the endocrine system like foreign matter in a watchworks, but fooling it into accepting new instructions that distort the normal development of the organism. . . . From the standpoint of human pathology, the environmental endocrine hypothesis could turn out to be the most significant environmental health hypothesis since the discovery of chemical mutagenesis." Krimsky examines the scientific roots, public response, and implications in the context of the development of ideas in science. It is all the more interesting that the book appears so early in the scientific history of the field: it was not until 1980 that the proceedings of the first meeting held on this topic were published and the compounds associated with ambient hormonal activity were termed environmental estrogens (3). Since then, as attention to this area of investigation has grown, these compounds have been variously called endocrine disrupting chemicals (4), xenoestrogens (5), environmental hormones (6, 7), hormonally active agents (8), and environmental signals (9).

The field of research dealing with the environmental endocrine hypothesis is, as with most new fields, rife with debate, inconsistencies, and controversy. This may, to some extent, be a result of the multidisciplinary nature of the topic. Meetings on endocrine disrupting chemicals often include ecologists (theoretical, field, economic), chemists (synthetic, combinatorial, analytic, modeling), endocrinologists (molecular, steroid biochemistry, clinical), toxicologists (global and organismic, mechanistic, regulatory and industrial), zoologists (representing phyla from worms to whales), policy wonks and mavens, and often, but not always, the media. The regulatory and media interest in the topic often move at a faster pace than the science. On the other hand, the challenges posed by this important area of investigation have led to novel approaches and findings driven, or at least influenced, by the multidisciplinary nature of the work, the intensity of the debates, and the interest of the public. These concerns were given public voice with the release of the Emmy-award winning documentary, *Assault on the Male* by Deborah Cadbury (1993), and the publication of the influential book, *Our Stolen Future* in 1997 (10).

It is also the case that the environmental endocrine hypothesis resides at the boundary of endocrinology and toxicology, challenging the common wisdom of both fields. For example, Crews *et al.* (11) outlined some of the salient points that distinguish environmental endocrine disruption from other toxicological approaches. They contrast the "traditional toxicological approach," which utilizes a carcinogenic model and mortality or acute toxicity, with the "endocrine disrupter approach," which relies on a developmental model and delayed dysfunction. They also look for a common element between the effective concentration of endogenous hormones compared with exogenous xenoestrogens found in the environment singly or in mixtures.

The multidisciplinary nature of environmental endocrine research is difficult to comprehend with a single review article. Several recent reviews have looked at different aspects of this area of research. For example, reviews on the environment and male reproductive health (12), screening methods for endocrine disrupting chemicals (13), and endocrine disruption in wildlife (14, 15) summarize much of what is known in the field. LeBlanc (16) took an ecological approach based on work with invertebrates and raised the possibility that lower organisms may serve as sentinel species for human health effects if we can interpret their signals. He described the importance of ecological networks that may provide early response signs to biologically active environmental contaminants.

Also, published proceedings from some of the seminal meetings on the subject provide important sources of information as well as historical perspectives. These include the three meetings on Estrogens in the Environment, the first in 1979 (3); the second, Estrogens in the Environment II: Influences on Development in 1985 (17); and the third, Estrogens in the Environment III: Global Health Implications in 1994 (18). The Wingspread Meeting on the Human-Wildlife Connection (4), in 1992, highlighted the important associations between human and wildlife health.

This present article is a brief review of selected literature concerning primarily estrogens and estrogenic chemicals, synthesizing what is known to date and offering a central thesis for this emerging area of research. It will attempt to illuminate patterns in our environment that are relevant to the endocrine system and its function. A key pattern resides in signaling systems in developmental and evolutionary biology as well as endocrinology. Thus, this review will both return to, and refine, the concept of environmental signaling that our laboratory introduced 2 yr ago (9). This term describes what is now known about environmental endocrine science, acknowledges an informative evolutionary link, and

anticipates additional signaling pathways that may include other hormonal activities as well as activities related to the nervous and immune systems.

Science and medicine have benefited from discovering patterns in observed phenomena and refining the recognized patterns into theories or syndromes (*e.g.*, the androgen insensitivity syndrome). If chemicals from many sources are indeed adding to the hormonal burden of humankind, one may use a mechanism-based pattern-recognition approach to gain understanding. This review attempts to provide a context in which to reconcile how apparently unrelated environmental chemicals might alter reproductive function. Again, as Hertz said in 1958, in discussing the addition of hormones to our environment, "... we have to give very serious consideration to its implications for our subsequent development and growth and possibly reproductive functions."

II. Environmental Hormones

As seen in Fig. 1, the steroid/thyroid/retinoid or nuclear receptor gene family encodes for a large number of receptors. It is thought that while thyroid hormone and retinoid receptors evolved coordinately, they diverged from the steroid hormones early in evolution. Moreover, the hypothesis has been advanced that ligand binding is an evolutionarily late event in the development of the receptors (19). Both functionally and phylogenetically, this is an important concept. The information content of chemicals destined to become ligands could be refined and diversified in concert with the gain of function in the receptor proteins that would recognize them.

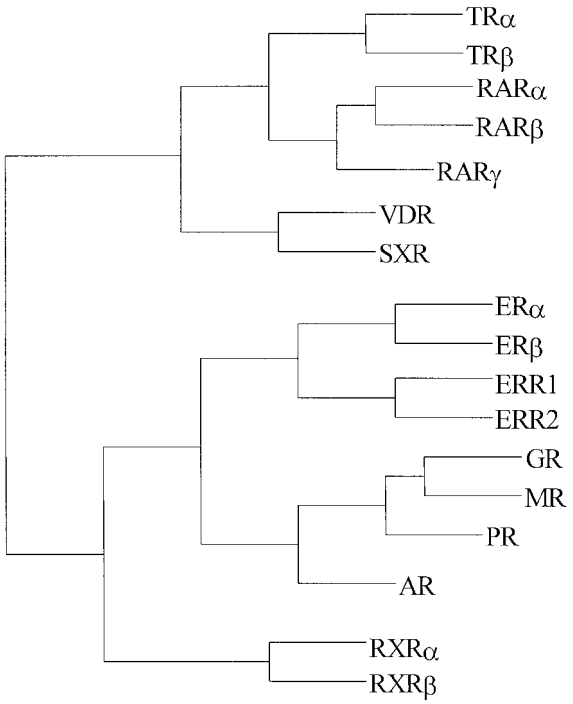

FIG. 1. Evolutionary relationships between selected members of the steroid/thyroid/retinoid gene family of nuclear receptors. Most of the known environmental chemicals with hormonal activity derive that activity through interaction with one or more of these receptors. Abbreviations used are: TR, thyroid hormone receptor; RAR, retinoid receptor; VDR, vitamin D receptor; SXR, steroid xenobiotic receptor; ER, estrogen receptor; ERR, estrogen-related receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; AR, androgen receptor; RXR, retinoid orphan receptor. Another ER form, ERγ has recently been reported (20). [Derived from (19).]

The family of receptors is defined by both structural and functional homologies. While the functions and ligands of many receptors in the group are known, there are a large and growing number of receptors with no known function or ligand—the so-called orphan receptors. The orphan receptor, SXR (steroid/xenobiotic receptor), recognizes many classes of xenobiotic chemicals and activates a response that results in the expression of xenobiotic metabolizing enzymes, providing a link between the internal and external environment (17). If SXR functions as a "xenobiotic sensor" as proposed, the gradient between endogenous hormones and exogenous environmental signaling chemicals may not be as great as we thought.

In addition to SXR, other nuclear receptors have been shown to bind environmental chemicals (Table 1 and Refs. 21–23). Recent reports of synthetic environmental chemicals that activate the retinoid receptor system raise the possibility of environmental retinoids (24). Chlorinated hydrocarbons, such as some polychlorinated biphenyls (PCBs), have long been conjectured to bind the thyroid hormone receptor on theoretical grounds (25), but this has not, as yet, been demonstrated. Zoeller *et al.* (21) recently reported that some PCBs clearly activate the thyroid hormone system without a direct demonstration of thyroid hormone receptor binding. Studies also demonstrate binding activity of environmental agents to thyroid hormone binding protein similar to T_4, but not to the thyroid hormone receptor (22, 25).

As yet, no synthetic environmental chemicals have been reported that function as androgens. However, a growing number of pesticides have been recognized recently as androgen antagonists. The antiandrogenicity of dichlorodiphenyltrichloroethane (DDT) metabolites and some insecticides

TABLE 1. Environmental hormonal activities

Hormonal activity	Environmental	
	Hormone	Antihormone
Estrogen	Yes, many[a]	Yes, few[a]
Progestin	?	?
Androgen	Yes, few[b]	Yes, many[c]
Gluccocorticoid	?[d]	?
Mineralocorticoid	?	?
Retinoid	Yes, one	?
Thyroid	?[e]	?

[a] See representative structures in Fig. 5.

[b] Androstenedione, the product of bacterial metabolism of stigmasterol; see Fig. 3.

[c] See representative structures in Fig. 2.

[d] Arsenic is reported to block the GR activation at the receptor binding level (23).

[e] PCB congeners elicit a thyroid hormone-like response, but no binding data for the thyroid hormone receptor is available (21). One study that evaluated binding of chlorinated hydrocarbons to the thyroid hormone receptor and thyroid binding proteins did not demonstrate specific receptor binding, while binding to transthyretin was of the same affinity as T_4 (22).

(26–29) also points out the structural diversity underlying the antihormonal activities of environmental compounds. Figure 2 shows the structures of those environmental antiandrogens compared with the pharmaceutical antiandrogen, hydroxyflutamide. Very recently, the herbicide linuron was shown to be a competitive binding ligand for the androgen receptor in rats or humans, and it altered androgen-dependent gene expression in castrate rats (30). The organophosphate insecticide, fenitrothion, was also shown to be a competitive reversible inhibitor of the androgen receptor (31). The *in vitro* potency of fenitrothion as an antagonist (K_B, 2.18×10^{-8} M) was comparable to that seen for the pharmaceutical antiandrogen flutamide. This value was approximately 8- to 35-fold greater than that determined for *p,p'*-dichlorodiphenyl ethylene (DDE) (28, 32) and linuron (33).

