Large Effects from Small Exposures. I. Mechanisms for Endocrine-Disrupting Chemicals with Estrogenic Activity

Wade V. Welshons,[1] Kristina A. Thayer,[2] Barbara M. Judy,[1] Julia A. Taylor,[1] Edward M. Curran,[1] and Frederick S. vom Saal[2]

[1]Department of Veterinary Biomedical Sciences and [2]Division of Biological Sciences, University of Missouri-Columbia, Columbia, Missouri, USA

Information concerning the fundamental mechanisms of action of both natural and environmental hormones, combined with information concerning endogenous hormone concen[...] very high doses in toxicological studies of EEDCs thus can dramatically underestimate bioactivity. Specifically: *a*) The hormonal action mechanisms and the physiology of delivery of EEDCs predict with accuracy the low-dose ranges of biological activity, which have been missed by traditional toxicological testing. *b*) Toxicology assumes that it is valid to extrapolate linearly from high doses over a very wide dose range to predict responses at doses within the physiological range of receptor occupancy for an EEDC; however, because receptor-mediated responses saturate, this assumption is invalid. *c*) Furthermore, receptor-mediated responses can first increase and then decrease as dose increases, contradicting the assumption that dose–response relationships are monotonic. *d*) Exogenous estrogens modulate a system that is physiologically active and thus is already above threshold, contradicting the traditional toxicological assumption of thresholds for endocrine responses to EEDCs. These four fundamental issues are problematic for risk assessment methods used by regulatory agencies, because they challenge the traditional use of extrapolation from high-dose testing to predict responses at the much lower environmentally relevant doses. These doses are within the range of current exposures to numerous chemicals in wildlife and humans. These problems are exacerbated by the fact that the type of positive and negative controls appropriate to the study of endocrine responses are not part of traditional toxicological testing and are frequently omitted, or when present, have been misinterpreted. *Key words:* dose response, endocrine disruptors, estrogen action, estrogen receptors, fetal development, inverted U, MCF-7 cells. *Environ Health Perspect* 111:994–1006 (2003). doi:10.1289/ehp.5494 available via *http://dx.doi.org/* [Online 2 February 2003]

During the past decade a number of pesticides, industrial by-products, manufactured products such as plastics, and natural chemicals have been shown to disrupt the endocrine system. These chemicals are referred to as endocrine-disrupting chemicals (EDCs). These chemicals have received considerable attention, in part because endocrine disruption is a relatively unstudied area in toxicology and is only recently being taken into account in risk assessment. The focus here is on EDCs with estrogenic activity (EEDCs), which are chemicals that act as hormone mimics via estrogen receptor mechanisms; this is currently the largest group of known endocrine disruptors. The main purpose of this article is to present an overview of the mechanisms of hormone action that provide the basis for understanding how EEDCs have the potential to be biologically active at low, environmentally relevant doses. Our strategy is to discuss the receptor mechanisms mediating responses to a natural hormone, 17β-estradiol (E_2), and then to use this information as the basis for describing the low-dose effects of chemicals that disrupt the normal functioning of this hormonal system, either by mimicking, modulating, or antagonizing the activity of the hormone. We have chosen to use estrogen as our example because there is more known about the biology of estrogens and xenoestrogens than other components of the endocrine system for which there is evidence for disruption by environmental chemicals; however, the information presented here is applicable to endocrine disruptors that interfere with other hormonal systems.

We will begin by briefly reviewing information concerning the relationship between dose, receptor occupancy, and responses (such as cell proliferation) after binding of E_2 to estrogen receptors (ER-α) in cultured human MCF-7 breast cancer cells. A number of specific factors influence the dose of an EEDC that reaches the target cells to produce a response. These factors include route of administration, absorption, distribution, metabolism, rate of clearance, plasma transport, cell uptake, affinity for estrogen receptor subtype in the cell, and the interaction of the ligand–receptor complex with tissue-specific factors comprising the transcriptional apparatus. This mechanistic information provides the basis for establishing the dose at the target site in cells (nuclear receptors associated with DNA or more recently identified receptors associated with the cell membrane) for an EEDC required to elicit a biological response similar to that produced [...] these factors would encompass physiologically based pharmacokinetic information (*1*), as well as quantitative structure-activity relationships (QSAR) (*2,3*). We have previously discussed the factors that influence access of E_2 and EEDCs from blood to estrogen receptors in cells elsewhere (*4–6*). Our primary focus in this review is on the latter part of the overall process that occurs once an estrogenic chemical has reached the nuclear estrogen receptor.

Dose ranges. We have separated dose-specific effects into three general categories: the physiological dose range for estrogenic activity, the toxicological dose range for acute toxicity, and the environmentally relevant dose range related to current exposures. The physiological dose range (of estrogenic activity, whatever the source) is defined by the normal concentration range of an endogenous hormone. More specifically, with regard to steroid hormones, the physiological concentration refers to the amount of free (unbound to plasma proteins and unconjugated) endogenous hormone that the EEDC is mimicking or antagonizing. The free hormone concentration is generally considered to be the biologically active portion of total hormone concentration in blood (*7,8*) and most accurately predicts biological activity (for example, free triiodothyronine and free thyroxine, as opposed to total hormone concentration, are routinely used for clinical diagnosis). The toxicological dose range is identified by some measure of toxicity, such

as death in the extreme case, a decrease in body weight, or malformations in a developmental study. The environmentally relevant dose range can be established for chemicals where there is information concerning levels monitored in air, food, or water or, less commonly, if there is information based on monitoring of biological tissues in wildlife or human populations.

It is important to note that during fetal and early postnatal life, the pharmacokinetics of chemicals and drugs are markedly different relative to adulthood, and pregnant and non-pregnant females also differ in this regard. Therefore, dose ranges in pregnant females and fetuses cannot be assumed to be the same as in adults and should be evaluated separately.

Low-dose range. The physiological and the environmentally relevant dose ranges typically fall well below the toxicological dose range based on using established protocols for examining acute toxic effects of chemicals. Exceptions would be instances of industrial accidents or workplace exposure, such as the Yu-Cheng incident in Taiwan involving accidental exposure to acutely toxic doses of polychlorinated biphenyls (PCBs) (*9*) or exposure to synthetic estrogens by workers in pharmaceutical plants (*10*).

At a meeting hosted by the National Institutes of Health (NIH) at the request of the U.S. Environmental Protection Agency (U.S. EPA), devoted to the low-dose issue (*11*), low dose was defined as doses below the range typically used in toxicological studies, where the dose range seldom extends more than 50-fold below the maximum tolerated dose (MTD) in an animal (*12,13*). The physiological and the environmentally relevant ranges we describe here fall within this low-dose range defined at the NIH meeting. For example, the MTD for the plastic monomer bisphenol A is 1,000 mg/kg/day (*14*). The U.S. EPA calculated a reference dose (RfD) based on a LOEL (lowest-observed-effect level) of 50 mg/kg/day; this was because a no-observed-adverse-effect level had not been determined, and adverse responses occurred at the lowest dose tested. The RfD of bisphenol A based on application of a safety factor of 1,000 was calculated to be 50 μg/kg/day (*15*).

The environmentally relevant amount of bisphenol A, however, has recently been determined on the basis of direct measurement in the blood of human fetuses at term. Parent (unconjugated, aglycone) bisphenol A concentrations ranged from 0.2 to 9.2 ng/mL, with a mean ± SD of 2.9 ± 2.5 ng/mL (*16*).

Developmental exposures. Although the issues discussed in this review apply to exposure to endocrine disruptors at any time in life, it is generally accepted that EDCs have the greatest impact when exposure occurs during development (*17,18*). In describing the *in vivo* effects of EDCs, we will emphasize effects of endocrine disruptors on fetal development. During fetal life, endogenous hormones regulate the differentiation and growth of cells, and developmental processes appear to have evolved to be exquisitely sensitive to changes in hormone concentrations. A consequence of this evolved strategy of development being epigenetic (that is, based on signals that cells are exposed to rather than due to a fixed genetic program) is that even in animals that are genetically identical, small fluctuations in endogenous hormonal signals during development provide the basis for significant variability in phenotype (*19*). This provides the mechanism via which even slight alterations in hormonal activity due to exposure to EDCs during very brief critical developmental periods in fetal life can potentially lead to irreversible changes in the course of differentiation of cells. These cellular changes are associated with permanent alterations in gene activity and organ function (*20,21*).

