



## Articles

### Environmental Xenobiotics May Disrupt Normal Endocrine Function by Interfering with the Binding of Physiological Ligands to Steroid Receptors and Binding Proteins

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The disruption of the reproductive system of male and female animals in the wild has been attributed to environmental chemicals (xenobiotics). The effects seen mirror alterations one might anticipate if the steroid hormone-dependent processes that regulate these systems were impaired. To determine whether xenobiotics (present at a concentration of 100  $\mu$ M) exert their action through steroid-mediated pathways, we examined their ability to inhibit the binding of [ $^3$ H]physiological ligands (present at a concentration of 7 nM) to the androgen and estrogen receptors, rat androgen-binding protein (ABP), and human sex hormone-binding globulin (hSHBG). The  $\gamma$ - and  $\delta$ -isomers of hexachlorocyclohexane, congeners of dichlorodiphenyl-trichloroethane (DDT; *p,p'*-DDT; *p,p'*-DDE; *o,p'*-DDT), dieldrin, atrazine, and pentachlorophenol, caused a statistically significant inhibition of specific binding of [ $^3$ H]5 $\alpha$ -DHT to the androgen receptor that ranged from 100% (*p,p'*-DDE) to 25% (dieldrin). Methoxychlor, *o,p'*-DDT, pentachlorophenol, and nonylphenol significantly reduced [ $^3$ H]17 $\beta$ -estradiol binding to the estrogen receptor by 10, 60, 20, and 75%, respectively. The binding of [ $^3$ H]5 $\alpha$ -DHT to ABP was inhibited 70% by the  $\delta$ -isomer of hexachlorocyclohexane, but the  $\gamma$ -isomer did not reduce binding significantly. Methoxychlor, *p,p'*-DDT, atrazine, and nonylphenol reduced [ $^3$ H]5 $\alpha$ -DHT binding to ABP by approximately 40%. Nonylphenol reduced the binding of [ $^3$ H]5 $\alpha$ -DHT to hSHBG by 70%. Hexachlorocyclohexane reduced [ $^3$ H]5 $\alpha$ -DHT binding to hSHBG by 20%, but the stereospecific effects observed with ABP did not occur. *o,p'*-DDT and pentachlorophenol resulted in a statistically significant 20% inhibition of [ $^3$ H]5 $\alpha$ -DHT binding to hSHBG. Some xenobiotics resulted in dissociation of [ $^3$ H]ligands from their binding proteins that was statistically identical to that caused by the unlabeled natural ligand, whereas others resulted in slower or more rapid dissociation rates. **Key words:** androgen-binding protein, androgen receptor, estrogen receptor, sex hormone-binding globulin, xenobiotic. *Environ Health Perspect* 105:294-301 (1997)

During the past 50 or more years, vast quantities of diverse synthetic chemicals (xenobiotics) have entered the environment because of efforts to increase agricultural productivity and because of modern industrial processes. These chemicals include herbicides, insecticides, fungicides, styrenes, polychlorinated biphenyls, and penta- to nonylphenols (1-3). Some xenobiotics have been shown to disrupt normal endocrine functions, which leads to aberrant development of female and male reproductive tissues and results in decreased fertility or sterility (1-3). An early documented case concerning the effects of these endocrine-disrupting agents on reproduction was the report that newly hatched herring gull chicks collected in the Lake Ontario area, a region highly contaminated with the pesticide DDT, had altered reproductive systems

(4). Male chicks had oviducts and gonads resembling ovaries and the oviduct system of female birds developed abnormally (4). The reduction in penis size and serum testosterone levels in juvenile alligators populating Lake Apopka in Florida has been attributed to the contamination of the lake by the DDT metabolite *p,p'*-DDE (5).

A number of experimental studies reinforce the concept that environmental xenobiotics can have detrimental effects on the reproductive health of animals (6-9). Circumstantial evidence is accumulating that environmental xenobiotics may be disrupting reproductive processes in human males (10-13). Sharp (14) has postulated that these disorders in male reproductive function arise from *in utero* exposure of male embryos to environmental estrogens.

Indeed, many of these effects resemble those that have been identified in the male offspring of humans and animals that were treated during pregnancy with the potent synthetic estrogen diethylstilbestrol (DES), (15-18). Many of the environmental reproductive toxicants are thought to be estrogen agonists or antagonists (1). However, the report of Kelce et al. (19), indicating that some xenobiotics may be acting as androgen antagonists, and our data (see below), indicating that many common xenobiotics preferentially inhibit steroid binding to the androgen receptor, require that the estrogen-mimetic concept of xenobiotic action be expanded to hormone mimetics.

Although many xenobiotics in the environment have only weak hormonal activity (20), their lipophilic nature and long half-lives (21-22) allow them to accumulate and persist in fatty tissues of the body, thus increasing their concentration and bioavailability. Although it appears that xenobiotics have hormonal effects at concentrations that are orders of magnitude higher than that required of physiological hormones, our wide exposure to large numbers of these compounds may have additive effects. This concept is strengthened by the observation that mixtures of xenobiotics have been shown to have synergistic effects in *in vitro* and *in vivo* model systems (23).

The endocrine-disrupting effects of many xenobiotics can be interpreted as interference

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with the steroidal regulation of the normal development and function of the male and female reproductive tracts. The initial step in the mechanism of action of steroid hormones is binding of the steroid to its receptor or binding protein (24). To determine whether xenobiotics might act through steroid receptor or steroid binding protein pathways, we examined the ability of selected xenobiotics to inhibit the binding of [<sup>3</sup>H]17β-estradiol to the estrogen receptor or to inhibit the binding of [<sup>3</sup>H]5α-dihydrotestosterone (5α-DHT) to the androgen receptor, androgen-binding protein (ABP), and sex hormone-binding globulin (SHBG). Our data indicate that xenobiotics interact selectively and specifically with steroid receptors and binding proteins. Thus, the endocrine-disrupting effects of environmental xenobiotics may occur through multiple steroid signaling pathways.

## Materials and Methods

### Selection of Xenobiotics

Xenobiotics were selected based on published data that they had endocrine-disrupting, estrogenic, or antiandrogenic effects or that they bind to estrogen or androgen receptors (1,6,19,25). All xenobiotics were prepared as 1.0 mM stock solutions in absolute ethanol and used directly or diluted in absolute ethanol for use.