While no synthetic environmental compounds have yet been reported to have androgenic activity, the work of Howell and colleagues has elucidated a route to significant androgenic contamination of the environment. Twenty years ago, Howell and colleagues (34) described masculinization of female mosquito fish, *Gambusia affinis holbrooki,* which were caught downstream from the effluent discharged from a paper mill in Cantonment, Escambia County, Florida. The females exhibited male secondary sex characteristics such as a male sex organ or gonapodium as well as male sexual behavior. The authors remarked that this fish species was known to be sensitive to the masculinizing effects of androgens and proposed, as one of three hypotheses, that an androgenic material was produced in the effluent. In subsequent studies (35), Howell's group determined that the masculinizing agent was indeed androgen, but androgen from a very unusual source. While steroidal androgens were not actually found in the paper waste, the plant sterol, stigmasterol, was. It was further determined that *Mycobacterium smegmatis*, which produces androgens as metabolic products, had formed extensive colonies in the effluent path. These bacterial "mats" were utilizing the plant cholesterol (stigmasterol) as a carbon source and metabolizing the plant sterol to a potent androgen, androstenedione (Fig. 3). This is a well established pathway in microbial metabolism and is known in the laboratory. The environmental implications for the reproductive system of vertebrates of this bacterial biochemistry were not established before the work with mosquito fish. Since these initial findings, masculinization of mosquito fish downstream from pulp and paper mills has been described in many other areas of the United States (36).

The production of biologically active androgens by bacterial mats has added a new dimension to the field of environmental endocrine science. So has a recent report that nontoxic concentrations of arsenic can function as a glucocorticoid receptor (GR) modulation factor by selectively inhibiting GR-mediated transcription through altered nuclear function rather than a decrease in hormone-induced GR activation or nuclear translocation (23). This raises the possibility that metals may indeed interact at fundamental levels with the cell processes associated with hormones and their receptors. These findings suggest that the demonstration of the effect of environmental chemicals on the GR may now require consideration of other heavy metals. Previous studies have shown that arsenite, cadmium, and selenite interact with the cysteines of the ligand binding domain of the GR and inhibit binding of dexamethasone to the receptor (37).

Finally, a wholly new kind of ligand-ER complex has been reported that may change the ways we think that environmental factors mimic hormones as well as shed light on the interaction between arsenite and GR mentioned previously. The heavy metal, cadmium (Cd), has been shown to mimic the effects of estradiol in estrogen-responsive breast cancer cell lines, both in cell proliferation and regulation of gene expression (38). Cadmium has recently been demonstrated to activate ERα by interacting with the ligand-binding domain of the receptor (39). These results led the authors to assert that "the heavy metal, cadmium, is a new environmental estrogen."

Indeed, much of the focus in environmental endocrine science has been on those chemicals that mimic the female sex hormone, estradiol-17β. The first synthetic chemical found to

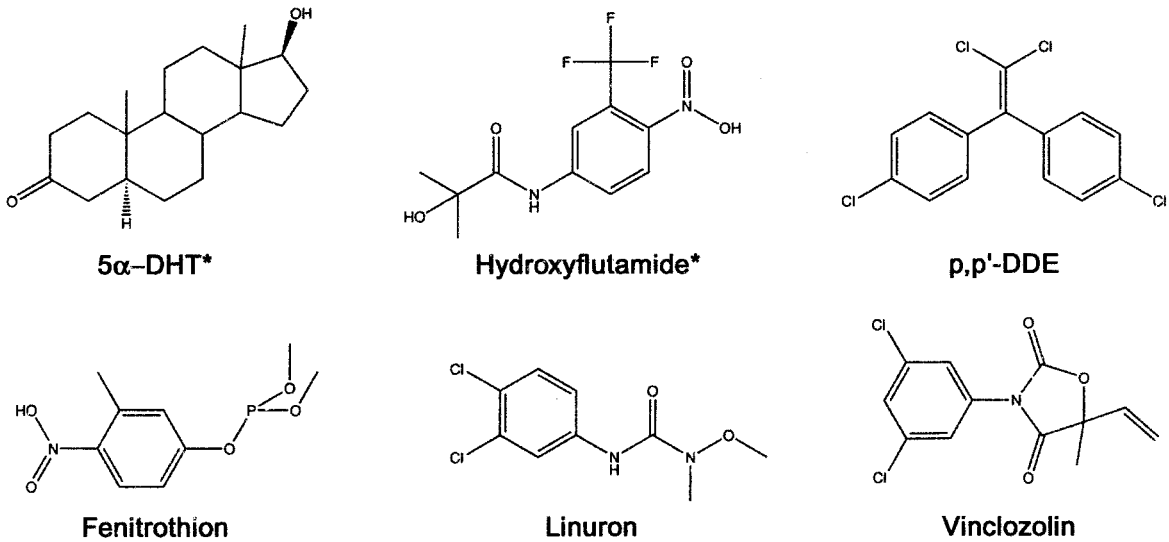

FIG. 2. Structural diversity among environmental chemicals reported to be antiandrogenic. The steroidal androgen, 5α-dihydroxytestosterone (5α-DHT) and its pharmaceutical antagonist, hydroxyflutamide, are shown for comparison. *p,p'*-DDE is a persistent contaminant, while the remaining are currently used pesticides: fenitrothion, an insecticide; linuron, an herbicide; and vinclozolin, a fungicide.

mimic the activity of an endogenous steroidal hormone was an estrogen. In 1933, Dodds and colleagues (40) described 1-keto-1:2:3:4 tetrahydrophenanthrene (Fig. 4) as "the first compound of known chemical constitution found to have definite oestrus-exciting activity" (40). Dodds continued to examine the structural basis for estrogenicity and, in a short landmark paper (41), described the first synthetic estrogen without the phenanthrene nucleus, the ring structure common to steroids (Fig. 4). In this paper, he evaluated a series of diphenyl compounds and concluded that only those with two hydroxyl groups in the para positions would be active as estrogens. The compound, di-(p-hydroxyphenyl) dimethylmethane, called bisphenol A, or BPA, may be the first synthetic selective estrogen receptor modulating (SERM) chemical reported. Dodds' experiments represent a pharmacological breakthrough in rational chemical synthesis, opening many routes to the same biological function exhibited by a variety of chemical structures, *e.g.*, estrogenicity, as determined using the ovariectomized rat vaginal cornification assay introduced just 8 yr earlier.

These early structural studies culminated in 1938 with the discovery of diethylstilbestrol or DES (42). DES, a derivative of the stilbene nucleus (Fig. 4), was shown to be significantly more efficacious than all the previous compounds tested by Dodds. As it was also orally active, relatively stable, and less expensive to produce than isolated or synthesized steroidal estrogens, DES quickly became the synthetic estrogen of choice for medicine and, later, agriculture. Its medical uses included estrogen replacement therapies, lactation suppression, postcoital contraception, pregnancy maintenance, and prostate cancer therapy. In agriculture, DES was used to chemically caponize chickens and stimulate growth in cattle.

While the widespread use of DES in cattle feed lots led to the introduction of tons of potent estrogens into the ecosystem, BPA was destined for far greater use. BPA was found to be an efficient cross-linking chemical and came to be used widely in the production of plastic polymers, primarily polycarbonates. It is somewhat ironic that two synthetic chemicals, the potent estrogen, DES, and the weak-acting estrogen, BPA, which have been so important to our understanding of environmental estrogens can be traced to one laboratory, that of Sir Charles Dodds.

Many structurally diverse chemicals have been reported to function as estrogens (Fig. 5). As with all steroid hormones, 17β-estradiol contains the three-ring phenanthrene; for estrogenicity, the first or A ring must contain a phenolic hydroxyl group. The pharmaceutical estrogens DES and ethinyl estradiol are as potent as the parent compound, estradiol. In

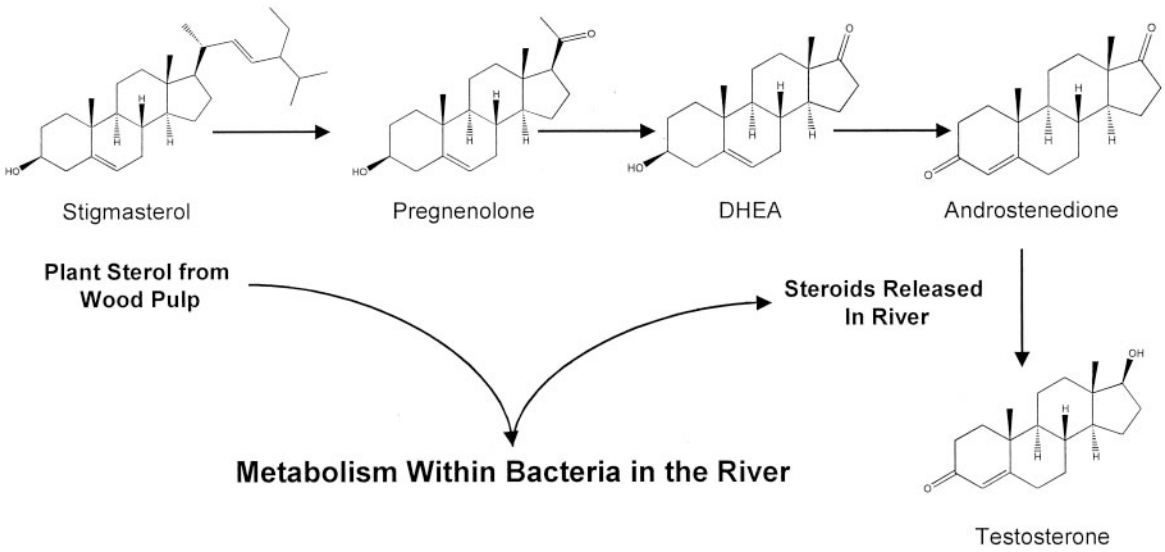

FIG. 3. The production of androgenic compounds by bacteria. Stigmasterol, a major plant sterol found in wood pulp, is efficiently metabolized to androgenic steroids such as androstenedione by the bacteria, *Mycobacterium smegmatis*. *M. smegmatis* form extensive colonies, or "bacterial mats," at the effluent site of pulp and paper mills. The natural plant sterol, stigmasterol, contained in the pulp effluent is converted by *M. smegmatis* into androstenedione, which is released into the river or stream. Female mosquito fish exposed to these androgens develop male structures. (See Refs. 34, 35, and 36 for details.)

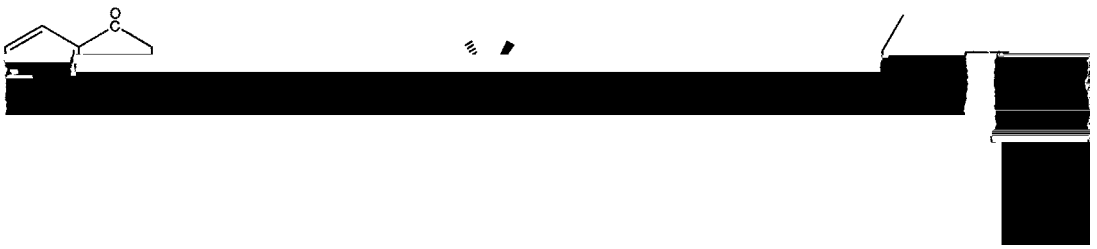

FIG. 4. First synthetic estrogenic chemicals or selective estrogen receptor modulators (SERMS). The laboratory of Dodds *et al.* reported the synthesis and bioassay of 1-keto-1:2:3:4 tetrahydrophenanthrene in 1933 (40), bisphenol A in 1936 (41), and DES in 1938 (42). Each successive compound was more estrogenic than the preceding one.