Implications. We will review mechanistic information showing that failure to apply fundamental principles of hormone receptor biology to dose selection in toxicological studies can potentially lead to a huge error in estimating risk associated with exposure to doses below the NOEL (no-observed-effect level) determined in traditional toxicological studies. These issues are problematic for toxicology, because they challenge the traditional use of extrapolation from high-dose testing to predict responses at much lower environmentally relevant doses. Additionally, these data also provide evidence that some traditional assumptions used in risk assessment for systemic (noncarcinogenic) toxicants, such as the assumption of a threshold (*22*) and a monotonic dose–response relationship (*23*), cannot be uniformly applied to EDCs (*24,25*). We will relate our findings regarding effects of very low doses (within the range of human exposure) of bisphenol A (the monomer used to manufacture resins and polycarbonate plastic and used as an additive in many other products) and methoxychlor (a currently used insecticide) to current methods of risk assessment for systemic toxicants. The classification of EDCs as systemic toxicants is due to an absence of data and is not based on findings of no genotoxic effects, particularly for estrogenic EDCs (*26*). Because estrogen is implicated in a number of cancers, both as an initiator and promoter, environmental chemicals that mimic estrogen cannot be ruled out as carcinogens. In particular, research is needed to determine whether exposure to EDCs during early life is related to the development of cancer later in life (*26,27*). A recent example of a relevant finding is that at very low doses (0.1–10 nM, 0.023–2.3 ng/mL), bisphenol A induces pro-

liferation of human prostate cancer cells via binding to a mutant form of the androgen receptor found in some prostate tumors (*28*).

It has been known for decades that some environmental chemicals mimic the activity of endogenous hormones. However, the mechanistic information we provide here concerning the functioning of the hormonal systems being disrupted by these chemicals was, in general, not considered in designing toxicological studies conducted to assess safety. This is especially true with regard to doses administered, long-term consequences of exposure during sensitive periods in development, and types of end points examined. With regard to dose, if the mechanistic information concerning hormone action that we review here had been considered, the currently accepted practice of only testing very high doses to predict effects of doses thousands or even millions of times lower would have been recognized as inappropriate. The result would have been that doses of EDCs such as methoxychlor and bisphenol A far below those currently being described as safe would, in fact, have been predicted to produce biological responses, and much lower doses would have been tested. A recent dose range–finding study of the dietary estrogen genistein (*29*) has used a wide range of multiple doses including a low-dose range, and these studies illustrate the importance of this approach (*29,30*). On the basis of the information provided here, we propose that toxicological testing procedures incorporate a much wider dose range, take into account the heightened sensitivity and unique effects (some of which may not be apparent until adulthood) that can occur as a result of endocrine disruption in the fetus, and shift to measuring functional changes in organs (focusing on continuous variables), rather than low-frequency dichotomous variables such as malformations associated with acute toxicity.

Mechanisms of Estrogen Action Predict Low-Dose Effects of EEDCs

Although the mechanism of action of most toxicants is unknown, the mechanism of action for estrogens, including EEDCs, is already known in substantial detail; however, much remains to be learned. For an EEDC to exert a direct estrogenic effect in a cell, the cell must have estrogen receptors (whether the receptors are located in the nucleus, cytoplasm, or cell membrane). With regard to nuclear receptors, the most critical piece of information regarding the mechanism of action of an EEDC is defined by its binding affinity for the subtype of estrogen receptor (alpha or beta) present in the cell. Once affinity for the receptor is estimated, one can

immediately apply information from a vast literature concerning the interaction of estrogenic chemicals with receptors to understand a considerable amount about the mechanisms of action of the chemical. Understanding the mechanism of action for a toxicant allows the incorporation of this information into predicting appropriate doses to use in toxicological studies (*11*). In this section we will describe the relationship between dose, receptor occupancy, and responses, such as cell proliferation, after binding of E_2 to estrogen receptors (specifically, ER-α) in cultured human MCF-7 breast cancer cells. In a subsequent article (*31*), we will relate this information to the results of *in vivo* experiments showing that the bioactive concentration of E_2 in serum during development in mice and rats is very similar to the bioactive concentration that stimulates cell proliferation in human MCF-7 cells. This information will provide the basis for determining doses of EEDCs that produce effects similar to those caused by an increase in E_2 during development in mice, as well as effects caused by low doses of EEDCs administered at other times in life.

Lipophilic and hydrophilic hormones. Hormones do not act directly, but rather indirectly, through binding to specific receptor proteins. When these receptor proteins are occupied by hormone, they become the signal transduction system for inducing the hormonal response. Two basic transduction systems for hormones have been identified. Hydrophilic hormones, such as the hypothalamic and pituitary hormones, do not easily cross cell membranes, but instead bind to the extracellular domain of transmembrane receptors; binding of the hydrophilic hormone to the membrane-bound receptor results in activation of complex intracellular signaling pathways that can lead to rapid changes (in seconds) in cell function (*32*). The second transduction system is used by lipophilic hormones, including the sex steroids such as E_2, which are small (molecular weight of a few hundred daltons) lipophilic molecules that can diffuse into cells. These hormones bind to intracellular receptors and induce transcription of specific genes (a much slower process). These intracellular receptors act as ligand-dependent transcription factors and belong to the nuclear receptor superfamily that, in addition to estrogen receptors, includes receptors for triiodothyronine, retinoic acid, vitamin D_3, cortisol, androgens, progesterone, and aldosterone (*33–35*). In addition to acting via binding to nuclear receptors, there is now considerable evidence that estradiol interacts with transmembrane receptors to stimulate rapid responses in some cells (*36–39*).

Although hydrophilic and lipophilic hormones act through different receptor systems, both require receptor occupancy as a precursor to produce a response in target cells. There is a critical aspect of this issue with regard to the potential for species differences in the response to EEDCs. It is well known that the gene structure and ligand-binding properties of the classical estrogen receptor (ER-α) have been highly conserved (that is, have experienced relatively little change) among vertebrates separated for up to 300 million years of evolution. Thus, the binding of an estrogenic chemical to ER-α in fish, amphibians, reptiles, birds, and mammals (including humans) shows relatively little difference (*40–42*). Binding to the receptor is the initiating step in endocrine disruption by estrogenic chemicals. It is during events prior to and subsequent to receptor binding that species and tissue differences emerge in terms of differences in absorption and metabolism, as well as specific genes regulated by estrogen. There are also tissue-specific components of the transcriptional apparatus (receptor coregulators) involved in determining which genes are regulated by ligand-activated receptors (*43,44*).

Even within a specific tissue in a single organism, there are developmental changes in the genes regulated by specific hormones (*45*). In addition, with regard to unique developmental effects of EEDCs, there is evidence that the functioning of enzyme systems involved in metabolizing endogenous steroids, drugs, and EDCs differs during fetal life and in adulthood (*46,47*). Regardless of these species, tissue, and life stage differences, if a chemical can bind to estrogen receptors in fish, the evidence is that it will also bind to estrogen receptors in humans and other vertebrates. Until there are data to the contrary, one would expect that the possibility of endocrine disruption occurring in humans can be predicted by assessing binding of an estrogenic chemical to estrogen receptors in any vertebrate. With regard to estrogenic EDCs and their potential for disrupting embryonic development, the similarity between vertebrates with regard to the mechanism of action of estrogenic chemicals that act via binding to estrogen receptors argues strongly for the continued use of animal models to assess human risk (*40–42*). Within the field of comparative endocrinology, the finding of highly conserved molecules such as estradiol and the estrogen-receptor complex has led to the general assumption that it is the specific uses to which hormones and their receptors have been put that has changed throughout the evolution of multicellular organisms, not the hormones and receptors themselves (*48*).

Relationship between hormone concentration and receptor occupancy. There are four properties of receptors that predict responses to estrogen and other hormones. The first property is affinity of the ligand for the receptor, which must be high enough for a sufficient number of receptors to be occupied at the concentrations at which the natural or manmade estrogen is present. The second property is saturability. As binding of the hormone to its receptor shows the property of saturation, there is no further increase in number of occupied receptors as a function of increase in dose once all receptors are occupied. Likewise, biological responses to hormones saturate; interestingly, saturation of response frequently occurs considerably below 100% receptor occupancy in what has been traditionally termed "spare receptor" observations (we cover this in more detail below). The third property is ligand specificity, as all compounds that show hormonal activity (or receptor-mediated antihormonal activity) must bind to the hormone receptor, whereas compounds that at a given concentration do not have hormonal activity (or antihormonal activity) do not bind to the receptor. The fourth property is tissue specificity of receptor distribution. Tissues that respond to the presence of a hormone must have receptors for the hormone. If a given cell does not have receptors for the hormone, that hormone is "invisible" to that cell, and the cell can show no primary response to the hormone, although indirect (secondary) effects may be observed. At concentrations above those within a normal physiological range, hormones may bind to receptors for other hormones. For example, E_2 binds to androgen receptors at concentrations approximately 100 times higher than the concentrations required to occupy estrogen receptors and induce responses (*49*). The biological consequences of "cross-talk" with other receptors at high doses of a ligand have not been well characterized for most systems, but this likely contributes to qualitatively different effects at low (physiological) and high (toxicological) doses. We discuss dose–response issues in more detail below.