### Sources of Receptors and Binding Proteins

Because large quantities of the receptors and binding proteins were required to conduct these studies, we obtained tissue and serum containing these proteins from commercial and other sources. Cytosols prepared from frozen young rabbit uteri (PelFreez, Rogers, AR) and frozen prostates from 21-day old Sprague-Dawley rats (Harlan Bioproducts for Science, Indianapolis, IN) were the sources of estrogen and androgen receptor, respectively. The tissues were stored at -80°C until used. To prepare uterine cytosol, the frozen tissues were weighed and pulverized, and TE buffer (10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA) 1:10 wt/vol was added to the tissue fragments. The sample was then homogenized using a Polytron (Brinkman Instruments, Westbury, NY). The homogenate was then centrifuged for 1 hr at 249,000g; the supernatant is referred to as cytosol. Frozen prostates were weighed and homogenized (1:4 wt/vol) in TE buffer containing 1.0 mM phenylmethylsulfonyl fluoride and 1 mg/ml antipain; cytosol was prepared as for the uterus. Cytosol prepared from adult Sprague-Dawley rat epididymides homogenized in TE buffer (1:4 wt/vol) was the source of ABP. Postpartum human

serum obtained from Vanderbilt Hospital (Nashville, TN) was the source of SHBG.

### Sources of Xenobiotics and Steroids

Hexachlorocyclohexane [1α,2α,3β(α),4α,5α,6β-hexachlorocyclohexane], δ- and γ-isomers; *p,p'*-DDT; methoxychlor [1,1,1-trichloro-2,2 bis(*p*-methoxyphenyl)ethane]; dieldrin; and pentachlorophenol were obtained from Sigma (St. Louis, MO). Nonylphenol was obtained from Aldrich (St. Louis, MO). Atrazine [6-chloro-*N*-ethyl-*N'*-(1-methyl-ethyl)-1,3,5-triazine-2,4-diamine], *p,p'*-DDE, and *o,p'*-DDT, were from Chem Service (Westchester, PA). Unlabeled estradiol and 5α-dihydrotestosterone were from Steraloids (Wilton, NH), and [<sup>3</sup>H]17β-estradiol (87 Ci/mmol) and [<sup>3</sup>H]5α-DHT (130 Ci/mmol) were from Dupont/NEN (Boston, MA).

### Binding Assays

**Charcoal assay.** Binding to the estrogen receptor was assessed by incubating uterine cytosol with 7 nM [<sup>3</sup>H]17β-estradiol. Binding to the androgen receptor, ABP, and SHBG was assessed by incubating rat prostate cytosol, epididymal cytosol, or human serum, respectively, with 7 nM [<sup>3</sup>H]5α-DHT. Samples were incubated (at 4°C overnight for receptors, or for 4–6 hr for ABP and SHBG) with 7 nM [<sup>3</sup>H]ligand alone (total binding) or together with a 100-fold molar excess of the corresponding unlabeled ligand to determine nonspecific binding. All assay tubes were brought up to a final volume of 0.5 ml with TE. Xenobiotics were used as inhibitors at a concentration of 100 μM. This concentration is in the range of those used by others for inhibition studies with xenobiotics (19,23). When the ability of xenobiotics to inhibit the binding of [<sup>3</sup>H]steroids to their binding proteins was studied, 10 μl of ethanol (the same volume in which the xenobiotics were dissolved) was added to the total and nonspecific binding tubes. Thus, 2% ethanol was present in each 0.5 ml incubation. This amount of ethanol decreased total binding to the receptors by 2–3% and decreased total binding to ABP and SHBG by about 10% compared to samples that contained no ethanol. The specific binding that occurred in the presence of ethanol was set at 100% in those experiments in which data are plotted as a percentage of control.

After the incubations were concluded, binding of [<sup>3</sup>H]ligands to their binding proteins was conducted using either the charcoal assay (26) or sucrose gradient ultracentrifugation (26). The charcoal assay was performed by adding 0.5 ml of a dextran (0.05%) coated charcoal (0.5%) suspension in TE to each sample tube. The

tubes were then agitated on a vortex mixer for 6 sec and then either centrifuged immediately for 5 min at 1,500g (ABP and SHBG samples) or incubated on ice for 10 min and then centrifuged (receptor samples). The difference in postcharcoal incubation time is to take into account the rapid dissociation of ligand from ABP and SHBG (27) and its slower dissociation from the receptors (26). After centrifugation, the supernatants were decanted into scintillation vials and the radioactivity in the samples was counted.

**Sucrose gradient analysis.** For sucrose gradient analysis, samples were incubated as described above and extracted with pellets from 1 ml of charcoal suspension (28), and 300 μl aliquots of each sample were applied to low ionic strength (0.01 M KCl) 5–20% sucrose gradients and processed as previously described (28).

**Competition studies.** Samples (androgen and estrogen receptors, ABP, SHBG) were incubated with a saturating concentration of [<sup>3</sup>H]physiological ligand (7 nM) and increasing concentrations of unlabeled physiological ligand or xenobiotic competitor. After incubation at 4°C overnight (receptors) or for 4 hr (ABP and SHBG), the amount of bound radioactivity was determined using the charcoal assay procedure. The data were plotted as percentage of control (specific binding in the absence of competitor) versus the log of the competitor concentration. The concentration of competitor that inhibited specific binding by 50% is the IC<sub>50</sub>. To determine the relative binding affinity (RBA) of the xenobiotics as compared to the physiological ligand, the IC<sub>50</sub> for the physiological ligand was divided by the IC<sub>50</sub> of the xenobiotic and the dividend was multiplied by 100.

**Determination of dissociation half-time.** Samples were incubated with 7 nM [<sup>3</sup>H]physiological ligand alone or with a 100-fold molar excess of unlabeled physiological ligand (to determine nonspecific binding) at 4°C overnight (receptors) or for 4–6 hr (ABP, SHBG). At the conclusion of the incubation, unlabeled physiological ligand (35 μM) or xenobiotic (100 μM) was added to each sample. At various times after these additions, the amount of bound radioactivity was determined using the charcoal assay procedure. The data were plotted as specific radioactivity bound as a percentage of control versus time. A regression line was fitted to the points and the dissociation half-time (t<sub>1/2</sub>) was calculated as previously described (27).

### Statistical Analysis

The data were analyzed by one-way analysis of variance followed by Duncan's multiple-



range test (29). Results were considered significant when  $p < 0.05$ . The data are presented as the mean and the standard error of the mean.

## Results

### Studies on the Estrogen Receptor

To screen xenobiotics for possible interaction with the estrogen receptor, we determined their ability to inhibit 7 nM [ $^3\text{H}$ ]17 $\beta$ -estradiol binding to the estrogen receptor present in uterine cytosol using the charcoal assay procedure. Figure 1 shows our results. Specific estradiol binding to cytosol occurred, which was completely inhibited by 700 nM unlabeled estradiol. Unlabeled (700 nM) 5 $\alpha$ -DHT inhibited binding by about 25%. Of the xenobiotics tested (present at 100  $\mu\text{M}$ ), methoxychlor, *p,p'*-DDT, *p,p'*-DDE, and pentachlorophenol inhibited estradiol binding to a small (10%) but statistically significant extent. In contrast, *o,p'*-DDT and nonylphenol caused a dramatic decrease in [ $^3\text{H}$ ]17 $\beta$ -estradiol binding: 60 and 75%, respectively. Estradiol binding was not significantly decreased by the  $\gamma$ - or  $\delta$ -isomer of hexachlorocyclohexane, dieldrin, or atrazine.