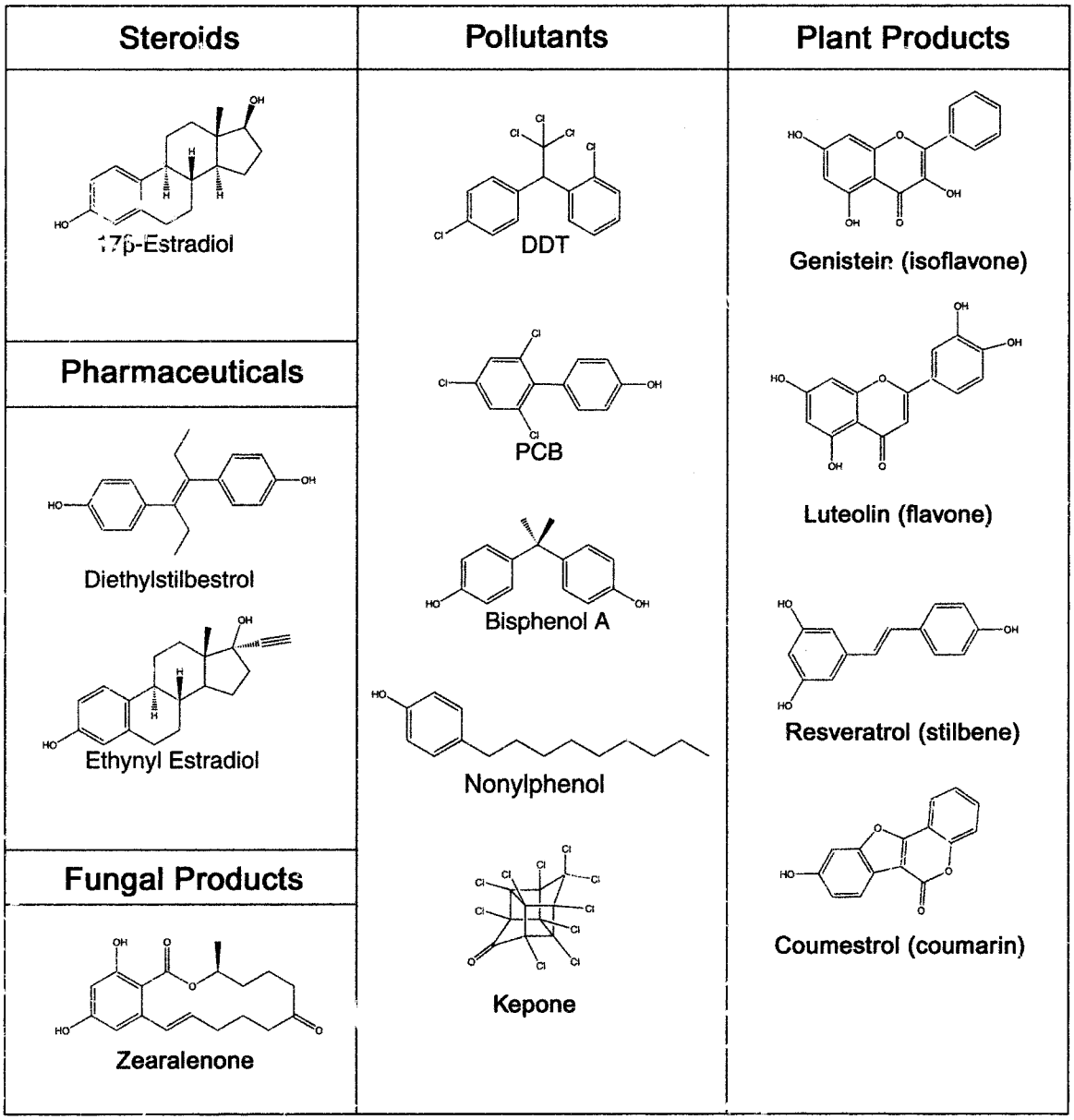

FIG. 5. Chemicals found in the environment reported to be estrogenic. This list is not comprehensive, but illustrates representative structures of estrogenic compounds from various sources. Information on these compounds is contained in the text.

viewing the structures of synthetic chemical contaminants, the most striking feature is, perhaps, the absence of a consistent structural motif. There is often, but not always, the presence of an aromatic ring or two. Several representative chemicals contain chlorine atoms. The role that chlorine plays in hormonal activity is still not clear. For instance Kepone, a structurally restricted, cubic molecule containing chlorine on every carbon but one, is known to be estrogenic (43).

In most cases these environmental chemicals were synthesized with no apparent hormonal intent. We can consider these chemical contaminants to be "inadvertent" estrogens or chemicals whose synthetic rational was unrelated to their ultimately determined hormonal activity. One of the best known examples is the pesticide, DDT. While the intended function of DDT was far removed from that of a vertebrate sex hormone, it was shown in 1950 by Burlington and Lindman (44) to estrogenize cockerels exposed to it. The estrogenic activity of DDT was rediscovered by Conney and colleagues in 1969 (45), while testing various pesticides as inducers of P_{450} enzyme activity. One of several comprehensive reviews on the estrogenicity of DDT and its congeners, written by David Kupfer (46), appeared 20 yr ago.

In addition to DDT, other chlorinated hydrocarbons, PCBs, have been shown to function as estrogens in both *in vivo* and *in vitro* assays (47). The degree of hydroxylation and the location of the chlorine and hydroxyl groups are important determinants in biological activity. In the case of substituted alkyl phenols, such as para-nonyl phenol, the potency of the compound as an estrogen has been shown to be related to side chain length and branching (48). However, all

known "inadvertent" estrogens are much less potent than the steroidal estrogen, 17β-estradiol.

The hormonal activities of environmental chemicals apparently reside in a functional attribute rather than a structural one. To discover the intrinsic function in chemicals, one could use an approach of functional toxicology or receptor-based toxicology (49). Chemicals could be screened through various hormone receptor activation assays, and the biological activity or function would be determined along with the potency of the compound relative to the parent hormone (Fig. 6; Refs. 49–51). As seen in Fig. 6, the receptor may behave as a signal integration unit and collect information from growth factors, other nuclear receptors, and a series of chaperone proteins and coregulator proteins. All of these signal inputs are routes for environmental chemicals to mimic or block hormones.

The total amount of environmental contamination with synthetic chemicals that give an estrogen signal is not known; however, 45,000 metric tons of the weak estrogen, p-nonylphenol, were produced in 1976, and by 1982 the total annual production of all alkyl phenol polyethoxylates was estimated at 140,000 metric tons. In 1993 BPA production in the United States was 640,000,000 kg; of that, 44,000 kg (0.10%) were reported recycled, land filled, incinerated, or released in the environment.

In addition to the many synthetic chemicals resulting from industrial practices, human activities have added in other ways to the hormonal burden on the environment. Many pharmaceutical chemicals were synthesized to function as estrogens, but their environmental impact was not considered. For example, DES, the potent synthetic estrogen, was used as a growth-promoting substance in cattle for more than 40 yr. In 1971 alone, Knight (52) estimated that 27,600 kg of DES were used for this purpose (52). While concern was expressed for the levels of synthetic hormones present in edible portions of beef, DES and its metabolites were also excreted into the ecosystem with unknown consequences. Metcalf (53) explored the fate of radiolabeled DES in a model ecosystem and reported that it was persistent and bioaccumulated. While the health outcomes are not known, studies in humans have demonstrated that after oral administration, the conjugated form of DES, DES glucuronide, is readily metabolized by intestinal bacteria and absorbed into the blood stream as the biologically active parent compound (54). These results suggest that conjugation of excreted environmental estrogens may not limit the efficacy of the hormone.

Another category of environmental contamination with synthetic estrogens is the excretion of components of pharmaceuticals used for contraception or hormone replacement in humans. There have been few studies on the levels of drugs in waste water and fewer on levels in drinking water. An early report in 1977 that 8.5 kg/day of salicylic acid, a metabolite of aspirin, was found in the waste water effluent

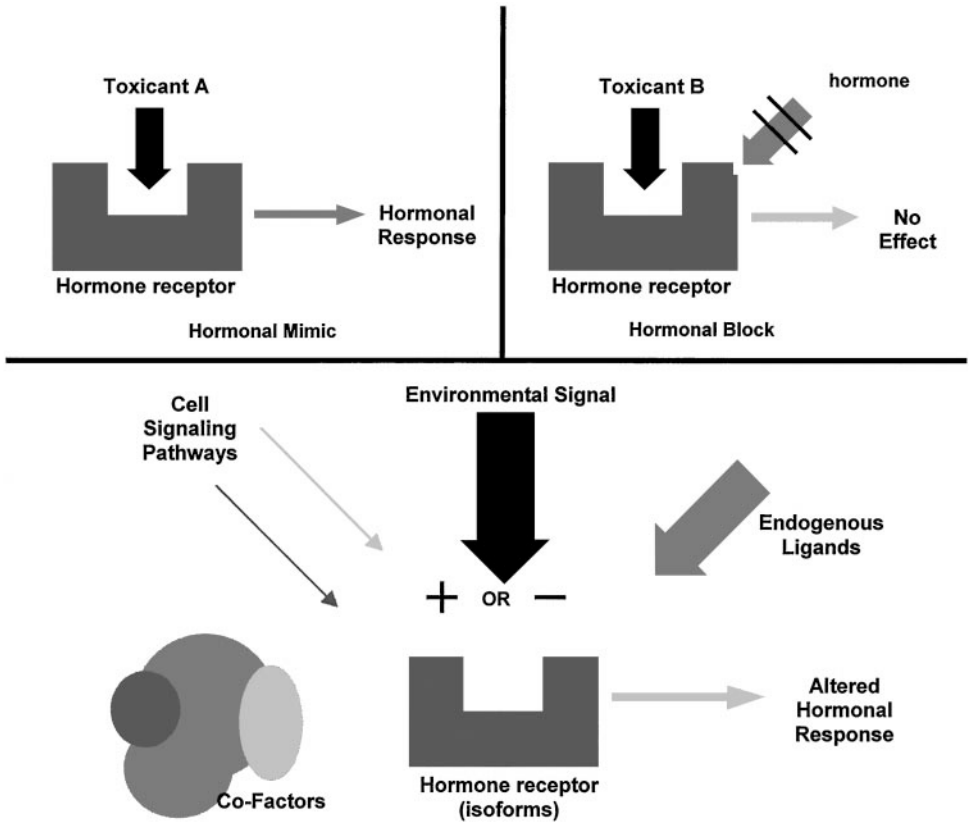

FIG. 6. Functional or receptor-based toxicology. The practice of using steroid hormone receptors to determine the hormonal or antihormonal activity of environmental chemicals is more than a decade old (49). The relatively simple concept presented then (*upper panels*), must now accommodate the advances in knowledge over the last 10 yr. These include the convergence of activating ligands and cellular signals on the ER (50), the multiple isoforms of the ER (51), contributions of coactivators and corepressors, and the gradation in response (50).

in Kansas City (55) prompted speculation that, "With millions of women taking oral contraceptives, some environmental contamination with estrogenic materials is a distinct possibility" (56). Very recently, an estrogenic component of commonly used oral contraceptives, 17α-ethinyl estradiol, was found in trace amounts in waste water effluent (57, 58). Estrogens and their glucuronides have been found in municipal sewage in Germany, Canada, and Brazil (59) and in surface and waste waters in The Netherlands (60).