Receptor occupancy is directly linked to responses, and responses to either a natural estrogen or an EEDC are brought about in relation to the number of occupied receptors. Above 10% receptor occupancy, and particularly above 50% receptor occupancy, which mathematically defines the K_d (the dissociation constant from the law of mass action applied to receptor–ligand binding kinetics) of the binding of hormone and receptor, receptor occupancy is never determined to be linear in relation to hormone concentration. Using a less stringent definition of linearity, proportionality between receptor occupancy and hormone concentration is observed below 10% receptor occupancy, and the relationship

between receptor occupancy and response (such as cell proliferation) is also only proportional below 10% receptor occupancy. We will thus consider that the relationship between receptor occupancy and hormone concentration, as well as between receptor occupancy and response, are approximately linear up to 10% receptor occupancy. At concentrations above the K_d, saturation of response occurs first, and then at higher concentrations, saturation of receptors is observed.

An example based on administration of E_2 to MCF-7 cells of the relationship between hormone concentration, receptor occupancy, and a response (cell proliferation) is presented in Table 1. The data in Table 1 show that as hormone concentration increases by factors of 10, receptor occupancy typically increases by the following relationship: *a*) If the hormone concentration is 1% of its K_d (% K_d: Table 1, middle column), the number of receptors occupied is also approximately 1% of total receptors. *b*) With a 10-fold increase in hormone concentration to 10% of the K_d, receptor occupancy increases to approximately 9%. *c*) The next 10-fold increase in hormone concentration is to the K_d and leads to 50% receptor occupancy. *d*) With another 10-fold increase in hormone concentration, 91% of receptors are occupied. *e*) Finally, another 10-fold increase in hormone concentration only leads to a small increase, from 91 to 99% receptor occupancy.

The importance of the data in Table 1 is that while at the lowest concentration referenced, a 10-fold increase in hormone leads to a 9-fold increase in receptor occupancy (from 1 to 9%), between the highest doses, a 10-fold increase in hormone concentration only leads to less than a 1.1-fold increase in receptor occupancy (from 91 to 99%). The practical result is that while at hormone concentrations below 10% receptor occupancy (10-fold below the K_d) receptor occupancy is close to proportional to hormone concentration, this is

not the case above this concentration. The view of the previously mentioned "spare receptor" hypothesis from this perspective is that a system such as this, which we assume evolved to be responsive to small changes in ligand concentration, could only operate in a portion of the binding range that was nearly linear (below 10% receptor occupancy), thus leading to the observation that there appeared to be receptors that were in surplus over those needed for responses, hence spare receptors. Surplus hormone receptors over the number of occupied receptors required for response (*50,51*) was recognized early in the study of the steroid receptors and steroid receptor-mediated action (*52*).

At the dose ranges of EEDCs used in current toxicity testing, chemicals are likely to be present within target cells at concentrations many orders of magnitude above their K_d for estrogen receptors. Within this dose range, changes in hormone concentration cannot have a detectable effect on receptor occupancy, because all receptors are saturated at 100% and no additional binding, which is required to result in an increase in response, can be observed. No primary hormonal effects can be observed in response to changes within this high-dose range, but only secondary effects not mediated by estrogen receptors.

Relationship between receptor occupancy and response. It is sometimes erroneously assumed that hormones act *in vivo* at their K_d (50% receptor occupancy). With a few exceptions, the physiological ranges for natural hormones (more specifically, the free, bioactive fraction (*7,8*) of the total circulating) are typically below the K_d. A biological basis for this observation may be that if natural hormone concentrations were at or above the K_d and thus near receptor saturation, even quite large changes in hormone concentrations would result in only a small change in occupied

receptors. This type of system would be relatively insensitive to changes in hormone concentrations and would require dramatic changes in hormone concentrations to elicit changes in response. Because very small changes in hormone concentrations, for example, a 50% increase, were associated with changes in responses in animal studies, it appears that the working range for hormones must be well below the K_d, and indeed the animal data support this hypothesis (*19,23,53,54*).

In many biological systems, saturation of response is observed well below saturation of receptors, and saturation of specific responses may even occur below the K_d. As indicated above, the spare receptor hypothesis is the term applied to this kind of observation (*55–58*) and has been described in detail, particularly on the basis of observations with transmembrane receptors. Specifically, transmembrane receptors show a much greater percent inhibition as the dose of ligand increases (~ 90%) than do nuclear receptors that are members of the nuclear receptor superfamily (~ 50%) (*59,60*). The potential contribution to nonmonotonic dose–response curves of the loss of receptors as dose of ligand increases is covered below.

There is only near-linearity of dose and occupancy up to a dose that results in 10% of receptors being occupied (below 0.01 nM for E_2), and the near-linear range between dose and response is even more restricted (shifted to the left). For example, although the K_d for E_2 binding to ER-α is approximately 0.1 nM, a significant increase in proliferation of MCF-7 estrogen-responsive breast cancer cells is seen with addition of 0.0004 nM E_2 to estrogen-free medium. Half-maximal proliferation is seen at 0.001 nM E_2, and near-maximum proliferation is seen between 0.01 and 0.1 nM. Thus, almost 91% of maximal cell proliferation is observed at a concentration 10-fold below the K_d, at a ligand concentration approximately 100-fold lower than 91% of receptor saturation (Table 1). The relationship between hormone response and receptor occupancy is not limited to permanent cell lines and has also been described for a number of estrogenic chemicals in primary rat uterine cells, where, as above, saturation of response occurs before saturation of receptor occupancy (*61*).

Interestingly, for E_2, the dose required to induce different responses in the same cell is not the same. For example, in GH$_3$ rat pituitary cells *in vitro*, proliferation of cells is half maximal at an E_2 concentration between 0.001 and 0.01 nM, whereas synthesis of prolactin is half-maximally induced at 0.1 nM (*62*). Progesterone receptors in MCF-7 cells require roughly 10 times more E_2 for induction relative to proliferation (*63*),

Table 1. Mathematical calculations of receptor occupancy versus hormone concentration for an example where the K_d = 0.1 nM.[a]

| | Estradiol concentration | | | Receptors | Cell proliferation, |
	(nM)	(ng/mL)[b]	Percent of K_d	occupied (%)[c]	(% of maximum response)[d]
	10	2.72	10,000	99	100
	1	0.272	1,000	91	100
K_d	0.1	0.0272	100	50	99
	0.01	0.00272	10	9	91
PR[e]	0.001	0.000272	1	1	50
	0.0001	0.0000272	0.1	0.1	9

[a]This K_d was chosen because it represents a midrange value commonly measured for the binding of estradiol to the estrogen receptor. [b]ng/mL = ng/g = µg/kg = ppb. [c]The mathematical relationship described here between ligand concentration and receptor occupancy applies to receptor–ligand interactions for all hormones, although each ligand will have a unique K_d associated with 50% receptor occupancy. [d]This column in the table represents a physiological response, in this example, the estrogen-dependent proliferation of MCF-7 human breast cancer cells. [e]The physiological range (PR), occurring at an EC$_{50}$ of 1 pM for cell proliferation, was determined from both *in vitro* stimulation of cell proliferation at 1 pM = 0.27 pg/mL (Figure 1) and free E_2 at EC$_{50}$ = 0.2 pg/mL (*54*), and *in vivo* studies where free E_2 = 0.21 to 0.54 pg/mL (*23,53*) and is within the range of 1% receptor occupancy. Note that here, as in many systems, response saturates (e.g., 99% response at 0.1 nM and 50% receptor occupancy) well before receptor occupancy saturates (e.g., 10 nM and 99% receptor occupancy).

similar to induction of prolactin in GH_3 cells. This relationship demonstrates that the activation of different genes requires different numbers of receptors to be occupied. Importantly, both of these responses saturate at a percent receptor occupancy far below receptor saturation, that is, spare receptor kinetics still apply.