Further evaluation of xenobiotic effects on estradiol binding to uterine cytosol was conducted using sucrose gradient analysis (Fig. 2). When uterine cytosol was incubated with 7 nM [ $^3\text{H}$ ]17 $\beta$ -estradiol alone, a single peak of macromolecular-bound radioactivity was detected. The peak had a sedimentation coefficient of about 10 S, in reference to a 4.6 S bovine serum albumin (BSA) sedimentation standard, which corresponds to that of the estrogen receptor on low ionic strength, 5–20% sucrose gradients (28). Incubation in the presence of 700 nM unlabeled estradiol eliminated the peak. These data confirm that the estradiol binding detected in uterine cytosol is to the estrogen receptor. As in the charcoal assay, nonylphenol was a highly effective inhibitor of [ $^3\text{H}$ ]17 $\beta$ -estradiol binding to the receptor and pentachlorophenol reduced [ $^3\text{H}$ ]17 $\beta$ -estradiol binding, but *p,p'*-DDT and *p,p'*-DDE had little detectable effect on peak heights.

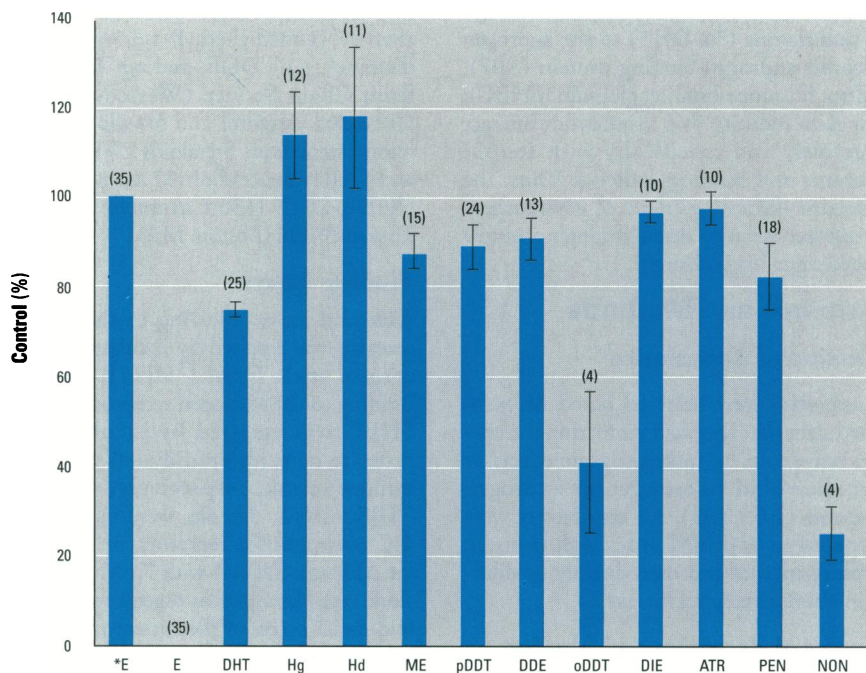
The ability of selected xenobiotics to cause dissociation of [ $^3\text{H}$ ]17 $\beta$ -estradiol from its receptor compared to the physiological ligand was tested. The addition of unlabeled estradiol (35  $\mu\text{M}$ ) to the estrogen receptor preparation that had been equilibrated with 7 nM [ $^3\text{H}$ ]17 $\beta$ -estradiol resulted in a time-dependent decrease in the amount of radioactivity associated with the receptor. The  $t_{1/2}$  of dissociation (Table 1) was calculated (27) to be about 40 hr, which is in agreement with literature values (26). Pentachlorophenol (100  $\mu\text{M}$ ) resulted in a

dissociation rate that was indistinguishable from that caused by estradiol (Table 1). When 100  $\mu\text{M}$  nonylphenol was used, dissociation of estradiol from its receptor was more rapid ( $t_{1/2}$  approx 7 hr; Table 1).

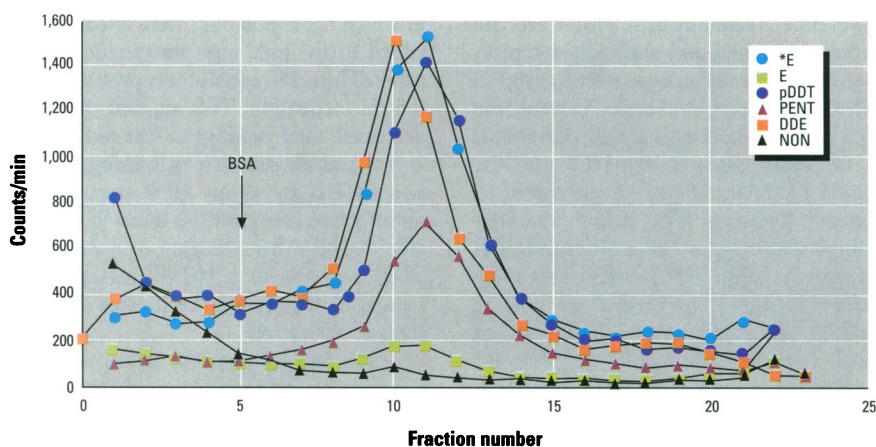
### Studies on the Androgen Receptor

In contrast to the minor effects of most of the xenobiotics tested in inhibiting estradiol binding to its receptor (Fig. 1), many xeno-

biotics inhibited [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to prostate cytosol to a statistically significant extent (Fig. 3). The most effective inhibitors were the DDT derivatives, *p,p'*-DDE, *o,p'*-DDT, and *p,p'*-DDT, which inhibited binding by 100, 90, and 80%, respectively. The hexachlorocyclohexane isomers, which inhibited binding by about 50%, were the next most potent inhibitors of [ $^3\text{H}$ ]5 $\alpha$ -DHT binding. Nonylphenol, which



**Figure 1.** Inhibition of [ $^3\text{H}$ ]17 $\beta$ -estradiol binding to the estrogen receptor (estrogen receptor source, uterine cytosol) as determined by the charcoal assay procedure. Abbreviations: \*E, specific binding of [ $^3\text{H}$ ]17 $\beta$ -estradiol; E, unlabeled estradiol; DHT, unlabeled 5 $\alpha$ -dihydrotestosterone; Hg, hexachlorocyclohexane- $\gamma$ ; Hd, hexachlorocyclohexane- $\delta$ ; ME, methoxychlor; pDDT, *p,p'*-DDT; DDE, *p,p'*-DDE; oDDT, *o,p'*-DDT; DIE, dieldrin; ATR, atrazine; PEN, pentachlorophenol; NON, nonylphenol. The data are plotted at control (%) in which the amount of specific binding of [ $^3\text{H}$ ]17 $\beta$ -estradiol = 100%. The mean and the standard error of the mean are plotted. The numbers in parentheses above the bars indicate the number of experiments in which samples were assayed in triplicate.