Studies on the effects on wildlife that live in ponds containing wastes from livestock are underway only now, but the total impact of natural and synthetic hormones discharged by cattle, hog, and chicken farming may be greater than previously believed. In a pilot study, Irwin and Oberdoerster (61) demonstrated feminization of turtles living in ponds that received waste runoff from cattle farms.

III. Environmental Estrogens

A. At the lab bench

While hormones in the environment seem to be a relatively new phenomenon and their biological effects uncertain, there are several well characterized examples of unexpected estrogenization of biological systems in cell culture, or, if you will, "inadvertent [estrogen] contamination in a controlled experimental micro-environment." In a laboratory setting, it was known for decades that cells derived from estrogen-responsive tissues were only weakly responsive to the mitogenic signal of estrogen in tissue culture (62, 63). In 1986, Berthois and colleaques (64) demonstrated that the commonly used pH indicator in tissue culture media, phenol red, was a weak estrogen. It was later shown that a contaminant of the phenol red preparation, bis-(4-hydroxyphenol)-[2-(phenoxysulfonyl)phenol] methane, was the actual estrogenic chemical (65). The unexpected estrogen in tissue culture media was sufficiently biologically active to stimulate cells in culture to proliferate maximally, often negating a detectable effect of the addition of estrogen to the media. In fact, most publications after 1986 that discuss estrogen action in cell or organ culture explicitly describe using phenol red-free media.

Five years later, Soto and colleagues (66) solved another problem encountered in the culture of estrogen-responsive cells. Many laboratories conducting *in vitro* experiments noted a marked difference in response to estrogens when they switched plasticware vendors. Soto *et al.* showed that some plastic petri dishes and tubes contained a residue of p-nonyl-phenol. This alkyl phenol contaminant was shown to be estrogenic, and the finding presaged by a few years the association shown by Sumpter's group (67) between p-nonyl-phenol contamination and feminization of fish in UK streams.

Another example of an unexpected estrogen at the lab bench is that of bisphenol A, a monomeric constituent of polycarbonate plastic, which, as previously described in this review, was originally synthesized as an estrogen. Feldman *et al.* (68) described an estrogen binding protein and an endogenous ligand in the yeast *Saccharomyces cerevisiae*. After an exhaustive set of studies, Feldman and colleagues (69) discovered that the estrogenic substance thought to be of yeast origin was actually bisphenol A, which is released from the polycarbonate flasks when they are autoclaved (69).

B. In animals other than humans

There are numerous reports of reproductive and developmental abnormalities in species ranging from snails to humans that have been associated with exposure to environmental hormones (primarily estrogens) (Table 2 and Refs. 15, 34, 70–84). With careful evaluation of the findings, it may be possible, over time, to discern an "environmental estrogen phenotype" that has two components—one developmental or "organizational" and the other, adult or "activational." This analysis will be the subject of a later review by our laboratory.

As an example of direct activational effects of environmental estrogens in wildlife, studies with cheetahs are informative. Cheetahs face extinction in the wild and exhibit reproductive failure and liver disease in captivity. Clinical symptoms such as hepatic venoocclusive disease found in 100 cheetahs in the Cincinnati Zoo suggested hyperestrogenization (85). HPLC and gas chromatography coupled with mass spectrometry revealed large amounts of the phytoestrogens, daidzein and genistein, in their diet. The authors estimated that the cheetahs consumed approximately 50 mg of the compounds per day. When four animals were given meat instead of soy-based diets, their liver function improved; thus, dietary estrogens were thought to be detrimental to this carnivorous species.

Thigpen and colleagues (86) showed that standard formula rodent diets can vary greatly in content of the phytoestrogens daidzein and genistein as well as in uterotrophic activity (86). In an earlier report (87) they show that 15-day-old weanling CD-1 mice fed an American Institute of Nutrition diet (AIN-76A) for 7 days had uterine weight gains close to that seen in mice fed a certified rodent chow containing 6 ppb of DES. The influence of differing dietary estrogen content on experimental results in rodents is an increasingly important variability factor in design of toxicology assays for environmental hormones as well as in more fundamental studies of hormone response in genetically manipulated mice. The experimental background levels of unintended estrogens are reminiscent of studies described earlier concerning estrogen-containing tissue culture media and plasticware.

Zearalenone, a fungal mycotoxin produced by *Fusarium*, binds the estrogen receptor (ER) (88) and is uterotropic in the newborn rat (89). Consumption of corn contaminated with *Fusarium sp* has been associated with estrogenic effects in poultry and livestock such as cloacae prolapse in turkeys (90), impaired fertility in cattle (91), and hyperestrogenicity in swine (92). The last disorder has been termed the "moldy corn syndrome." The exact extent of estrogenic mycotoxin contamination of human foodstuffs is not known, but is estimated to be 3 μg/person/day in North America.

C. In humans

There have been case studies in the clinical literature that illustrate the acute, reversible (activational) effects of exog-

TABLE 2. Examples of reproductive and developmental abnormalities attributed to endocrine disruption

Species	Observation	Contaminant	References
Mammals			
Humans	Gynecomastia, oligospermia, impotence, hypogonadism, decreased libido, reduced sperm counts and motility, menstrual cycle irregularities	DDT, kepone, oral contraceptive exposure, stilbene derivatives	(70)
Cattle	Infertility	Coumestrol	(71)
Sheep	Infertility, dystocia	Isoflavonoids, coumestans	(71)
Seals	Impaired reproductive functions	PCBs	(15)
Mink	Population decline, developmental toxicity, hormonal alterations	PCBs, dioxins	(72)
Rabbits	Infertility, failure of ovulation, failure of implantation	Isoflavonoids	(71)
Guinea pigs	Infertility	Isoflavonoids, coumestans	(71)
Mice	Proliferative lesions, reproductive tract tumors, infertility, inhibition of estrus, inhibition of ovulation	DES, isoflavonoids	(71,73)
Birds			
Japanese quail	Abnormal reproductive behavior, hematology, and feather morphology	*o,p′*-DDT	(74)
Gulls	Abnormal development of ovarian tissue and oviducts in male embryos	*o,p′*-DDT	(75)
Waterbirds	Egg shell thinning, mortality, developmental abnormalities, growth retardation	DDE, PCBs, AhR agonists	(72)
Reptiles			
Alligators	Abnormal gonads, decreased phallus size, altered sex hormone levels	*o,p′*-DDT, *p,p′*-DDE, dicofol	(76,77)
Red-eared slider turtle	Anomalous reproductive development	*trans*-Nonachlor, *cis*-Nonachlor, arochlor 1242, *p,p′*DDE, chlordane	(78)
Fish			
Mosquito fish	Abnormal expression of secondary sex characters, masculinization	Androstenedione	(34)
Roach	Hermaphroditism, vitellogenin in males, altered testes development	Sewage effluent mixture	(72)
Lake trout	Early mortality, deformities, blue sac disease	Dioxin, related AhR agonists	(72)
White sucker	Reduced sex steroid levels, delayed sexual maturity, reduced gonad size	Bleached kraft plup, mill effluent mixtures	(72)
Flatfish	Decreased hormone levels, reduced ovarian development, reduced egg/larvae viability	PAHs	(72)
Invertebrates			
Snails	Masculinization, imposex, formation of additional female organs, malformed oviducts, increased oocyte production	Tributyltin, bisphenol A, octylphenol	(79–82)
Marine copepods	Stimulate sexual maturation and egg production	Bisphenol A	(83)
Daphnia magna	Delayed molting time	PCB29, arochlor 1242, diethyl phthalate	(84)

Abbreviations: AhR, aryl hydrocarbon receptor; DES, diethylstilbestrol; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; PAH, polyaromatic hydrocarbons; PCB, polychlorinated biphenyl.

enous estrogen on the human male. The most informative is a case entitled, "The mortician's mystery: gynecomastia and reversible hypogonadotropic hypogonadism in an embalmer" (93), in which a 50-yr-old man presented with a progressive loss of libido, a decrease in testicular size and beard growth, and marked breast development. As these symptoms are associated with excess estrogen in a male, the patient, a mortician, was examined carefully for an estrogen producing tumor and excess serum steroidal estrogens. Failure to find a clinical answer suggested that there might be an exogenous or environmental source of estrogen exposure. Organic chemical extraction of the patient's serum revealed an unknown substance that effectively displaced radiolabeled estradiol from its receptor. A similar activity was discovered in the embalming cream used by the patient. When the source of environmental estrogen was removed, the patient experienced a significant restoration of libido, testes size, and sperm count, as well as reduction in breast size. The authors remarked upon the reversibility of the clinical symptoms and reached the following conclusion: "some principles observed in our patient may be generalizable to groups . . . although he presented with striking clinical findings, it is possible that lesser degrees of exposure to estrogen in . . . industrial exposures are more common and induce less profound disturbances of reproductive function, such as oligospermia in men and menstrual irregularities in women."

IV. Estrogens and Estrogenic Signaling

In 1979, the first Symposium on Estrogens in the Environment was held, ". . . to determine what an estrogen is and how it works, and what effect estrogenic substances might have on human health . . . since many chemicals with diverse

chemical structures, some of which are environmental contaminants, have been endowed with 'estrogenic' properties" (3). This seemed a fairly straightforward goal at the time; however, 20 yr later the attainment of that goal remains elusive.

One of the difficulties in the field of environmental endocrine research is semantic. What is an environmental estrogen? According to The American Heritage Dictionary of the English Language, ed 4 (2000), an estrogen is "any of several steroid hormones produced chiefly by the ovaries and responsible for promoting estrus and the development and maintenance of female secondary sex characteristics." The word, which first appeared in 1927, is comprised of the following components, "estr(us)" (again, from the same dictionary, "estrus [is] the periodic state of sexual excitement in the female of most mammals, excluding humans, that immediately precedes ovulation and during which the female is most receptive to mating; heat.") plus "o" (the combining form) and "–gen" ("producer; one that is produced."). Thus, estrogen is a word of recent origin with a functional definition, *i.e.*, something that produces a period of heat in a female—a signal.