Nonmonotonic Dose Response to Estrogens

Nonmonotonic (inverted-U) dose–response relationships: **in** vitro *effects of low and high doses of estrogens.* Responses to hormones, including estrogens, saturate as does receptor occupancy, and therefore cannot be linear as a function of an increase in dose within the high-dose range. Further, for many responses to a wide range of concentrations, across many powers of 10-fold, the dose–response relationship is nonmonotonic as well, with response decreasing at doses above those that initially reach a level of saturation. There are a number of published examples of this *in vivo* and *in vitro*. In male mouse fetuses, a very small increase in E_2 or a physiologically equivalent increase in estrogenic activity by an estrogenic chemical such as diethylstilbestrol (DES) resulted in prostate enlargement detected later in life (*23,64–66*). In marked contrast to these findings, consistent with numerous prior studies, administration of much higher doses of either natural or man-made estrogens during the prenatal or neonatal period of prostate development caused a reduction in prostate size relative to untreated males (*23,64,66–69*).

The lower doses of DES that resulted in an increase in prostate size (*23,64,65*) were predicted to increase total serum estrogenic activity within a physiological range, based on studies of the free concentration of DES in serum (*5*) and transplacental transport of radiolabeled DES in pregnant mice (*47*). Specifically, a low dose of DES of 0.02 μg/kg/day administered to pregnant mice was predicted to lead to an increase in free, bioavailable DES in the fetus that falls within the physiological dose range of free, bioavailable estrogenic activity during normal fetal development (*54*); this exposure led to the prostate enlargement response (*23*). This dose of DES, in the physiological range of estrogenic activity, falls within the low-dose range of exposure. In contrast, in the same studies, a 10,000-times higher dose of DES (200 μg/kg/day) resulted in gross abnormalities in the reproductive organs, including a marked reduction in prostate size (*23,64*). This dose of DES therefore falls within the toxicological dose range and represents a high-dose range of exposure.

There are many additional examples of nonmonotonic dose–response relationships.

For example, it has been known for some time that there are adverse effects at low and high doses, on either side of an optimum physiological range for normal development, for other ligands that bind to receptors in the steroid receptor superfamily, such as vitamin A and thyroid hormone. It is difficult to compile a literature focusing on inverted-U dose–response curves, as these types of dose–response functions are common in endocrine studies and are often not identified in titles or abstracts as a noteworthy finding. Among those that have been reported, nonmonotonic dose–response curves can occur at several levels of organization, ranging from the biochemical based on *in vitro* studies (*28,54,62,70–75*) to the organ or system level based on *in vivo* studies (*23,60,66,76–82*).

MCF-7 cell **in** vitro *model for inverted-U endocrine dose responses.* MCF-7 human breast cancer cells (*83*) are a permanent cell line that contains estrogen receptors. These cells have retained estrogen responsiveness for a sustained period of continuous cell culture and show estrogen-dependent stimulation of cell proliferation by natural and xenobiotic estrogens (*84–86*). In addition, the same chemicals that stimulate growth at lower concentrations can slow MCF-7 cell growth at higher concentrations (*72,73,* for example) and inhibit growth by acute cytotoxicity at high concentrations in the micromolar (ppm) range (Figure 1A). The dose–response range required to observe these dual effects by natural and xenobiotic estrogens can be very wide, spanning 1,000- to 100,000-fold for bisphenol A and octylphenol up to and exceeding 100 million-fold for DES and E_2 (Figure 1A) (*54*). These cell responses in tissue culture to very wide concentration ranges create a type of inverted-U dose response that can be used as an *in vitro* model.
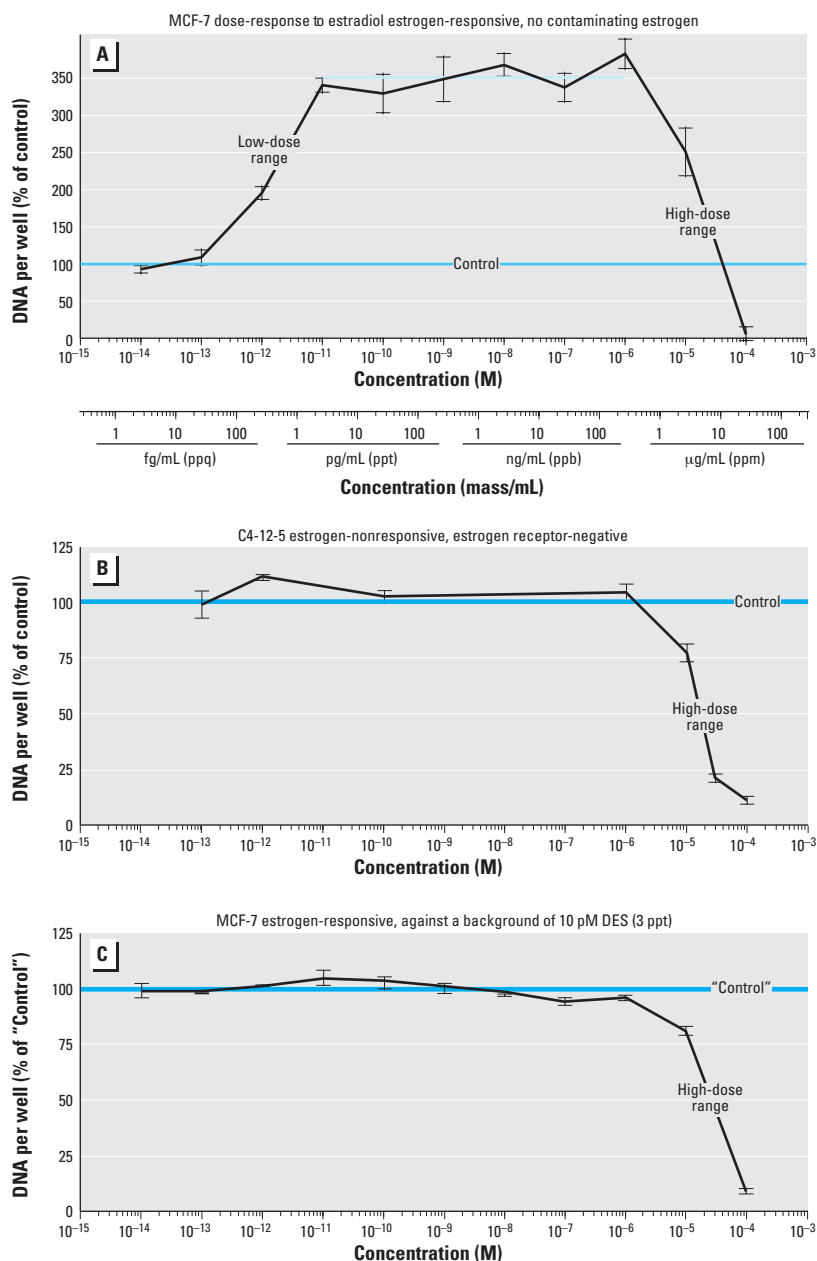
Low-dose stimulation of cell proliferation followed by high-dose cytotoxicity is illustrated in Figure 1A in estrogen-responsive MCF-7 cells. Growth was stimulated by E_2 in the concentration range from 0.1 pM to 100 pM. This low part-per-trillion (ppt) range is the physiological range for E_2 determined in studies of free estradiol in rats and mice from fetal life through adulthood (*23,53*); this is the low-dose range indicated in the figure. The cell growth response was saturated and did not increase with increased hormone concentration from 100 pM through to 1 μM. Above 1 μM (the high-dose range indicated in Figure 1A), however, cytotoxicity reduced the cell growth response to E_2, with inhibition of response to below the control level at 100 μM. The physiological dose range for E_2 action was approximately 100 million times lower (0.1–1.0 pg/mL culture medium; 0.1–1.0 ppt; the low-dose range) than the toxicological dose range that results in acute

toxicity (which occurred at 10–100 μg/mL culture medium, or 10–100 parts per million (ppm); the high-dose range).