**Figure 2.** Sucrose gradient analysis of estradiol binding to the estrogen receptor. Abbreviations: \*E, sample incubated with [ $^3\text{H}$ ]17 $\beta$ -estradiol alone prior to sucrose gradient analysis; E, unlabeled estradiol; pDDT, *p,p'*-DDT; PENT, pentachlorophenol; DDE, *p,p'*-DDE; NON, nonylphenol; BSA, bovine serum albumin.

reduced [ $^3\text{H}$ ]17 $\beta$ -estradiol binding to the estrogen receptor by 75%, had no effect on [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to androgen receptor (Fig. 3), nor did methoxychlor or estradiol. Dieldrin, atrazine, and pentachlorophenol resulted in a statistically significant 30–40% reduction in [ $^3\text{H}$ ]5 $\alpha$ -DHT binding (Fig. 3).

Two considerations prompted us to examine [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to prostate cytosol by sucrose gradient analysis. First was the report that the prostate contains a steroid-binding protein other than the androgen receptor that is fairly nonspecific and not readily saturable (30). This protein sediments on low ionic strength, 5–20% sucrose gradients at approximately 3.7 S (30) in contrast to the androgen receptor, which sediments at approximately 8–10 S (28). The second consideration was our

observation that in some experiments, *p,p'*-DDE present at a concentration of 100  $\mu\text{M}$  inhibited [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to prostate cytosol to a greater extent than did 0.7- $\mu\text{M}$  5 $\alpha$ -DHT. These data suggested that binding moieties other than the androgen receptor might be present in some prostate cytosol preparations.

Figure 4A shows the results of sucrose gradient analysis of [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to cytosol made from a batch of 15 prostates. A single symmetrical peak of bound [ $^3\text{H}$ ]5 $\alpha$ -DHT was detected that had a sedimentation coefficient of 8–10 S (Fig. 4A). This peak, which represents the androgen receptor, was preceded by exponentially decreasing amounts of radioactivity representing unbound ligand that was not removed by the charcoal extraction procedure. The 8–10 S

peak was eliminated when the sample was incubated with [ $^3\text{H}$ ]5 $\alpha$ -DHT and 0.7  $\mu\text{M}$  unlabeled 5 $\alpha$ -DHT (Fig. 4A) and it was greatly reduced when samples were incubated with 100  $\mu\text{M}$  *p,p'*-DDE or 100- $\mu\text{M}$  *p,p'*-DDT. No [ $^3\text{H}$ ]5 $\alpha$ -DHT binding was detected in the 4 S region of the gradient where specific or nonspecific binding to moieties other than the receptor would be present.

Analysis of a batch of cytosol prepared from 15 other prostates yielded the results shown in Figure 4B. In this case, macromolecular-bound [ $^3\text{H}$ ]5 $\alpha$ -DHT was detected in both the 4 S and 8 S regions of the gradient. Unlabeled 5 $\alpha$ -DHT (0.7  $\mu\text{M}$ ) eliminated [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to the androgen receptor present in the 8–10 S region and decreased, but did not eliminate, [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to the 4–5 S component(s). In contrast, *p,p'*-DDE (100  $\mu\text{M}$ ) eliminated [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to components in both regions of the gradient (Fig. 4B). Whether the 4–5 S component represents the 3.7 S low affinity, high capacity prostate binding protein reported by others (30) is not known. The presence of this unknown protein provides an explanation for our observation that 100  $\mu\text{M}$  *p,p'*-DDE reduced binding of [ $^3\text{H}$ ]5 $\alpha$ -DHT to some prostate cytosol preparations to a greater extent than did 0.7  $\mu\text{M}$  unlabeled 5 $\alpha$ -DHT.

The ability of *p,p'*-DDE to cause dissociation of [ $^3\text{H}$ ]5 $\alpha$ -DHT from the prostate androgen receptor was determined as described above for the estrogen receptor. Our data (Table 1) indicate that unlabeled 5 $\alpha$ -DHT and *p,p'*-DDE resulted in a similar pattern of dissociation of [ $^3\text{H}$ ]5 $\alpha$ -DHT from the androgen receptor and a  $t_{1/2}$  of greater than 15 hr, a value that is in agreement with the literature (31).

### Studies on Androgen-binding Protein

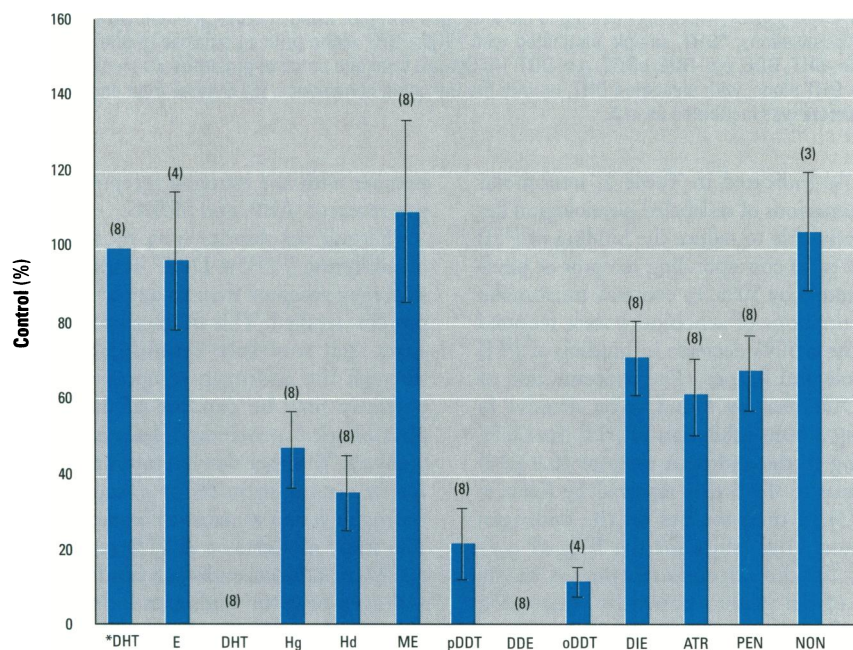
Charcoal assay analysis of [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to rat epididymal cytosol containing ABP indicated that several xenobiotics resulted in a statistically significant reduction in the amount of [ $^3\text{H}$ ]5 $\alpha$ -DHT bound to ABP (Fig. 5). The most effective xenobiotic inhibitor was the  $\delta$ -isomer of hexachlorocyclohexane, which caused a 70% decrease in bound [ $^3\text{H}$ ]5 $\alpha$ -DHT. In contrast, the inhibition caused by the  $\gamma$ -isomer of hexachlorocyclohexane did not reach statistical significance. Methoxychlor, *p,p'*-DDT, and atrazine all reduced [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to ABP by 40–50% (Fig. 5). *p,p'*-DDE reduced binding to ABP by 20%. Estradiol inhibited [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to ABP by about 60%.