To "induce estrus" is a behavioral and physiological process involving many organ systems and a commitment of time. As scientific knowledge of estrogen action has evolved, so has the functional definition. Over time, an estrogen has been defined in the scientific literature as a chemical capable of inducing vaginal cornification in an immature mouse; a chemical that increases uterine weight in an ovariectomized mouse; chemicals associated with proliferation of the uterine epithelium in castrate female mice; chemicals capable of stimulating an increased number of cells from estrogen target organs grown in tissue culture; chemicals that form ligands for the ER and displace radiolabeled estradiol from its binding; chemicals that regulate the expression of estrogen target genes; and, chemicals that transactivate ER-driven reporter genes in cells in culture. While one would think that any or all of these functional definitions would apply, the use of one or another has led to controversy. If a chemical binds the ER with a high affinity and specificity, is it an estrogen? Or must it also activate ER-regulated genes? Must it lead to a functional response? Hertz said, "Notwithstanding this complex array of variably associated effects of estrogens, the *sine qua non* of estrogenic activity remains the mitotic stimulation of the tissues of the female genital tract. A substance which can elicit this response is an estrogen; one that cannot do this is not an estrogen" (94).

This semantic problem is not unique to environmental estrogens. Semour Lieberman (95) recently posed the questions, "When is an estrogen an estrogen? When is it not?", whereby he revisited the concept of estrogenicity and the precision by which it should be defined. He was considering the use of the term estrogen to describe pharmaceutical and environmental compounds as well as natural hormones. In fact, Lieberman points out, we still do not know whether estradiol and estriol, two natural steroidal compounds, are really both estrogens, even though they have been called such for 60 yr, since behavioral estrus is induced by estradiol, not estriol. He raises the deliciously provocative possibility that estriol, the estrogen of pregnancy in humans, may actually have a different role than one might surmise from its classification as estrogen.

Therefore, when we say, for example, that plants make estrogen, precision requires us to say that plants make compounds that induce some responses traditionally associated with the steroid hormone, estradiol, either *in vivo* or *in vitro*. The language used to describe compounds that may alter the endocrine system presents a challenge in linguistic research as intriguing as much of the laboratory research in this area.

The signaling molecule, estradiol, regulates reproduction in many invertebrates and all vertebrates. Of invertebrates, Cnidarians (coral) (96, 97), crustaceans [water fleas (98, 99) and lobsters (100)], mollusks (snails) (101), and echinoderms (starfish) (102) are reported to produce estradiol. The phylogenetic distribution of estradiol production in the animal kingdom suggests that estrogenically active chemicals may be evolutionarily conserved signals. It also suggests the possibility that all animals are sensitive to estrogens, whether endogenous or environmental. In addition to the ligand signal, it appears that the signal recognition system is also widely distributed phylogenetically. As seen in Table 3 (20, 50, 51, 84, 103–116), ERs have been found in many vertebrate species. In those species in which it has been studied—including mammals, birds, and fish—both ERα and -β subtypes have been found. Very recently, a third distinct form of ER, ERγ, has been cloned from a teleost fish, the Atlantic croaker, *Micropogonias undulates* (20). This represents the first identification of a third classical ER in vertebrates. Phylogenetic analysis suggests that ERγ evolved through gene duplication from ERβ early in teleost lineage. As both ERβ and -γ bind 17β-estradiol with high affinity, the presence of three subtypes of ER in teleost fish suggests that the estrogen signal

TABLE 3. Selected examples of species containing ER or related receptors

Species	ER or ER-related molecules	References
Mammals		
Humans	ERα, ERβ	(50)
Cattle	ERα	(103)
Sheep	ERα, ERβ	(104)
Dogs	ER	(105)
Cats	ER	(106)
Rats	ERα, ERβ	(51)
Mice	ERα, ERβ	(50)
Birds		
European starlings	ERα, ERβ	(107)
Japanese quail	ERα, ERβ	(108)
Reptiles		
Alligator	aER	(109,110)
Red-eared slider turtle	ER	(111)
Fish		
Rainbow trout	rtERα, rtERβ	(112)
Tilapia	OaER	(113)
Atlantic croaker	ERα, ERβ, ERγ	(20)
Invertebrates		
Daphnia magna	EcR	(84)
Crayfish	EcR	(114)
Drosophila	EcR	(115)
Mosquito	EcR	(116)

Abbreviations: aER, alligator estrogen receptor; EcR, ecdysone receptor; ER, estrogen receptor; OaER, *Oreochromis aureus* estrogen receptor; rtER, rainbow trout estrogen receptor.

may be distributed in networks that we have not yet even considered. It remains to be demonstrated that ERγ exists in other vertebrate species, although in a study using the ERα knock out (ERKO) mouse, investigators explained their results in which catechol estrogens and methoxychlor, but not 17β-estradiol, stimulated uterine cell proliferation and lactotransferrin induction in the ERα minus mouse by raising the possibility of a third, or γ, form of the ER (117).

In studies reported so far, ER has not been found in invertebrate species. Other members of the nuclear receptor super gene family have been shown including the ecdysone receptor (Table 3) and several vertebrate "orphan receptors" such as COUP II (chicken ovalbumin upstream promoter II). This is an important area for further investigation and will help evaluate and refine the concepts of environmental signaling contained in this review.

For many years the characterization of an ER associated with the cell membrane has been sought as a means to explain the rapid responses seen after estrogen stimulation (118). There is now a growing body of literature describing the localization of ER molecules in the plasma membrane of estrogen target cells (119, 120). The membranes of endothelial cells are reported to contain ERα coupled to nitric oxide synthase as a functioning signaling module (121, 122). ER has also been shown in perimembrane activity in neural cells (123) and is thought to mediate rapid nongenomic estrogen signaling. The activation of coupled membrane receptors for estrogens and dopamine by environmental estrogens opens an exciting new dimension in environmental signaling (124). Finally, to explain the induction of mitochondrial gene expression with ethinyl estradiol (125), Chen and Yager (126) recently localized ERs -α and -β to the mitochondria of estradiol-treated cells (126). This provides yet another area of control for estrogenic signaling and locates a response module in the oxidatively active cellular organelle that may utilize the electron-donating capacity of catechol estrogens.

V. Environmental Signaling

We begin to understand the broad environmental signaling aspects of estrogenic chemicals when we consider chemicals produced by plants that evolved long before vertebrates and yet have been found to be hormonally active in numerous mammalian species (71). Subsequently, some of these chemicals have been termed estrogens, or phytoestrogens (literally, "plant estrogens"), as a result of their hormone signaling effects in animals. While plant estrogens probably did not originate as what we now think of as "estrogens," later vertebrate dietary exposure and response to these compounds led to the concept of phytoestrogens or plant chemicals with estrogenic activities (71). In a now classic report by Bennets *et al.* published in 1946 (127), compounds derived from plants such as subterranean clover were found to be capable of compromising the fertility of grazing sheep by overestrogenizing them.

Since the corollary evolution of animals has required the formation of internal signaling molecules of reproductive importance, it is tempting to speculate that estrogenic plant signals may have been internalized, or rather may have evolved, into the endocrine system, playing a crucial role in the coevolution of both major phyla. This also raises the question of interphyla cross-talk, *i.e.*, since animals recognize plant hormones, do plants recognize animal hormones? This question may be of environmental importance given the increasing burden of synthetic and other estrogenic compounds released into the environment.

Phytochemical signals, some of which are estrogenic, have evolved to benefit the plants that produce them. The most commonly studied phytochemicals are the flavonoids, including isoflavones and flavones, represented by genistein and luteolin, respectively. In fact, it has been shown that the isoflavones, like genistein, bind vertebrate forms of ERα or ERβ and alter the transcription of estrogen-responsive genes (128, 129). The report that isoflavones are better ligands for ERβ than ERα raises the possibility that environmental estrogens may exert greater effects on tissues or species with higher ERβ/ERα content (129).

The flavonoids represent a family of phytochemicals that have been thought to function to deter herbivores from eating the plant containing them, protect the plant from fungal and bacterial pathogens, and initiate symbiosis with nitrogen-fixing bacteria (130). It is this last function that is the defining signaling role for flavonoids.

Leguminous plants, such as soybean and alfalfa, produce such flavonoids. Symbiosis occurs as a result of complex signaling between host plant and bacteria, which is initiated by plant recruitment of bacteria to root hairs through the release of these small molecule polyphenolic compounds (131). *Rhizobium* bacteria exist as free-living organisms in the soil or as nitrogen-fixing symbionts of leguminous plants. In response to phytochemical signals (release of flavonoids), *rhizobia* infect the roots of host plants and induce the formation of specialized organs called root nodules (132). *Rhizobia* then colonize the root nodules and, in exchange for carbon nutrients from the host plant, provide the plant with a nitrogen source by changing atmospheric nitrogen into a nitrogen fertilizer (133). This is called "symbiotic nitrogen fixation."

For example, alfalfa, *Medicago sativa*, secretes the flavonoid luteolin, or 3′,4′,5,7-tetrahydroxyflavone, from its root hairs into the surrounding soil where the soil bacterium *Sinorhizobium meliloti* is located (134). Luteolin interacts with constitutively produced rhizobial NodD proteins, and this interaction activates transcription of a cassette of nodulation (*nod*) genes necessary for symbiosis (135). Host and bacteria specificity is necessary to maintain symbiotic partners, *e.g.*, alfalfa and *S. meliloti*, or soybeans and *Bradyrhizobium japonicum.* Each plant producing a unique profile of phytochemical signals achieves this specificity. For example, upon recognition of alfalfa's primary phytochemicals, luteolin and apigenin, *S. meliloti* NodD proteins activate *nod* gene transcription, but other flavonoids, such as chrysin and coumestrol, inhibit NodD-induced gene activation (141).

The ability of flavonoids to initiate, maintain, and regulate symbiosis requires unique signal recognition by, and activation of, the bacterial transcription regulator NodD. The ability of flavonoids to activate symbiosis is in part determined by the specific type of NodD protein within a *Rhizobium* species and, as illustrated above, is also regulated by the

specific phytochemical signaling molecules (136, 137). This suggests a complex interaction between flavonoids exuded by legume roots to recruit *Rhizobium* and those flavonoids found within the root that might function to negatively regulate this interaction. This balance of positive and negative inducing flavonoids serves a crucial function for the maintenance of symbiosis.