The acute cytotoxicity of E_2 in cultured MCF-7 cells did not depend on the presence of estrogen receptors. We have derived clonal cell lines from MCF-7, including cell line C4-12-5, which no longer express estrogen receptors and are completely estrogen nonresponsive and proliferate in the absence or presence of estrogen (*87*); re-expression of estrogen receptors in these clonal cell lines can lead to recovery of estrogen-dependent cell proliferation (*88*). As stated above, without receptors, these C4-12-5 cells are "blind" to the presence of the hormone. Cytotoxicity occurred within the same high-dose range of E_2 in the clonal C4-12-5 cells (derived from MCF-7 cells) that do not express estrogen receptors (Figure 1B) as in the parental MCF-7 cell (Figure 1A); however, the low-dose range effects to stimulate cell proliferation could not be demonstrated in the estrogen-nonresponsive cells (Figure 1B). These estrogen receptor–negative variants proliferate in the absence of estrogen, and in the absence of estrogen receptors, low doses of estrogen are the incapable of eliciting effects in these cells.

Importantly, stimulatory effects of estradiol in the low-dose range could also be obliterated in estrogen-responsive MCF-7 cells by the presence of a background or contaminating level of another estrogen such as DES (Figure 1C). Background estrogenic activity due to contamination by addition of DES at only 3 ppt (10 pM DES) completely obscured the low-dose range effects of E_2 on cell proliferation, but did not impair detection of the high-dose range, toxic effects observed above 1 μM E_2 (above 0.3 ppm; Figure 1C). Although this background contamination was created experimentally with 3 ppt DES, the presence of contaminating estrogens in the phenol red pH indicator dye included in most tissue culture media limited the recognition of and acceptance of estrogen-dependent cell proliferation by MCF-7 cells until 1985 (*63,89,90*). Unrecognized estrogenic contamination may interfere with any study, *in vitro* or *in vivo*, unless this possibility is excluded by the performance of appropriate controls.

Overall, both low-dose and high-dose effects by E_2 were observed in MCF-7 cells (Figure 1A). Demonstration *in vitro* of the low-dose effects of E_2, but not the high-dose effects, was obscured by testing in the absence of estrogen receptors (Figure 1B) or by testing in the presence of a low level of a contaminating estrogen (Figure 1C). The objective of appropriate control procedures discussed below is to allow one to distinguish whether negative results are due to an actual lack of activity of a compound, or rather due to

Figure 1. MCF-7 human breast cancer cell proliferation at low through high doses. (*A*) Stimulation of MCF-7 cell proliferation in estrogen-free medium by E_2 up to a dose at which E_2 is cytotoxic. Control line indicates estrogen-free medium. (*B*) Lack of response to E_2 by estrogen receptor–negative, estrogen-nonresponsive C4-12-5 cells derived from MCF-7 cells, in estrogen-free medium. Proliferation is independent of dose up to a dose that is cytotoxic. Control line indicates estrogen-free medium. (*C*) Lack of response to E_2 by estrogen-responsive MCF-7 cells to E_2 due to the presence of a background of 10 pM DES (3 ppt) added to the estrogen-free medium to mimic contamination and present in all dose groups. Proliferation is independent of dose up to a dose that is cytotoxic. "Control" line indicates estrogen-free medium plus the 3 ppt DES background. High-dose effects of E_2 are seen in *A*, *B*, and *C*, whereas low-dose effects are visible only in *A*, the dose response performed in estrogen-responsive MCF-7 cells examined in the absence of detectable background estrogen. In *A* the concentration range is shown simultaneously as molarity (M), as mass per milliliter, and as mass ratio (ppq: parts per quadrillion). Half-maximal stimulation of proliferative response occurred at approximately 1 pM E_2 in medium (0.272 ppt) in the low-dose range, whereas inhibition was induced at micromolar concentrations in the high-dose range. Estrogen-dependent cell proliferation and cytotoxicity were determined exactly as described in prior publications (*72,138,139*). Briefly, the very wide dose responses (*54*) were performed for E_2 by incubating the indicated cells in 24-well plates for 4 days in culture medium (phenol red-free medium, charcoal-stripped serum) plus E_2 at concentrations from 0.01 or 0.1 pM through 100 μM, with daily medium changes. Proliferation was determined by DNA assay at the end of the incubation, and results were expressed as percent of the control; control 100% values were 1.0, 3.7, and 5.5 μg DNA/well for *A*, *B*, and *C*, respectively. Values are the mean and standard error of measurements in replicate wells; *n* = 3.

unresponsiveness of a tissue, or contamination that is obscuring all responses.

Importance of Valid Positive and Negative Controls for Endocrine Responses

Although E_2 was clearly capable of exerting effects in the physiological, low-dose range (Figure 1A), demonstration of the low-dose effects was system dependent. Importantly, the inability to detect the low-dose effects of E_2 in Figure 1B and C was due to the experimental conditions and was not due to the absence of estrogenic activity by E_2 itself or due to an absence of the potential to show estrogen responses in uncontaminated MCF-7 cells with estrogen receptors. This conclusion will only be realized if specific positive and negative controls are included to allow for the correct interpretation of results. Without evaluation of the appropriate negative and positive controls, it is not valid to conclude that a chemical lacks low-dose estrogenic activity simply because it fails in assays that may be represented by the conditions in Figure 1B, where the test system is unresponsive, or in Figure 1C, where the test system is responsive but contaminated. In these examples, if the controls were omitted (or ignored), E_2 itself in its own physiological concentration range (as well as any other estrogenic chemical) would be wrongly identified as inactive in two out of three assay systems.

The positive and negative controls. Each panel of Figure 2 illustrates specific positive and negative controls relevant to each experiment in Figure 1; this includes use of an antiestrogen (AE), which is a competitive antagonist of estrogen action (*90,91*). These controls allow one to interpret the absence of detectable low-dose effects in Figure 1B and C, either as the lack of cellular responsiveness to estrogen generally, or as the presence of a masking estrogenic contamination.

A concentration of E_2 that saturates the proliferative response in the low-dose range is used as a positive control. This treatment demonstrates the presence of estrogen responsiveness in the assay relative to the negative control that is estrogen free (Figure 2A). An antiestrogen such as raloxifene or ICI 182,780 is used to confirm a baseline for estrogen receptor activation in the negative control treatments; there should be no reduction in response by the antiestrogen because no receptor-mediated responses have been initiated in the absence of estrogen (Figure 2A). If an inhibition of response is observed in the presence of antiestrogen with no intentional addition of estrogen (Figure 2C), then the conclusion is that estrogenic stimulation is occurring in the system from contamination. Another important issue is that when high

doses of a chemical are being examined for estrogenic activity, after demonstrating that addition of antiestrogen inhibits the response, competitive reversal of this inhibition of response by co-incubation with an excess of estrogen (for example, 10 nM E_2) (Figure 2C) added with the antiestrogen is in turn used to distinguish antiestrogenic activity from toxicity due to the combined action of the test chemical and antiestrogen. This last step is the final element in discriminating between antiestrogenic activity of a compound and acute toxicity (*91*).

Interpretation of the controls. In Figure 2A, the positive control E_2 at 100 pM stimulated response, and of equal importance, exposure to an antiestrogen at 100 nM (AE) in the absence of any E_2 did not reduce the proliferative response below the control level of growth. The interpretation drawn from the controls in Figure 2A is that *a*) the MCF-7 cell system was estrogen responsive, and importantly, *b*) under the negative control growth conditions, there was no detectable background estrogenic contamination. In this system, both low- and high-dose effects of E_2 were observed (Figure 1A).

Figure 2B shows the same controls applied to C4-12-5 cells, a clonal variant of MCF-7 cells that lacks estrogen receptors. Positive control E_2 did not stimulate cell proliferation, and furthermore, the antiestrogen did not inhibit proliferation of the C4-12-5 cells (Figure 2B). The interpretation of these controls is that the C4-12-5 cells are estrogen nonresponsive, showing responses neither to low-dose estrogen nor to antiestrogen. Importantly, even though the cells were not responsive in the low-dose range of exposure, the proliferation of the estrogen receptor–negative C4-12-5 cells was still inhibited by E_2 in the same high-dose range that inhibited proliferation of the estrogen-responsive

MCF-7 cells (Figure 1B); only high-dose toxic effects of E_2 were observed, and these are clearly not mediated by nuclear estrogen receptors.