Sucrose gradient analysis indicated that macromolecular-bound [ $^3\text{H}$ ]5 $\alpha$ -DHT in epididymal cytosol sedimented at 4.6 S as

**Table 1.** The effect of various compounds on the half-time of dissociation of [ $^3\text{H}$ ]physiological ligand from its receptor or binding protein.

Protein	Compound						
	E	DHT	NON	PEN	ME	Hd	DDE
ER	40 hr	–	7 hr	40 hr	–	–	–
AR	–	15 hr	–	–	–	–	15 hr
ABP	–	7 min	–	–	–	70 min	–
SHBG	–	85 min	85 min	5 min	ND	–	–

Abbreviations: E, 17 $\beta$ -estradiol; DHT, 5 $\alpha$ -dihydrotestosterone; NON, nonylphenol; PEN, pentachlorophenol; ME, methoxychlor; Hd, hexachlorocyclohexane- $\delta$ ; DDE, *o,p'*-DDE; ER, estrogen receptor; AR, androgen receptor; ABP, androgen-binding protein; SHBG, sex hormone-binding globulin. Blank spaces indicate that these comparisons were not made. ND, no detectable dissociation during the time studied.



**Figure 3.** Inhibition of [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to the androgen receptor (androgen receptor source, rat prostate cytosol) as determined by the charcoal assay procedure. Abbreviations: \*DHT, specific binding of [ $^3\text{H}$ ]5 $\alpha$ -DHT to the receptor; E, estradiol; DHT, 5 $\alpha$ -dihydrotestosterone; Hg, hexachlorocyclohexane- $\gamma$ ; Hd, hexachlorocyclohexane- $\delta$ ; ME, methoxychlor; pDDT, *p,p'*-DDT; DDE, *p,p'*-DDE; oDDT, *o,p'*-DDT; DIE, Dieldrin; ATR, atrazine; PEN, pentachlorophenol; NON, nonylphenol. Specific binding of [ $^3\text{H}$ ]5 $\alpha$ -DHT = 100%. The mean and the standard error of the mean are plotted. The numbers above the bars indicate the number of experiments in which the samples were assayed in duplicate.



would be expected for ABP (32). This binding was completely inhibited by unlabeled 5 $\alpha$ -DHT, reduced by hexachlorocyclohexane- $\delta$ , but not affected by hexachlorocyclohexane- $\gamma$  (Fig. 6).

When the  $\delta$ -isomer of hexachlorocyclohexane was used in dissociation studies, we noted that the dissociation rate ( $t_{1/2}$  approx 70 min) was slower than the  $t_{1/2}$  of about 7 min obtained when unlabeled 5 $\alpha$ -DHT was used (Table 1). This latter value is similar to what we have previously reported (27,32).

### Studies on Sex Hormone-binding Globulin

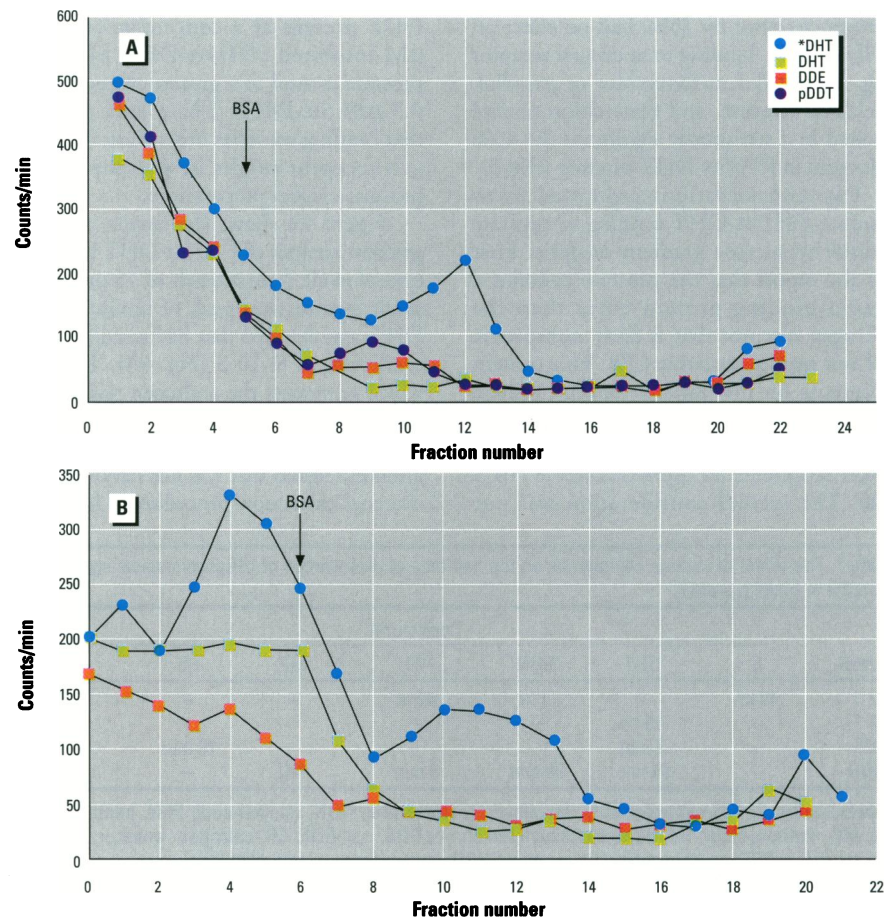
When the binding of [ $^3$ H]5 $\alpha$ -DHT to human postpartum serum containing SHBG was examined using the charcoal assay procedure, we noted that the xenobiotics hexachlorocyclohexane- $\delta$  and  $\gamma$ , *o,p'*-DDT, pentachlorophenol, and nonylphenol caused a statistically significant reduction in specific binding (Fig. 7). Nonylphenol resulted in a 70% decrease in [ $^3$ H]5 $\alpha$ -DHT binding, whereas the other xenobiotic competitors reduced binding by about 20–30%. SHBG was the least responsive protein to the inhibitory effects of xenobiotics on 5 $\alpha$ -DHT binding. Methoxychlor, *p,p'*-DDT, *p,p'*-DDE, dieldrin, and atrazine were ineffective at inhibiting [ $^3$ H]5 $\alpha$ -DHT binding to SHBG. Estradiol inhibited [ $^3$ H]5 $\alpha$ -DHT binding to SHBG by 85% (Fig. 7).