Some pollutants and organochlorine pesticides affect endocrine signaling in animals and human cell culture systems by weakly binding to ERs and modulating their ability to turn on transcription of estrogen-responsive genes (63, 138). These same environmental contaminants may interfere with plant-rhizobial signaling. This hypothesis is based on studies showing that endocrine disrupting chemicals, as well as phytochemicals produced by leguminous plants as a signal to *Rhizobium*, are both able to bind ERs in animals and affect the transcriptional activation of responsive genes (15, 129, 139). Some of the same phytochemicals that are able to bind the ERs and activate transcription are also able to cause transcription of responsive nodulation genes by interacting with the *Sinorhizobium meliloti* NodD protein. The NodD protein and ERα share not only similarities in compounds that they are able to bind or respond to, but may also share a degree of sequence homology in their ligand-binding regions. One study has reported that two regions of NodD1 share 45% and 35% amino acid homology with two regions in the hormone-binding domain of the mammalian ERα (140). Therefore, these two distinct proteins may share an evolutionary connection in sequence as well as the ability to bind estrogen-like compounds. Functional similarities and transcriptional effects are also shared by ERα and NodD; each protein binds a specific phenolic hormonal ligand, and this binding alters expression of key target genes leading to morphogenetic and metabolic responses.

The tightly controlled expression of these target nodulation genes allows *Rhizobium* to respond to the specific phytoestrogen signal emitted by the host plant and begin the process of symbiosis (130, 141). In light of the genetic and functional similarities between ERα and NodD, endocrine disrupting chemicals that are able to bind ERs and modulate signaling may employ the same mechanism to modulate the ability of *S. Meliloti* NodD to respond to the phytoestrogen signal, luteolin. Therefore, our laboratory used a construct containing key nodulation genes linked to a reporter gene (135) to study the effect of endocrine disrupting chemicals on signaling between the NodD protein, a proposed evolutionary relative of the ERs, and its natural phytoestrogen ligand. Fox *et al.* (Fox, J. E., M. Starcevic, K. Y. Kow, M. E. Burow, and J. A. McLachlan, submitted) show that under these conditions, DES, but not 17β-estradiol, inhibits luteolin-NodD-induced gene activation. Similar levels of inhibition have been seen with known endocrine disrupting chemicals. These results raise the possibility that endocrine disruption may be seen in symbiotic environmental signaling systems

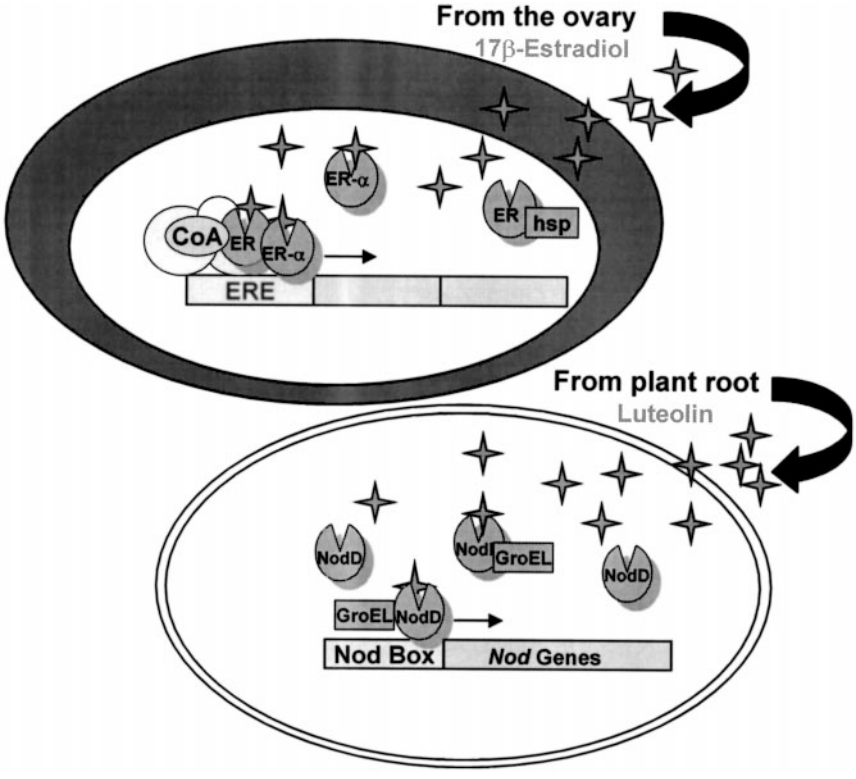

FIG. 7. Functional analogy between estradiol endocrine signaling and luteolin symbiotic signaling. The *upper panel* depicts the ovarian secretion of 17β-estradiol, the signaling molecule that activates the transcriptional apparatus controlled by the ER in association with a specific DNA recognition motif, estrogen response element (ERE), in the estrogen target cell (*e.g.*, uterine cell). The *lower panel* depicts the secretion by the plant (alfalfa) root of luteolin into the surrounding soil. This provides the signal that activates the transcriptional apparatus controlled by the interaction of the NodD binding protein and the DNA recognition motif called a Nod Box contained in the promoter region of target genes. Luteolin-NodD association activates the transcription of multiple bacterial nodulation genes.

that exist between organisms rather than within them. Figure 7 shows a stylized model of the functional analogy that may be derived from endocrine signaling by estradiol and symbiotic signaling by luteolin.

Another symbiotic relationship between plants and microbes involves flavonoid signaling and arbuscular mycorrhizal fungi (142). This important mutual interaction in the rhizosphere is ancient (>400 million years old) and universal (representing fungal colonies in the roots of most vascular plants) (143). Flavonoids such as quercetin and kaempferol are known to stimulate the hyphal growth of the fungus, *Gigaspora margarita* (144). Thus, as with rhizobial bacteria, root phenolics are secreted secondary plant metabolites that function as growth or differentiation signals for mutualistic fungi.

Since many phytochemicals that participate in plant-microbe symbiosis are also estrogenic in vertebrate systems (145), one might ask what the effect of "vertebrate estrogens" and antiestrogens are in the root-mycorrhizal signaling system. Using a transformed organ culture of carrot roots, Poulin *et al.* (144a) were able to demonstrate hyphal growth in *G. margarita* and *Globus intraradices* induced by the flavonoids, quercetin, or biochanin A, respectively. Using a highly specific antiestrogen, EM-652 [Dovalla-Bell *et al.* (145a)], they were further able to demonstrate a dose-related reduction in the biochanin A signal. 17β-Estradiol produced a 2.4-fold increase in hyphal growth, but was effective only at the highest concentration (5 μM).

Thus, as pointed out by Baker (145), consideration of the evolutionary role of signaling molecules used by plants to alter the behavior of microbes can provide insights into the mechanism of action of estrogenic compounds at many phylogenic levels. This approach should be informative for elucidating the chemical structures and functions inherent to environmental agents that behave in a hormonally active fashion.

Since several hormonally active xenobiotics are chlorinated hydrocarbons, the principle of environmental signaling suggests a search for naturally occurring signaling molecules that contain chlorine. In fact, a potent developmental signal, differentiation-inducing factor-1 (DIF-1) is a chlorinated alkyl phenone produced by the slime mold, *Dictyostelium*. This chlorinated signaling molecule is released by the ameba and induces it to differentiate into stalk cells (146). DIF-1 regulates the central cell fate decision during *Dictyostelium* development (147). With DIF-1 the cells differentiate into stalk cells, and without it, they become spores (148). The DIF-1 levels rise during cellular differentiation (149). As the cells differentiate they produce an inactivating enzyme, DIF-1 dechlorinase, which prevents further increases in the signal (150).

As in the legume-rhizobial bacteria system, the DIF-1 system provides a signal-dependent mechanism for cellular aggregation and differentiation. In the case of *Rhizobium* the process results in a root nodule, while in *Dyctyosteleum*, it results in a fruiting body. It is interesting to speculate that the signaling properties seen in vertebrate estrogenic signaling may be related to evolutionarily ancient systems developed in soil bacteria and slime molds.

Similarly, for chemicals such as PCBs or other chlorinated hydrocarbons, environmental bacteria can play a role in bioactivating hormonally inert compounds into more estrogenic forms by first dechlorinating and then hydroxylating the parent compound (151). The extent of such conversion is not known, but it provides another avenue for the production of hormonally active environmental compounds.

The conversion, then, of nonhormonally active compounds in the environment to active "hormones" is an important issue to consider both in the context of environmental endocrine disrupting chemicals as well as in naturally occurring hormonally active environmental signaling systems. Are there adaptive benefits to *Gambusia* exposed through generations to testosterone or, as is more likely, are there more proximate targets for utilization of the androgen produced by the bacterial mats? While plant sterols serve as a mere carbon source for organisms that do not respond to the androgenic metabolic products, organisms sharing the same environment, such as mosquito fish, interpret these metabolic products as "masculinizing signals" that alter their body plans and endocrine systems.

From a toxicological view, the insights into the structure-function relationships of modern day contaminants may be significantly advanced through evolutionary considerations of the ancient signaling molecules known to elicit differentiation responses. Likewise, we must add to our calculations of distribution of natural and synthetic chemicals the role played by microbial conversion.

VI. Estrogens and Fetal Development

A. Effects of estrogens and estrogenic chemicals on development of males

Many of the reports regarding effects of environmental estrogens on wildlife have dealt with altered sexual development. Hydroxylated PCBs have been shown to sex reverse male-determined turtle embryos in much the same way estradiol does (152). Fry and Toone (75) have shown that DDT will feminize gulls in ovo. The reduced phallus size and altered estrogen-to-testosterone levels seen in alligators in Lake Apopka, Florida, suggest a developmental exposure to environmental estrogen or antiandrogens (76).

These results in wildlife raise the possibility that prenatal estrogen levels in humans might be associated with later genital anomalies in the male offspring. One way to explore that issue is to ask what the estrogen status was during the fetal development of men with retained or cryptorchid testes. When maternal pregnancy conditions were noted for men with crytorchidism it was found that the clinical conditions reported for the mother during the fetal life of the men—obesity, hyperemesis, first pregnancy, or hypertension—were each associated with elevated estrogen levels in their mothers (153).

The term "organizational effects" describes the persistent developmental effects of hormones while "activational effects" describes the acute, reversible effects of hormones. Studies on developmental exposure to the potent estrogenic chemical DES may shed light on the mechanisms underlying the apparent "organizational" effects of estrogens.

1. DES as a model for developmental estrogenization. Studies in our laboratory and others have helped to define a phenotype typical of male mice exposed *in utero* to DES and other estrogens. The structural or functional changes associated with the phenotype include undescended testes, cysts of the epididymis, prostatic lesions, distended seminal vesicles, retained Müllerian ducts, reduced fertility, and abnormal spermatogenesis (even in a scrotal testis). In a smaller number of cases, the occurrence of testicular cancers was noted (154–157). The severity of these changes was dose-dependent as were the appearance of all the lesions in the suite. It was subsequently shown that the epididymal cysts were of Müllerian duct origin (158); it was apparent that the enlarged prostatic utricle was also the Müllerian contribution to the prostate gland.