Finally, as can be seen in Figure 2C, even in the same MCF-7 cells that were responsive within the low-dose range in the full dose response (Figure 1A), a very slight background level (contamination) of an estrogenic chemical was sufficient to eliminate detection of the low-dose stimulating effect of estradiol, if treatments are compared only with a negative control that is presumed, without testing, to be estrogen-free. In Figure 2C, it can be seen that the positive control E_2 added to the "Control" medium did not stimulate further growth, and without further information, the system would be incorrectly interpreted as nonresponsive in the low-dose range (Figure 1C). Incubating cells in the "Control" medium plus antiestrogen, however, inhibited cell proliferation, indicating the potential for an estrogen receptor–driven stimulation of cell growth. Competitive reversal of the antiestrogen effect with a surplus of E_2, indicated by the light blue bar in Figure 2C, confirmed that the inhibition was antiestrogenic and not due to nonspecific toxicity.

The interpretation of the dose–response experiment (Figure 1C) is now that the MCF-7 cells were fully responsive to E_2 in the low-dose range but were already maximally stimulated by background estrogenic contamination in the presumed negative control. DES at only 3 ppt was sufficient to fully mask the low-dose effects of E_2; only high-dose, toxic effects of E_2 could be observed (Figure 1C). In the absence of the appropriate controls, or if the controls were misinterpreted or ignored, E_2 itself, an unquestioned estrogen, would be incorrectly identified from Figure 1B or C as an inactive chemical in the low-dose range (its physiological range), but

not in the high-dose range, with respect to estrogen-dependent cell proliferation.

Implications. Positive and negative controls such as those described above are needed for adequate interpretation of EEDCs in the context of low-dose effects, nonlinear saturation of response, and reversal of response that can generate a nonmonotonic dose–response relationship. Of great importance, research on low-dose effects requires a new level of understanding of ambient estrogenic activities, and controls are absolutely required to assess these activities experimentally. Ambient estrogenic activities for *in vitro* studies consist of contaminants in air, media, or plastic, whereas *in vivo*, ambient estrogenic activities could include variable background levels of endogenous hormone as well as activity from a variety of external sources such as feeds. Appropriate controls are not typically included in toxicological tests conducted for regulatory purposes.

Relevant to this discussion are findings that the concentration of E_2 in cell culture medium that results in proliferation at approximately 50% of maximum is very close to the concentrations of free serum E_2 during development in mouse and rat fetuses (0.2–0.3 pg/mL) (*23,53*). Even slight variations in the levels of estradiol have been related to differences in the course of development in mice, rats, and gerbils (*19,23,92–94*). For example, we experimentally increased the free serum estradiol concentration in male mouse fetuses from the control level of 0.2–0.3 pg/mL (via a Silastic capsule containing estradiol implanted in the pregnant dam). This 0.1 pg/mL increase in free serum estradiol resulted in a marked change in development of the urogenital system in the male fetuses (*23*).

Taken together, these findings indicate a very high degree of sensitivity (well below a part per trillion) of both human and rodent tissues to E_2 both *in vitro* and *in vivo*. This high degree of sensitivity to very small perturbations in E_2 provides the basis for concern about the use of appropriate controls to test for background contamination by estrogenic chemicals in studies with animals. Estrogenic contamination can occur via the food (*95,96*), caging (*97*), or bedding (*98*), as well as in studies with cultured tissue via components of media (*63*), or plastic tubes and cultureware (*99,100*). Although there have been studies that have examined the effects of components of diets on steroid synthesis in humans (*101*), this issue has not been a focus of toxicological studies involving EEDCs. Our recent findings show that in mice maintained on different types of commercial animal feeds during pregnancy, serum estradiol levels in fetuses are markedly different (unpublished observation).
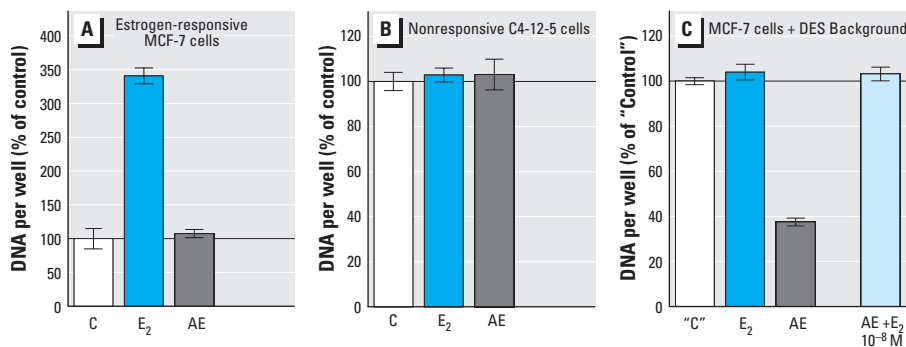
Figure 2. The relevant controls for the dose responses of Figure 1A–C. (*A*) Estrogen-responsive MCF-7 cells in estrogen-free medium. (*B*) Estrogen receptor–negative, estrogen-nonresponsive C4-12-5 cells derived from MCF-7 cells, in estrogen-free medium. (*C*) Controls. Estrogen-responsive MCF-7 cells in the presence of a background of 10 pM DES (3 ppt) added to the estrogen-free medium and present in all media and treatments including controls. Abbreviations: AE, 100 nM antiestrogen (raloxifene or ICI 182,780); AE + E_2 10^{-8} M, 100 nM antiestrogen (raloxifene or ICI 182,780) plus E_2 at 10^{-8} M; C, control estrogen-free medium; "C", estrogen-free medium plus 3 ppt DES; E_2, 100 pM E_2. Values are the mean and standard error of measurements in replicate wells; n = 3.

Endocrine Mechanisms Mediating Errors in Estimating Low-Dose Responses from High-Dose Studies

The default risk assessment assumes linearity of dose response. Major errors in assessing risk can be made when linearity of response and the preceding receptor occupancy is assumed across the entire dose range, which is the current assumption used in risk assessment. Although almost everyone involved in risk assessment recognizes that the assumption of linearity is invalid (even for cancer) (*102*), the application of safety factors that results in linear extrapolation across a wide dose range remains the default for current risk assessment. For example, safety factors (used to calculate a "safe" dose for human exposure) of 10-fold each are often used to estimate each of the following: human risk from animal studies, to account for variability within the human population, when the lowest dose tested results in an adverse response (termed the LOEL), and most recently, as an added safety factor for protecting children. Application of these 10-fold safety factors results in linear extrapolation from a LOEL or NOEL (determined by testing a few very high
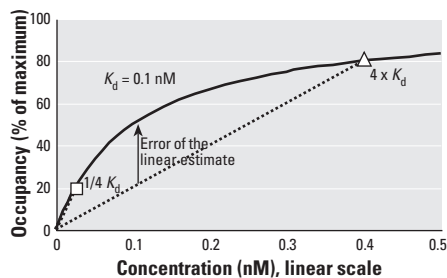
Figure 3. Error in predicting actual receptor occupancy based on linear estimation applied to a saturating test dose. Receptor occupancy (solid line) is graphed against a linear scale of ligand concentration from 0 to 0.5 nM, where the K_d for ligand binding = 0.1 nM. Linear estimations to zero concentration (dotted lines) are shown originating from single measurements at two test doses, one below the K_d (square point of origin, at 1/4 K_d = 0.025 nM) and one above the K_d (round point of origin, at 4 × K_d = 0.4 nM). This assumes no background-contaminating estrogenic activity from either endogenous or exogenous sources other than the chemical being tested. Where the test dose used as the origin of the linear estimation is below the K_d, the linear estimation is very close to actual occupancy. Where the lowest test dose used as the origin of the linear estimation to zero dose is above the K_d, the linear estimation deviates substantially from actual receptor occupancy, indicated as "Error of the linear estimate." The fold-underestimate of occupancy, and therefore underestimate of response for receptor-mediated events, increases as the origin of measurement increases above the K_d and is calculated in Table 2 for a number of EEDCs where the origin is 10,000-fold above the K_d, which could not be shown to scale on this figure.

doses) to arrive at a safe dose. Thus, in practice, the model upon which risk assessment is practiced assumes that this linear extrapolation procedure is valid and will result in calculation of a dose that is safe for humans exposure.

Error of a linear estimate relative to actual receptor occupancy. When a linear extrapolation model is applied to a saturating, receptor-mediated response to estimate the risk of an adverse response, this linear estimate results in a false assumption concerning the actual reduction in response (and thus risk) that occurs with decreasing dose. The error we refer to is illustrated in the simplified graphic example in Figure 3. The use of 10-fold safety factors to estimate occupancy of receptors (and subsequent responses) on the basis of results from animal studies assumes a linear relationship between dose and response, even though this may not be overtly acknowledged. We will initially discuss the theory behind the error that occurs on the basis of extrapolation from very high to very low doses assuming a linear function and then provide examples from actual data for DES, genistein, and bisphenol A obtained from *in vitro* studies using MCF-7 cells. The error we refer to here based on receptor occupancy is in reality lower than the error based on actual responses, as responses can saturate at lower concentrations than those required to achieve receptor saturation (Table 1). Therefore, our calculations of error in Table 2 are, in fact, conservative.