When the binding of [ $^3$ H]5 $\alpha$ -DHT to serum components was examined by sucrose gradient ultracentrifugation, label was bound to a moiety sedimenting at 4.6 S, which is the appropriate sedimentation coefficient for SHBG (33). As expected, unlabeled 5 $\alpha$ -DHT and nonylphenol inhibited [ $^3$ H]5 $\alpha$ -DHT binding to SHBG, whereas atrazine did not (Fig. 8).

When dissociation studies were conducted, the addition of unlabeled 5 $\alpha$ -DHT to SHBG samples that had been equilibrated with [ $^3$ H]5 $\alpha$ -DHT resulted in a  $t_{1/2}$  of dissociation of approximately 85 min, a  $t_{1/2}$  similar to literature values (27). Nonylphenol resulted in a similar dissociation half-time, but pentachlorophenol resulted in a more rapid dissociation rate ( $t_{1/2}$  approx 5 min). Methoxychlor, which had no effect on steroid binding to hSHBG, did not effect the dissociation of [ $^3$ H]5 $\alpha$ -DHT from SHBG during the time period studied (Table 1).

### Concentration of Ligand Causing a 50% Inhibition of Binding to Receptors and Binding Proteins

To further evaluate the affinity of xenobiotics for steroid receptors and binding proteins, competition studies were conducted using multiple concentrations of competi-



**Figure 4.** Analysis of [ $^3$ H]5 $\alpha$ -DHT binding to the androgen receptor using sucrose gradient ultracentrifugation. Abbreviations: \*DHT, sample incubated with [ $^3$ H]5 $\alpha$ -DHT alone prior to sucrose gradient analysis; DHT, 5 $\alpha$ -DHT; DDE, *p,p'*-DDE; pDDT, *p,p'*-DDT. (A) Cytosol from one batch of prostates was incubated with [ $^3$ H]5 $\alpha$ -DHT alone, with unlabeled DHT, or with the indicated xenobiotics. (B) Cytosol from another batch of prostates was incubated as in A.

tors. As indicated in Table 2, nanomolar concentrations of unlabeled physiological ligand and were able to reduce the binding of [ $^3$ H] ligand to its corresponding receptor or binding protein by 50%. In contrast, micromolar concentrations of xenobiotics were required to cause a 50% decrease in binding of [ $^3$ H] physiological ligands. The concentration of *p,p'*-DDE that we found to be effective in causing a 50% inhibition of [ $^3$ H] 5 $\alpha$ -DHT binding to the androgen receptor (6.8  $\mu$ M) is similar to the 5  $\mu$ M reported by Kelce et al. (19) in their studies on the androgen receptor. Our values for the  $IC_{50}$  of *o,p'*-DDT for the estrogen receptor is in the range of the values reported by others (23), using different xenobiotics, for this receptor. We are not aware of published data on the inhibition of physiological ligand binding to ABP or SHBG by xenobiotics.

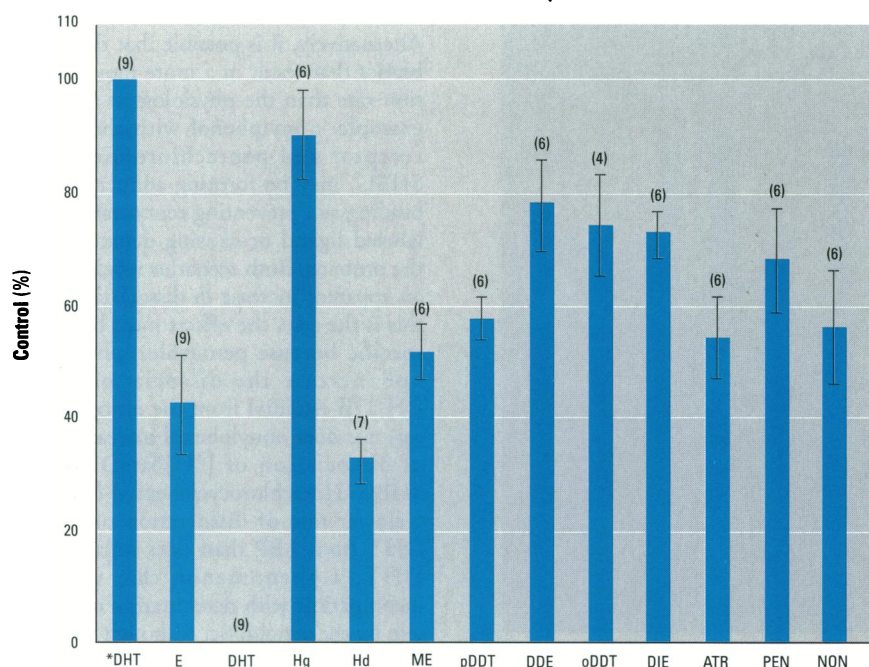
### Discussion

Our experiments demonstrate that xenobiotics interact in a specific and differential

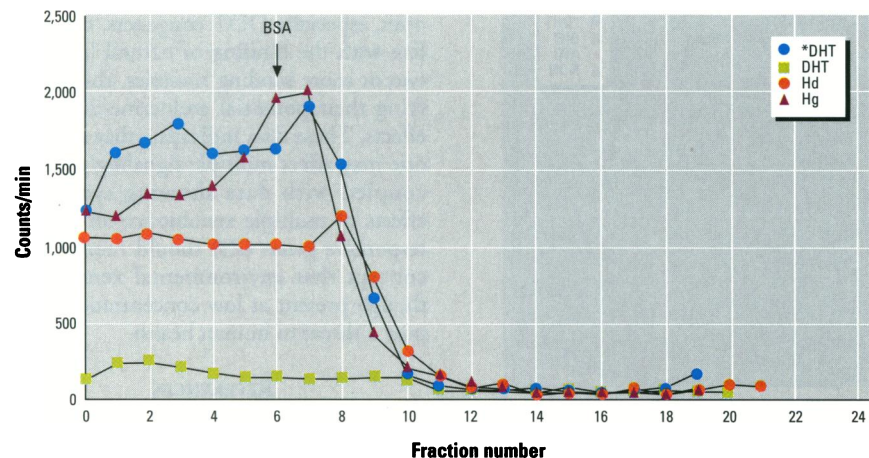
manner with the estrogen receptor, androgen receptor, ABP, and SHBG.