The results of Sharpe *et al.* (159) and vom Saal and associates (160) and others who have studied the effects of steroidal and environmental estrogens on the genital tract of the fetal male rodent have provided further confirmation that estrogen can induce long-term functional changes. More recently, studies with the ERα null mouse (161) have added strong support to the concept that male genital tract development may have an estrogen component.

The similarity in the morphogenesis of the reproductive system in mammals as diverse as the mouse and human provides comparative insights into the spectrum of effects associated with *in utero* estrogen exposure. Mice and human fetuses progress from an "indifferent" stage of internal genitalia in which both the presumptive male (Wolffian duct) and female (Müllerian duct) reproductive organs coexist regardless of the genetic sex of the fetus to the definitive structure of the appropriate gender. This process is under the control of hormones from the fetal testes after differentiation of the fetal gonad. The configuration will be female unless the fetal testis intervenes by secreting Müllerian Inhibiting Substance (MIS) to induce regression of the Müllerian duct and testosterone to maintain the Wolffian duct (162). In mice, DES exposure *in utero* results in the retention of both male and female genital ducts, thus forming a male pseudohermaphrodite or a genetic male with functioning testes and a male genital tract as well as a female genital tract. Failure of testicular descent is also commonly observed.

Studies in organ culture confirm the retention of female genital anlage in the DES-exposed tissues. They also extend the *in vivo* observations to demonstrate that the DES effect is not on the synthesis or secretion of MIS from the fetal testes, but in the Müllerian duct resistance to the apoptotic signal of MIS (163).

2. Features in the human male. Genital tract defects similar to those seen in DES-treated mice were also observed in men whose mothers had taken DES (164, 165). A group at the University of Chicago reported that DES-exposed men had a higher incidence of undescended (cryptorchid) testes and epididymal cysts than comparable unexposed men. Gill and colleagues (166, 167) went on to confirm and extend these studies and showed, in addition, a higher incidence of hypoplastic testes and abnormal sperm. In one study reporting testicular cancer in one DES-exposed man, the possibility of cancer of the testis as a result of prenatal exposure to DES was raised by Gill *et al*. (167). A few other case reports of testicular cancer (seminoma) and epididymal cysts in prenatally DES-exposed men have been reported (168).

Comparison of mouse and human data demonstrates the importance of understanding the timing of biological events involved in the development of the reproductive tract of each. For example, when comparing the total dose of DES administered during pregnancy to the mouse, as compared with the human, to produce retained testes, the Relative Potency Index (RPI) was more than 80. However, when the dose comparison was made during the biologically relevant period for testicular descent in both species (days 14–16 of gestation in the mouse and weeks 7–27 in the human), the RPI was between 1 and 2 (169).

Thus, the male offspring of DES-exposed pregnancies of both mice and humans share some defects in common, including undescended testes, epididymal cysts, and sperm abnormalities. A recent study by the Wilcox group confirmed the occurrence of structural abnormalities in DES-exposed men but found that there was not a significant difference in fertility between the study participants and control subjects (170). While retention of the female genital anlage, the Müllerian duct, was a prominent feature in DES-exposed male mice, no report from similarly exposed men has addressed this issue. One might expect some element of Müllerian duct retention in the human male, since the hormone responsible for regression of the female duct is also thought to play a role in testicular descent, a defect common to both species.

One of the earliest reports of adverse effects of prenatal exposure to DES on male progeny was a single case of pseudohermaphroditism in a male infant. The child's mother had been given high doses of DES during pregnancy (50 mg/day commencing in the sixth week of gestation; 200 mg/day by the eighth week and for the duration of the pregnancy) (171). The genital lesions in the boy included hypospadias and testes apparently devoid of germ cells. The period of gestation during which exposure occurred appears important since Davis and Potter (172) observed no abnormalities in the external genitalia of four male infants whose mothers were treated after the first trimester with high doses of DES. Finally, in parallel with the studies on the prenatally DES-exposed mouse, the Müllerian duct derivative in the prostate, the prostatic utricle, was hypertrophic and contained areas of squamous metaplasia, suggesting that the fetal Müllerian derivatives responded the same in both species to the estrogenizing effect of DES *in utero* (173).

B. Molecular mechanisms for the developmental actions of estrogen

The developmentally estrogenized male phenotype—retained or cryptorchid testes, decrease in sperm number, increase in abnormal sperm, retained Müllerian ducts, epididymal cysts, hypospadias, and prostatic disease—has been seen, in whole or in part, in mice, rats, hamsters, and humans exposed to estrogens *in utero*. The genes involved in the process of male genital tract morphogenesis are only now being identified. The acute or persistent modulation of the expression of developmentally critical or hormone-respon-

sive gene in the male genital tract by estrogenic compounds is currently ongoing in numerous laboratories.

1. Cryptorchidism. Emmen *et al.* (174) have recently shown that the cryptorchidism associated with prenatal treatment with various estrogens in mice may be the result of estrogen-related inhibition of insulin-like factor 3 (Insl3), produced by the fetal testes. Insl3 had been shown earlier to affect testicular descent through signaling from the fetal testes to the gubernaculum. Insl3 mutant mice exhibit bilateral cryptorchidism; this is thought to occur as a result of altered development of a component of the genital mesentery, the gubernaculum, which retains an elongated "female" structure (175, 176).

2. Hypospadias. Hypospadias, a defect of the external male genitalia associated with prenatal estrogen treatment, has also recently gained molecular dimensions. In this case, the Yamada group (177) has reported that the development of the external genitalia of the mouse involves signaling by fibroblast growth factor (FGF) during formation of the genital tubercle. FGF 10 knock-out mice show abnormal development of the glans penis, suggesting an important role for that signaling molecule in the induction of hypospadias.

3. Müllerian duct retention. The molecular mechanism for Müllerian duct retention associated with DES is becoming clearer. While it had been shown that the effect of DES on Müllerian duct retention resides at the level of the duct rather than the fetal testis (163), the molecular alteration in the duct has recently been shown to be a failure of the MIS receptor in the fetal duct to respond to the peptide (178). The molecular mechanisms associated with Müllerian pathogenesis after prenatal exposure to DES is starting to be understood. Ma *et al.* (179) studied the localization of the Hox genes related to morphogenesis of the fetal Müllerian duct in the mouse. By concentrating on Hoxa, they determined the longitudinal distribution of these genes along the developing genital tract and were able to relate changes seen in Hoxa-10 gene disruption to that seen in prenatal exposure to DES.

4. Molecular feminization of the developmentally estrogenized male tissues. Androgen-dependent secretory proteins, SVS-IV, V, and VI, have been characterized from the rat (180, 181) and mouse (182) seminal vesicle. In both the rat and mouse, SVS-IV was under control of androgen and, therefore, not expressed in female or castrate male tissues. An estrogen-dependent uterine secretory protein (183) and the gene encoding it were identified as lactotransferrin, a member of the transferrin gene family (184). Lactotransferrin is under powerful control by estrogen, is located in the uterine epithelium, and varies with estrogen levels during the estrous cycle (185). Lactotransferrin was expressed in mammary gland and leukocytes but was not regulated by estrogen in these tissues (185). Lactotransferrin was not expressed in ovariectomized female mice or in male control mice. Male mice that were castrated and treated with estrogen did not express lactotransferrin in their seminal vesicles (186).

When the seminal vesicles of prenatally DES-exposed males were analyzed, lactotransferrin mRNA was detected. The level of expressed lactotransferrin message increased after castration of the male and exceeded that of the uterus when each was estrogen stimulated. These results comprise the first demonstration of hormonally altered sexual development at the gene level. Further analysis of the seminal vesicles of DES-treated mice demonstrated that while they retained the cytoarchitecture associated with the male organ, they expressed epithelial gene products associated with the uterus, *i.e.*, lactotransferrin and ERα (187). In fact, virtually all the cells of the DES-exposed mouse seminal vesicle epithelium express antigens recognized by antisera to lactotransferrin and ERα.

Additional experiments were conducted to assess whether prenatal exposure to DES had feminized the seminal vesicle cells or, alternatively, blocked the masculinization of the organ at the molecular level. It was shown that DES-exposed mouse seminal vesicles were competent to express the seminal vesicle specific protein SVS-IV under androgen control as well as lactotransferrin. In fact, the same cell was often able to make both products (188).

VII. Mechanisms in Altered Fetal Development

Estrogens are considered reversible cellular signals. Administration of estrogen to an ovariectomized mouse results in organ growth, cell proliferation, and target gene expression in the uterus (189). When estrogen is withdrawn, uterine size and weight as well as expression of estrogen-regulated genes return close to the unstimulated state.

On the other hand, when estrogens are given to newborn mice, at least one gene under estrogen control is expressed persistently, even in the absence of estrogen (190). The estrogen treatment that results in persistent expression of lactotransferrin also results in epithelial cancers of the uterus in a majority of the mice (191). This leads to the question, how does a reversible signal become irreversible in the absence of detectable gene mutation? As described earlier, during the process of cell differentiation, genes are differentially turned on or off. For the most part, a key event in establishing the pattern of gene expression in a cell is the methylation or demethylation of regulatory elements of the gene.

Five CpG sites available for methylation occur in a region upstream from the ERE in the mouse lactotransferrin promoter. In the developmentally estrogenized mouse, two sites remain unmethylated, while in the corresponding control, only one CpG site remains unmethylated (192). It has been shown previously that a 1-bp change in methylation pattern can have a strong effect on expression of the gene (193). Adult mice treated with the same dose of DES for the same period of time did not have a change in the DNA methylation pattern of the lactotransferrin gene. This is consistent with the inability of such treatment in the adult to persistently change expression of the gene. The actual mechanism underlying the molecular feminization of genes by estrogen has still not been elucidated. However, other studies in our laboratory on the control of lactotransferrin expression may prove informative. We have shown that developmental exposure to estrogens resulted in the persistent overexpression of lactotransferrin in the uterus of females (190). The cellular secretory protein is expressed at the mRNA and protein

levels as if the mouse is receiving injections of estrogen, even 3 weeks after ovariectomy. More recently, we have shown that the persistent expression of a normally hormone-regulated gene may be the result of developmental imprinting (192). A fundamental event in cell differentiation involves programming genes to be differentially regulated later in life. One mechanism for developmental gene programming is selective DNA methylation or demethylation of its promoter.