For simplicity here, in the discussion below we will not discriminate between dose administered and dose at the estrogen receptor in target cells and will simply refer here to a test dose. The reason for this is that for *in vitro* studies conducted in serum-free medium, the administered dose and the dose available to bind to estrogen receptors are very similar (*4*). *In vivo* this is obviously not the case due to absorption, metabolism, clearance, plasma binding, etc., all of which are far more complicated to study in developing fetuses than in adults (*54*). It is nonetheless the basis of modern endocrinology that a dose at target does exist, whether or not it can be easily determined, and that this dose determines the response and its magnitude relative to the receptor occupancy it can generate. Our discussion here is meant to apply to the dose at target.

It is important to note that during fetal and early postnatal life, the pharmacokinetics of chemicals and drugs are markedly different relative to adulthood. In addition, pregnant and nonpregnant females also differ in this regard. Data from studies with adult animals thus cannot be used to predict the pharmacokinetics of chemicals in pregnant females and fetuses (*16,103,104*). Thus, evidence that a

particular chemical is cleared rapidly in a nonpregnant adult cannot be used to discount the possibility of achieving a much higher dose at target in fetuses and neonates (*46*). Unfortunately, for most chemicals, there are no pharmacokinetic data and thus no basis for predicting dose at target for the most susceptible subpopulation: pregnant females and their fetuses.

The test dose for purposes of our discussion here is a high dose administered in toxicological experiments that is used to predict responses at much lower doses. As shown in Table 1 and Figure 3, the relationship between hormone concentration and receptor occupancy is approximately linear at low receptor occupancy (Figure 3, test dose example at 1/4 K_d). As the test dose exceeds the range of approximate linearity, for example, a test dose at 80% receptor occupancy (Figure 3 at 4 × K_d), the linear model (linear extrapolation from test dose to zero dose) will clearly underestimate actual receptor occupancy and will thus underestimate the actual responses that would occur at lower doses (Figure 3, arrow labeled "error of the linear estimate"). This deviation from linearity has great importance with regard to the strategy of using very high doses of EEDCs in toxicological studies and extrapolating to predict responses at much lower doses.

Table 2 presents specific quantitative information for a number of chemicals. With regard to understanding the error that can occur in estimating the potential for low-dose responses on the basis of extrapolating from high to low doses across a wide dose range, we will describe an *in vitro* experiment in which bisphenol A was examined in MCF-7 cells as an example. For our example here, the test dose for bisphenol A (shown in Table 2, row 1) is 844,000 ppb (844 mg/kg), chosen for its relation to K_d for ER-α and for proximity to test doses administered in prior *in vivo* toxicological studies of bisphenol A (again, using this as the dose at target) (*14*). Under the assumption that the test dose of 844,000 ppb is within a linear response range and therefore within a linear receptor occupancy range for direct hormonal effects, reducing the dose by 50% (to a dose of 422,000 ppb) would lead to the prediction that receptor occupancy would also drop by 50% (Table 2, row 2). In fact, because the test concentration is so much higher than the K_d, virtually no actual change in receptor occupancy occurs (the actual change in receptor binding in MCF-7 cells would be from 99.99 to 99.98% with this 50% reduction in dose), and no change in response mediated by these receptors would be detected.

When one administers a dose of bisphenol A that is 10-fold lower than the test dose (84,400 ppb or 84.4 mg/kg), receptor

occupancy still only drops from 99.98% to 99.90% in MCF-7 cells (Table 2, row 3), and again, this change is not likely to be a detectable decrease in binding. This decrease in dose also would thus not be likely to lead to a detectable decrease in response mediated by these receptors. Even at a dose of 844 ppb, which is a dose 1,000 times lower than the test dose of 844,000 ppb, 90.91% of receptors will still be occupied in MCF-7 cells. On the basis of the information presented in Table 1, one would not expect to approach the region of maximum detectability for a change in response until doses that resulted in less than 50% receptor occupancy (the K_d) were reached. In addition, on the basis of results in Table 1, it is apparent that responses can occur at concentrations in the range of 1% receptor occupancy. As shown in Table 2, at the concentration of bisphenol A that results in approximately 1% receptor occupancy (0.844 ppb), or 1 million times lower than our initial test dose, the linear extrapolation model would have predicted negligible receptor binding, and thus no response, based on a test dose of 844,000 ppb.

Nonmonotonic dose–response curve, response to endogenous hormone, and an assumed threshold dose all increase the magnitude of the error of a linear estimate. Our calculations are based on receptor occupancy, which is a physical chemical parameter subject to less between-species variation and greater precision of measurement than is the measurement of response. Cellular responses, however, occur at doses associated with very low receptor occupancy: the cell in essence amplifies the receptor signal. Therefore, use of receptor occupancy is in fact conservative relative to the ultimate physiological responses on which risk assessment would be based. For example, if these calculations were based on the EC_{50} (effective concentration 50%; 50% response) for a specific cell response such as cell proliferation that is 10- to 100-fold lower than the K_d (Table 1), then the underestimate of the potential for a response would be 10- to 100-fold higher, or

up to 1,000,000-fold, instead of the 10,000-fold in this example.

Incorporation of additional features of real-world risk assessment will further add to the error, not reduce it. A nonmonotonic dose response, specifically the inverted U, can substantially increase the error of the linear estimate based on a high-dose reference point (that is well below the maximum response because of the inverted-U dose–response curve). This is illustrated qualitatively in Figure 4A, where the error of the linear estimate for response is compared with that for an inverted-U dose response from a reference point above the dose that results in the maximum response. To avoid the possibility of this type of error, it is necessary to examine a much wider range of doses than is typical in toxicological studies involving animals.

Finally, as illustrated in Figure 4B, the default risk assessment applied to EEDCs assumes the existence of a threshold. But when xenoestrogen activity is added to a natural system that is already responding to endogenous estrogen such as estradiol, any threshold in estrogenic response must already be exceeded by the endogenous hormone. This absence of a threshold in response to exogenous estrogen has been experimentally confirmed in an experiment concerning the regulation by estrogen of sex determination in reptiles (*22*). The assumption of no response up to an assumed threshold above the zero EEDC dose, when this is not the case, will result in a great, potentially infinite error if linear extrapolation is used instead of actually determining the shape of the dose–response curve (Figure 4B).

Figure 4B also depicts the error associated with examining a test chemical with estrogenic activity, such as bisphenol A, that adds to an existing background level of endogenous estradiol, which is variable because of endogenous and exogenous factors (*19*). Variation in endogenous estradiol is related to variation in phenotype in rodents (*105*), supporting the hypothesis that endogenous estrogen is already above threshold for estrogen-mediated

responses (*22*). There can thus be no threshold for responses to exogenous EEDCs. This finding is important, as background levels of endogenous estradiol markedly alter the response of fetuses to endocrine disruptors administered to pregnant mice and rats, including EEDCs such as bisphenol A (*93,94*). This issue is also relevant with regard to comparing effects of EEDCs at different life stages. During fetal life in males and females, pregnancy, or proestrus in females, estradiol levels are significantly higher than during postnatal life in males or prior to puberty and during diestrus in females (*53*). These marked differences in the background levels of estradiol will obviously influence responses to low doses of EEDCs. The importance of endogenous estradiol levels in the response to low doses of EEDCs, which has been ignored in toxicological studies and in the models used in risk assessment, is covered in more detail below.

Implications for current risk assessment. For an EEDC such as bisphenol A, with a relative estrogenic activity approximately 10,000-fold less than E_2 in MCF-7 cells [but not necessarily other tissues where it is much more active; (*64*)], the range of estrogenic activity of this chemical equivalent to that of physiological E_2 would be approximately 0.05–30 ppb (0.05–30 ng/mL) within target cells. There are now numerous published reports that bisphenol A shows estrogenic activity at and below this concentration in a variety of cell culture systems (*4,28,100, 106–112*). For example, Gupta (*64*) reported that a 50-pg/mL (50 ppt) dose of bisphenol A significantly stimulated prostate gland formation and growth of the fetal mouse prostate in primary culture, similar to a 0.5-pg/mL dose of DES. Bisphenol A stimulated human prostate cancer cells to proliferate at a dose of 1 nM (~ 0.23 ppb) (*28*).