Several xenobiotics were more capable of inhibiting [ $^3$ H]5 $\alpha$ -DHT binding to the androgen receptor than to any of the other proteins studied. This finding strongly suggests that multiple xenobiotics acting through the androgen receptor-mediated pathway may be capable of disrupting physiological processes regulated by this pathway. Whether these compounds would act *in vivo* as agonists or antagonists of androgen action remains to be determined. The study of Kelce et al. (19) reports that *p,p'*-DDE inhibits androgen binding to the androgen receptor, androgen-induced transcriptional activity, and normal male prepubertal development. Our data indicate that *p,p'*-DDE was the most potent of the xenobiotics tested at inhibiting [ $^3$ H]5 $\alpha$ -DHT binding to the androgen receptor. Our study indicates that *p,p'*-DDT and *o,p'*-DDT are also potent inhibitors of [ $^3$ H]5 $\alpha$ -DHT binding to the androgen





**Figure 5.** Inhibition of [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to androgen-binding protein (ABP; source, cytosol from rat epididymides) by xenobiotics as determined by charcoal assay. Abbreviations: \*DHT, specific binding of [ $^3\text{H}$ ]5 $\alpha$ -DHT; E, estradiol; DHT, 5 $\alpha$ -dihydrotestosterone; Hg, hexachlorocyclohexane- $\gamma$ ; Hd, hexachlorocyclohexane- $\delta$ ; ME, methoxychlor; pDDT, *p,p'*-DDT; DDE, *p,p'*-DDE; oDDT, *o,p'*-DDT; DIE, dieldrin; ATR, atrazine; PEN, pentachlorophenol; NON, nonylphenol. Specific binding of \*DHT = 100%. The mean and the standard error of the mean are plotted. The numbers above the bars indicate the number of experiments in which the samples were assayed in triplicate.



**Figure 6.** Sucrose gradient analysis of [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to androgen-binding protein (ABP; source, cytosol rat epididymides). Abbreviations: \*DHT, cytosol incubated with [ $^3\text{H}$ ]5 $\alpha$ -DHT alone; DHT, 5 $\alpha$ -dihydrotestosterone; Hd, hexachlorocyclohexane- $\delta$ ; Hg, hexachlorocyclohexane- $\gamma$ .

receptor. *o,p'*-DDT is usually considered to be estrogenic (34), but our data suggests that it could also be expected to act in an androgenic or antiandrogenic manner.

Both the  $\delta$ - and  $\gamma$ -isomers of hexachlorocyclohexane are potent inhibitors of [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to the androgen receptor. These compounds bind to rat ABP, but not to human SHBG, in a stereospecific manner. ABP and SHBG are products of the same gene (35), but they

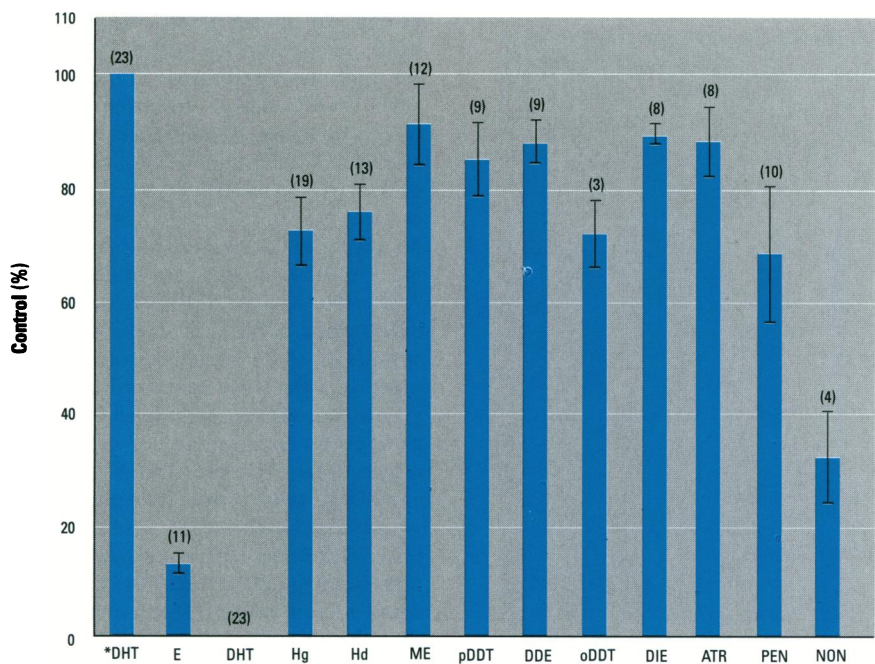
differ considerably in the oligosaccharides attached to them (36). Differences in steroid-binding between rat ABP and human SHBG have been observed (27). In addition to differential binding of hexachlorocyclohexane isomers to ABP and SHBG, other binding differences between the proteins were noted. Methoxychlor and *p,p'*-DDT caused approximately a 40% reduction in binding to ABP, but had no statistically significant inhibitory effect on

binding to human SHBG. On the other hand, nonylphenol was a better competitor for androgen binding to SHBG than to ABP. This finding is consistent with our observation (Fig. 5) and reports in the literature (27) that human SHBG binds estradiol better than ABP in rat epididymal cytosol. Whether the effects of xenobiotics on androgen binding to human ABP are more similar to those on binding to rat ABP or to human SHBG remains to be determined. ABP and SHBG were long considered to be simply steroid transport proteins in the testis-epididymis and in the general circulation, respectively. Evidence now exists demonstrating cell-surface receptors for these proteins (37–38). ABP and SHBG bind to their receptors and activate adenyl cyclase (39). Xenobiotics that bind to these proteins could prevent them from binding to their receptors and/or they could inhibit normal signal transduction, thus disrupting the action of these proteins.