The altered methylation pattern associated with estrogen treatment during differentiation of uterine epithelial cells provides a mechanism for irreversible expression of a normally reversible signal or provides one possible route for the change from epigenetic to genetic in hormonal carcinogenesis. The generalization of this mechanism to hormonal carcinogenesis requires studies on methylation and expression of different genes that are important to cell differentiation or proliferation. It has been shown that breast cancer cells have silenced genes that otherwise would function as tumor suppressor genes and that the silencing is a function of altered methylation (194). It is not known whether estrogens play any role in the methylation or demethylation of these genes.

Genomic imprinting refers to a very specific phenomenon: "the non-Mendelian inherited epigenetic form of gene regulation that results in monoallelic expression" or, "genomic imprinting is an epigenetic form of gene regulation that results in the expression of only one parental allele" (195). The elements of genomic imprinting are the persistent change in gene expression that is accomplished in an epigenetic fashion, and the silencing of a gene from one parent, but not the other. The process of cell differentiation is based on differential gene expression or the sequential turning on and off of different genes to establish a stable phenotype. While this process may not be monoallelic, it is both epigenetic and inherited. In developmental toxicology, the term "gene imprinting" refers to persistent changes in gene expression that occur through nonmutagenic mechanisms.

The same conundrum has appeared in the molecular biology of cancer, in which "silencing" of tumor suppressor genes has been associated with the cancer process. Since tumor suppressor genes are in many cases genomically imprinted monoallelic genes, the process fits the specific definition. However, in cases where the neoplastic transformation process involves persistent activation or repression of nongenomically imprinted monoallelic genes, the term gene imprinting is useful.

The terminology is especially useful when considering mechanisms of hormonal carcinogenesis. It is well known that treatment of various species, including humans, with exogenous estrogen is associated with tumors in different organs (196). Estrogens have been largely assumed to function in the tumorigenic process as a secondary stimulus or promoter, due to the paucity of evidence that estrogens or estrogenic chemicals are point mutagens (197). In spite of the failure to conclusively demonstrate that estrogenic chemicals, unlike other carcinogenic chemicals, do not form covalent adducts to DNA, nor induce structural DNA mutations, it has been shown that estrogens of various structures and biological potencies can function as carcinogens. This evidence includes neoplastic transformation of cells in culture in the absence of enhanced cell proliferation (198–200), carcinogenic effects in the adult hamster kidney (201), or neonatal mouse (191), and hamster (202) uteri.

In fact, very little is known regarding the role of estrogens or other hormones in gene imprinting. A case can be made, however, that estrogen-associated signaling pathways may contribute to DNA methylation or demethylation. For example, our laboratory showed earlier that estrogen-like effects were induced in uterine cells in culture (203) or in the mouse uterus *in vivo* (189) by epidermal growth factor (EGF). Subsequently, we demonstrated that the EGF signal required the presence of a functioning ER in cell culture for the expression of the growth factor effect (204). This study was subsequently confirmed in mice lacking the ERα gene (205). The demonstration of cross-talk between membrane receptors and steroid hormone receptors has also been shown for IGF-I and ER, dopamine and PR, and both IGF-I and EGF and ER in cell culture. The studies of receptor cross-talk have established the ER as an important signal transduction molecule in peptide hormone signaling systems. We have recently demonstrated additional evidence of this signaling convergence by showing that the cell survival pathway associated with phosphatidylinositol-3 kinase and AKT also requires a functioning ER for its action (Burow, M. E., B. N. Duong, D. E. Frigo, S. Elliott, C. B. Weldon, B. M. Collins-Burow, J. Alam, B. S. Beckman, and J. A, McLachlan, submitted). Thus, pathways involved in cell proliferation, differentiation, and survival apparently converge on the ER.

Finally, it has been shown previously that both EGF and estrogen increase the levels of c-*fos* or c-*jun* in target cells or tissues (206). The response of the immediate early genes to estrogen also proceeds, apparently, via an ER. In the case of developmental estrogenization, c-*fos* and c-*jun* were persistently expressed. Recently, a demonstration that cells constitutively overexpressing c-*fos* up-regulated cytosine methyltransferase, the enzyme involved in DNA methylation, has suggested to the authors that fos may be one regulator of the process (207).

In summary, we suggest a mechanism whereby estrogen either directly or through related signaling pathways plays a role in programming or imprinting genes involved in cell proliferation, differentiation, or survival. This happens in such a way that the genes are either persistently over- or underexpressed or will respond to a later cue, such as another hormone leading to altered cell function and ultimately disease. Given that the process of gene imprinting is a normal one seen in cell differentiation and genomic allelic silencing, one may speculate that for estrogens or estrogenic chemicals, the distinction between physiology and pathology is blurred. The case for estrogen-associated gene imprinting is strengthened by two recent papers in which it was shown that specific genital tract neoplasms associated with developmental exposure to DES can be generationally transmitted to both female (208) and male (73) progeny.

Cellular imprinting by estrogenic compounds may arise through at least two mechanisms. In one case, the estrogenic chemical may directly imprint the gene through a process leading to persistent genetic change, probably at the level of DNA methylation. On the other hand, altering components of signaling pathways at key points in cell differentiation such that altered gene expression would ensue could form a

biochemical memory. The most likely genes to be important to this process would be those involved in response to secondary hormonal cues. Thus, when a gene programmed to respond to estradiol at puberty is misprogrammed or reimprinted by developmental exposure to a hormonally active chemical, it will respond abnormally to the secondary cue, resulting in a functional cellular abnormality. It is this concept of epigenetic memory that is most compelling in dissecting the mechanism of action of hormones during development. Vitellogenin, or egg yolk protein, has been well studied in this way (209–216). Prior estrogen stimulation in chickens or frogs results in an accelerated induction of vitellogenin following a second stimulus. The molecular "memory" apparently is coded in the conformation of the chromatin site associated with vitellogenin. While studies of this type have not been thoroughly done with mammals, it provides an additional model with which to view the possible imprinting effects of estrogen during development.

VIII. Lessons Learned

In environmental endocrine science, we have made a series of observations that, at first, seemed unconnected. However, now, as the observations start to establish a pattern, we can begin to discern the linkages between them. In the last 20 yr we have discovered the intrinsic biological signaling properties of numerous synthetic environmental chemicals. We are also beginning to learn about the complex network of signaling molecules that facilitate information flow in the communication system of ecological life. In the same time period, cell and molecular biology has elucidated many of the signaling molecules necessary for intra- and intercellular communications. The similarities between the signaling strategies adopted by the internal and external world are probably more than coincidental if the evolution of the signaling systems followed, in any way, the convergent pathways suggested in this review.

Environmental signals are chemical messenger molecules functioning in a communication network linking numerous species. One may speculate that the functional aspects of this more globally distributed network might have provided a framework or blueprint to build the internal communication networks of animals, which we call their endocrine systems. As such, similarities in response to such signals in some cases should not be unexpected. Indeed, a central strategy for all life forms is the transmission of important characteristics to their offspring. This also is a form of information transfer in which the signal is embedded in the physical entity being transmitted. Thus, the transmission of genetic information and the utilization of signaling molecules and pathways are intrinsic to life from bacteria to humans. As de Loof (217) states, "communication is the one essential property for life." Chemicals that alter either or both levels of information flow can have consequences that may be deleterious to the individual or population.

From the study of embryos and evolution the following three patterns emerge:

1. The structural diversity of environmental hormones may reflect the evolutionary background of these chemicals as plant signaling molecules or differentiation specific signals for organisms that do not require an endocrine system. Moreover, signals developed for one communication system may be functionally misinterpreted by another system.

2. Evolutionarily important signals are likely to be those related to reproduction or differentiation of the species and its cells. This information is most crucial to the survival of that species and, likely conserved as pathways, most often misinterpreted. Chemicals synthesized to disrupt the reproductive capacity of insect pests stand a good chance of affecting cell differentiation in unintended species and in unintended ways.

3. Molecules with high informational content can induce long-term changes in a communication system if the information is disseminated at inappropriate times. Hormones that may alter the processing of information by imprinting a response pathway or imparting memory functions in a cell can be expected to have long-term effects on developing organisms. The effect will be related to the number and types of programming mistakes induced.

The patterns underlying evolution and embryogenesis are being uncovered through systematic inquiry using the techniques of molecular and cell biology. As patterns begin to emerge in environmental endocrine science, recognition of similarities to those associated with evolution and development should provide insights to mechanisms and outcomes. Without pattern recognition, there is not the ability to predict, and without prediction there is not the possibility to prevent. If male fruit bats are lactating in Malaysia (218), look for the environmental hormone. If there is a dramatic increase in the cases of premature breast development in Puerto Rico (219), look for the environmental hormone. And, if a 50-yr-old mortician presents with gynecomastia and hypogonadotrophic-hypogonadism with no estrogen-producing tumor (93), look for the environmental hormone.

It has not always been recognized as such, nor has it been applied to environmental endocrine science, but making public health predictions based on environmental chemical confusion or environmental signal misinterpretation has a long and mostly successful history in environmental health sciences and toxicology. One informative example will suffice. From the Middle Ages until as recently as 1959, an acute behavior disorder could result from eating moldy rye bread; the fungal product associated with this condition is a neuroactive compound in humans called ergot and the condition is known as ergotism (220). In the Middle Ages and later, ergotism was known as St. Anthony's fire since humans consuming infested rye flour behaved as if they were on fire and were thought to be possessed by the devil. This level of knowledge was consistent with the unpleasant consequences usually visited on such individuals. As the association between mold in bread and disease in humans was made, the scientific explanation, the human body's misreading of the fungal signal, provided a course of public health action—prevent mold from developing in rye flour or if it does, don't make bread from it.

As the principles underlying environmental endocrine science are developed or discovered, the opportunity to apply them to understand and anticipate the environmental component of human reproductive diseases and developmental

disorders should excite endocrinologists. From an environmental stewardship perspective, the evolving concept of environmental signals can provide insights with which to address the impact of hormonally active chemicals on humans and the ecosystems that they share with other species. Disruption of this apparently broad communication system has the potential for global change that transcends the endocrine system.

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Note Added in Proof

Embryos. A very recent study on sex reversal in humans describes a plausible molecular mechanism for a female phenotype in genetic males. Overexpression of WNT-4, a putative sex-determining gene, due to a duplication of 1P31-P35 led to female development in an XY individual. The authors propose that this genetic imbalance is associated with conditions including ambiguous genitalia and failure of testicular descent (221).

Evolution. An elegant case is made for the elaboration of the steroid receptor family through gene duplication and ligand exploitation in which the metabolic intermediates of hormones become ligands. Analysis of the evolution of steroid receptors and endocrine complexity concludes that the estrogen receptor was the first steroid hormone receptor and, if so, may explain why so many different taxa may be sensitive to environmental pollutants that interact with the estrogen receptor (222).

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