The currently accepted LOEL dose of bisphenol A of 50 mg/kg/day (*15*) was reported from high-dose toxicological studies (*14,113*). This study is typical in that it used doses 50,000–500,000-times higher than the

Table 2. Error in estimating responses to low doses, in the physiological range of estrogenic activity, for estradiol, DES, genistein, and bisphenol A as a result of assuming linearity across the entire dose–response curve with regard to predicted versus actual estrogen receptor occupancy.

Row	Estradiol (ppb)	DES (ppb)	Genistein (ppb)	Bisphenol A (ppb)	Actual receptors occupied (%)	Occupied receptors predicted by linear model (%)	≈ Fold underestimation of response by linear extrapolation[a]
1 test dose[b]	272	568	475,000	844,000	99.99	100	1
2	136	284	238,000	422,000	99.98	50	2
3	27.2	56.8	47,500	84,400	99.90	10	10
4	2.72	5.68	4,750	8,440	99.01	1	100
5	0.272	0.568	475	844	90.91	0.1	900
6 K_d[c]	0.0272	0.0568	47.5	84.4	50	0.01	5,000
7	0.00272	0.00568	4.75	8.44	9.09	0.001	9,000
8	0.000272	0.000568	0.475	0.844	0.99	0.0001	10,000

[a]Fold underestimation of response by linear extrapolation is the actual receptors occupied divided by the predicted receptors occupied. [b]The dose in row 1 is referred to in the text as the "test dose," at a dose 10,000-times higher than each K_d; calculated from K_d values of 0.1 nM (0.0272 ppb) for estradiol (approximate), 0.212 nM (0.0568 ppb) for DES, 176 nM (47.5 ppb) for genistein, and 370 nM (84.4 ppb) for bisphenol A (4,5). [c]Row contains concentrations at the respective K_d of each compound.

2- and 20-µg/kg/day doses we administered to pregnant mice on the basis of our calculation of an amount of bisphenol A that our preliminary findings accurately predicted would be bioactive in male mouse fetuses (*4*). The

Figure 4. (*A*) The error due to assuming that the dose–response curve is linear (dotted line) when, in fact, the dose–response curve is nonmonotonic and forms an inverted U (solid blue line). The error in estimating actual responses that will occur at doses below the test dose in a toxicological study increases as the concentration of the test dose increases relative to a test dose that would result in a maximum response. This figure shows that the magnitude of the error in estimating responses at doses below the test dose for an EEDC (using linear extrapolation) is greater when the dose–response curve is nonmonotonic relative to the error when the dose–response curve is monotonic (Figure 3). (*B*) This figure depicts the error associated with examining a single dose of a test chemical (triangle) with estrogenic activity, such as bisphenol A, that adds to an existing background level of endogenous estradiol, which is variable because of endogenous and exogenous factors. In the current model used in risk assessment, a linear extrapolation (dotted line) from the test dose (triangle) to an assumed threshold dose (circle) is used, based on the assumption there will be an absence of response at this assumed threshold dose. In this figure, the assumption is that endogenous estrogen is already above threshold for the estrogen receptor–mediated response to the EEDC (vertical dashed line). There can thus be no threshold for the response to the exogenous EEDC. The assumption of no response at the assumed threshold EEDC dose, when this is not the case, will result in a great error, potentially infinite, in estimating the response at this dose, if linear extrapolation from a high test dose is used instead of actually determining the shape of the dose–response curve.

transplacental transport of bisphenol A has now been studied in greater detail in rodents (*103,114–116*), and the doses we used would result in unconjugated bisphenol A levels in mouse fetuses that are within the range measured in human umbilical cord blood (*16,103*).

Effects using low doses of bisphenol A, which are in the new low-dose range below the LOEL based on testing very high doses, have now been reported in rodent studies on mammary gland (*117*), vagina (*118*), prostate (*4,64,65,119,120*), sperm production (*121,122*), epididymis (*64,121*), rate of embryonic development (*123,124*), pituitary response to E_2 (*109*), and rate of growth and timing of puberty in females (*93,125*). There are also reports of effects of bisphenol A in mollusks, fish, and frogs at very low concentrations, including below 1 µg/L (1 ppb) (*126–132*). Even though a few studies have reported no effects of low doses of bisphenol A, the weight of the evidence now clearly supports that such effects occur in both vertebrates and invertebrates.

It is also interesting that in two highly publicized studies using low doses of bisphenol A (*133,134*), no effects of bisphenol A were found; in addition, no effects of their positive control chemical, DES, were found. Although DES at the dose used was questioned as a valid positive control by one of the groups (*135*), its validity as a positive control estrogen at the low doses used in these studies was fully endorsed by the National Institute of Environmental Health Sciences Low-Dose Peer Review Panel (*11*). In each of the two studies, the control animals were obese (30% over normal body weight) relative to mice used in prior studies that used the same strain and age and that had shown effects of fetal exposure to bisphenol A and positive control chemicals (*4,136*), including the same low dose of DES (*23,64,82*) used by Ashby et al. and Cagen et al. (*133,134*). The fact that the control animals in both the Ashby and Cagen studies were obese and had enlarged prostates and then did not respond to either bisphenol A or the positive control DES suggests that the interaction of components of the diet with manmade chemicals, such as bisphenol A, is an issue that requires further study; our recent studies have confirmed this prediction (unpublished data). This also serves as an example of the importance of attending to information provided by the appropriate negative and positive controls (Figure 2), which these authors ignored (*11*).
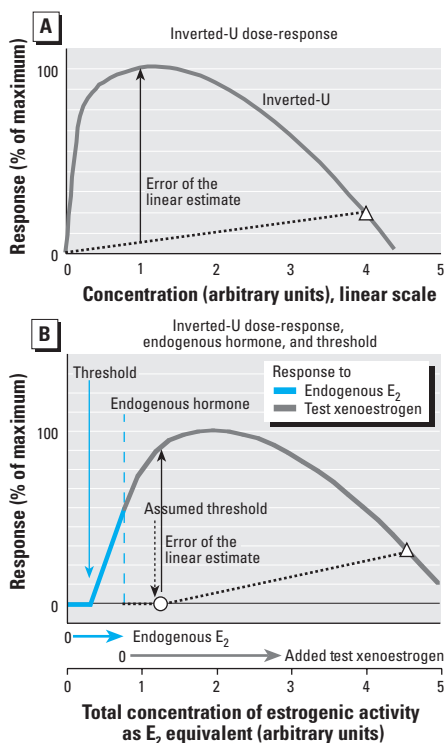
Conclusions

Information about the mechanism of action of EEDCs, together with information concerning mechanisms of hormone action, predict that current risk assessment assumptions

can lead to a dramatic underestimation of responses (and thus risk) associated with exposure to low doses of EEDCs, particularly during development when the effects of very small changes in hormonal activity are permanent (*54,64*). The practice of examining only a few very high doses and then extrapolating to predict effects of doses thousands or millions of time below those being studied is especially problematic for endocrine disruptors. The necessity for including low doses in the physiologically relevant range of estrogenic activity, as opposed to only very high doses, when testing for effects of endocrine disruptors is dictated by *a*) evidence that estrogenic chemicals (as well as other hormone mimics or chemicals that otherwise interfere with endocrine function) can produce nonmonotonic dose–response curves where responses both increase and decrease across the dose range, and *b*) the theoretical absence of a threshold for environmental chemicals that operate via receptors (such as the estrogen receptor) for endogenous ligands, such as E_2; the threshold issue is covered in more detail elsewhere (*13,22*). In addition, controls valid for the positive determination of endocrine responsiveness must be included, and when included, interpreted appropriately, particularly when results that are apparently negative are obtained. The potential for error inherent in drawing strong positive conclusions from purely negative data has clearly not been appreciated by some toxicologists (*133,134*), as well as regulators responsible for assessing this information.

Taken together, the above *in vitro* findings show the substantial error that occurs as a result of extrapolating on the basis of findings using very high doses to predict effects at environmentally relevant doses, which are often thousands or millions of times lower than doses being tested. Responses to low doses of EEDCs should be determined by testing a much wider range of doses than the 50-fold range common in toxicological studies today (*13*), including doses in the environmentally relevant range, and by accounting for all sources of estrogenic activity (endogenous and exogenous) and their interactive effect (*137*).

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