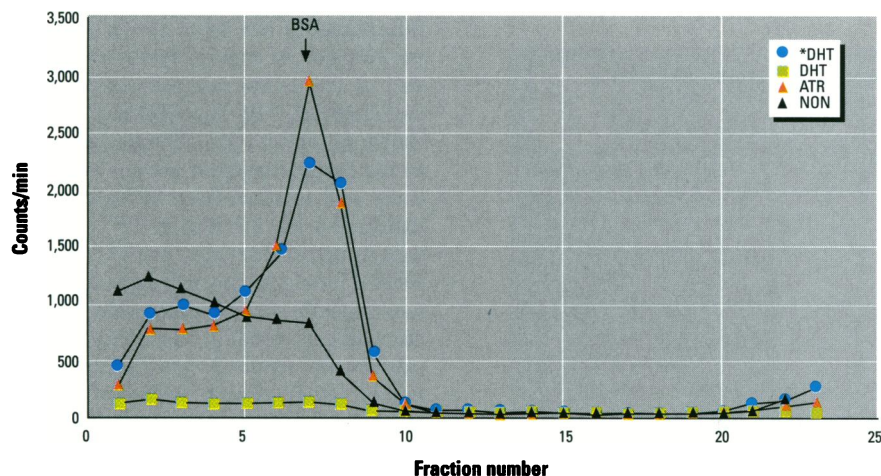
Of the xenobiotics tested, fewer interacted with the estrogen receptor than with the androgen receptor or ABP. Nonylphenol was a highly potent inhibitor of [ $^3\text{H}$ ]17 $\beta$ -estradiol binding to the estrogen receptor and its inhibition was not statistically different than that caused by the known estrogen mimetic, *o,p'*-DDT. Nonylphenol has been shown to induce proliferation of human breast cancer cells and to trigger mitotic activity in the rat endometrium (40). Nonylphenol has been shown to inhibit [ $^3\text{H}$ ]17 $\beta$ -estradiol binding to the estrogen receptor present in rainbow trout and to stimulate vitellogenin gene expression in this species (41). Because nonylphenol does not inhibit [ $^3\text{H}$ ]5 $\alpha$ -DHT binding to the androgen receptor, it may cause the *in vitro* effects solely through the estrogen receptor. However, since the experiments reported used human or fetal bovine (40,41) serum, both of which contain SHBG (42), in cultures with the cells, some effects of nonylphenol acting through SHBG cannot be discounted. *In vivo* endocrine-disrupting effects of nonylphenol in species that possess SHBG are likely to be mediated through both the estrogen receptor and SHBG.

We also studied the ability of xenobiotics to influence the half-time of dissociation of [ $^3\text{H}$ ]ligand from the proteins. The data that we obtained indicate that while some xenobiotics resulted in dissociation rates identical to those caused by the physiological ligand, others resulted in more rapid or in slower rates. These data suggest that some xenobiotics have a three-dimensional structure that permits access to the binding site equivalent to that of the natural steroid, while the conformation of other





**Figure 7.** The effect of xenobiotics on binding of [<sup>3</sup>H]5α-DHT to human sex hormone-binding globulin (hSHBG; source, human serum). Abbreviations: \*DHT, [<sup>3</sup>H]5α-DHT, E, estradiol; DHT, 5α-dihydrotestosterone; Hg, hexachlorocyclohexane-γ; Hd, hexachlorocyclohexane-δ; ME, methoxychlor; pDDT, p,p'-DDT; DDE, p,p'-DDE; oDDT, o,p'-DDT; DIE, dieldrin; ATR, atrazine; PEN, pentachlorophenol; NON, nonylphenol. Specific binding of \*DHT = 100%. The mean and standard error of the mean are plotted. The numbers above the bars indicate the number of experiments in which the samples were assayed in triplicate.



**Figure 8.** Sucrose gradient analysis of [<sup>3</sup>H]5α-DHT binding to human sex hormone-binding globulin (hSHBG; source, human serum). Abbreviations: \*DHT, serum incubated with [<sup>3</sup>H]5α-DHT alone prior to gradient analysis; DHT, 5α-dihydrotestosterone; ATR, atrazine; NON, nonylphenol.

**Table 2.** Relative binding affinity (RBA) and IC<sub>50</sub> of xenobiotics for steroid receptors and binding proteins

Protein	Competitor					
	DHT	E	DDE	oDDT	Hg	Hd
ER	—	100 (2.7 nM)	—	0.007 (40 μM)	—	—
AR	100 (1.1 nM)	—	0.016 (6.8 μM)	—	—	—
ABP	100 (9 nM)	—	—	—	—	0.025 (38 μM)
SHBG	100 (1.2 nM)	—	—	—	0.0007 (160 μM)	—

The numbers in parentheses indicate the molar concentrations of competitors that reduced specific binding by 50% (IC<sub>50</sub>); blanks indicate that these comparisons were not made. Abbreviations: DHT, 5α-dihydrotestosterone; E, estradiol; DDE, p,p'-DDE; oDDT, o,p'-DDT; Hg, hexachlorocyclohexane-γ; Hd, hexachlorocyclohexane-δ; ER, estrogen receptor; AR, androgen receptor; ABP, androgen-binding protein; SHBG, sex hormone-binding globulin.

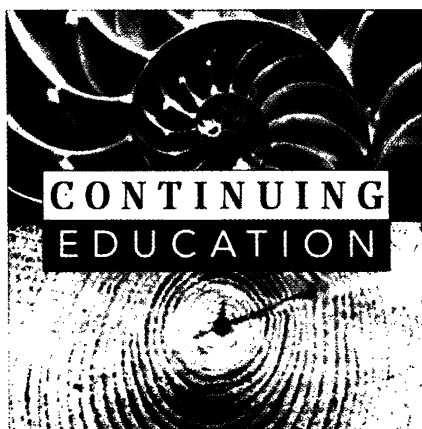
xenobiotics facilitates or hinders this access. Alternatively, it is possible that those xenobiotics that result in a more rapid dissociation rate than the physiological ligand, for example, nonylphenol with the estrogen receptor and pentachlorophenol with SHBG, may be forming adducts with the binding site preventing reassociation of the labeled ligand or causing denaturation of the proteins. Both scenarios would result in an apparent increase in dissociation rate. If this is the case, the effects must be protein-specific because pentachlorophenol does not increase the dissociation rate of [<sup>3</sup>H]17β-estradiol from the estrogen receptor, nor does nonylphenol increase the rate of dissociation of [<sup>3</sup>H]5α-DHT from SHBG. Hexachlorocyclohexane-δ results in a slower rate of dissociation of [<sup>3</sup>H]5α-DHT from ABP than does unlabeled 5α-DHT, a phenomenon that would be incompatible with denaturation of the protein by the xenobiotic. Although we cannot discount the possibility that the apparent inhibition of binding caused by some xenobiotics is actually the result of the denaturation or adduct formation, the multiple point competition data are consistent with our contention that several xenobiotics compete with physiological ligands for sites on steroid receptors and binding proteins.

Our data indicate that some xenobiotics, especially DDT congeners, can interfere with the binding of natural ligands to two or more binding moieties, thus amplifying their potential endocrine-disrupting effects. These data indicating that a xenobiotic may affect multiple signaling pathways coupled with data showing synergistic effects of multiple xenobiotics on estrogen responsive genes (23) should reinforce the concept that environmental xenobiotics, though present at low concentrations, may pose a threat to human health.

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