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ESTROGENIC BEHAVIOR OF 2(*o*-CHLOROPHENYL)-2-(*p*-CHLOROPHENYL)-1,1,1-TRICHLOROETHANE AND ITS HOMOLOGUES*

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Abstract—Eight chlorinated hydrocarbons were tested for their ability to compete with ^{3}H-estradiol-17β for specific binding proteins in uterine cytoplasm from immature rats. The binding was assayed on 5–20% sucrose density gradients. 2(*o*-Chlorophenyl)-2-(*p*-chlorophenyl)-1,1,1-trichloroethane (o,p'-DDT) and 2(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-1,1-dichloroethylene (o,p'-DDE) ($1{\cdot}4 \times 10^{-4}$ M) competed with ^{3}H-estradiol-17β ($8{\cdot}7 \times 10^{-9}$ M) for binding to the "8 S receptor" in the cytoplasm. 2,2-Bis-(*p*-chlorophenyl)-1,1,1-trichloroethane (p,p'-DDT), 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene (p,p'-DDE), 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethane (p,p'-DDD), 2(*m*-chlorophenyl)-2-(*p*-chlorophenyl)-1,1-dichloroethane (m,p'-DDD), 2(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-1,1-dichloroethylene (o,p'-DDD) and bis-(*p*-chlorophenyl)-1,1-dichloroacetate (DDA) did not compete at $1{\cdot}4 \times 10^{-4}$ M. Transfer of the receptor-bound compounds into the nuclei was examined using similar competition techniques. o,p'-DDT and o,p'-DDE competed with estradiol binding in the nuclei. These studies indicate that o,p'-DDT and o,p'-DDE at high concentrations act as estrogens as measured by their ability to compete with estradiol-17β for binding to the uterine cytopoasmic receptor and in the transfer and binding of estradiol in the nuclei of uterine cells.

Endocrine reproductive processes are affected in many species of animals by ingestion of DDT pesticides. In birds, this is primarily manifested by egg fragility from reduced thickness and concentrations of calcium in egg shells [1–3]. Chlorinated hydrocarbons have been implicated in the endocrine reproductive processes of mammals in two ways. First, many members of this family of pesticides increase hepatic steroid metabolism, thus lowering endogenous levels of steroids [4–6]. Second, several halogenated hydrocarbon pesticides appear to possess estrogenic activity. o,p'-DDT§ which comprises about 20 per cent of the commercial DDT formulations is most often implicated as an estrogen. This activity has been demonstrated by examining its capacity to increase uterine wet weight, RNA and glycogen synthesis, and to suppress luteinizing hormone concentrations in plasma [7–9]. o,p'-DDT and methoxychlor decrease the uptake *in vivo* of ^{3}H-estradiol-17β into rat uteri [10]. Pretreatment with carbon tetrachloride, producing hepatic injury, however, inhibits the estrogenic effect of o,p'-DDT on the uterus, suggesting that a metabolite of o,p'-DDT may be responsible for its estrogenic action [10]. o,p'-DDT injected into neonatal female rats decreases the age at which vaginal opening occurs and induces persistent vaginal estrus after a period of normal estrous cycles. The subsequent sterility is associated with the development of follicular cysts in the ovaries and a reduction in the number of corpus lutea [11]. These effects are dose dependent, with 100 μg daily for 3 days producing the defect [12]. Singh [13] has reviewed the evidence suggesting that this syndrome in rats may be similar to the polycystic ovary syndrome seen in some women. Sex steroids and nonsteroidal estrogens, such as diethylstilbestrol and clomiphene citrate given to neonatal rats are also known to produce this syndrome [14, 15]. The present study was designed to examine whether o,p'-DDT and some of its homologues have intrinsic estrogenic activities in a cell-free system prepared from immature rat uteri. We utilized the generally accepted criterion that estrogens bind to the uterine cytoplasmic receptor forming an estrogen–receptor complex as a first step in the uterine response to estrogen, and that this complex is subsequently transferred to the nucleus [16–18].

EXPERIMENTAL

Sprague-Dawley rats (Simonson Laboratories, Gilroy, Calif.) 22–23 days of age, were killed by cervical dislocation. Uteri were rapidly removed, stripped of adhering fat and homogenized in 0·04 M Tris-HCl, 1·5 mM EDTA, pH 7·4, at 4° in motor-driven ground glass homogenizers (Kontes). To examine cytoplasmic

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§ o,p'-DDT, 2(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-1,1,1-trichloroethane; p,p'-DDT, 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane; m,p'-DDD, 2(*m*-chlorophenyl)-2-(*p*-chlorophenyl)-1,1-dichloroethane; p,p'-DDD, 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethane; o,p'-DDD, 2(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-1,1-dichloroethylene; o,p'-DDE, 2(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-1,1-dichloroethylene; p,p'-DDE, 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene; and DDA, bis-(*p*-chlorophenyl)-1,1-dichloroacetate.

binding *in vitro*, the cytosol was obtained by centrifuging the homogenate at 105,000 g_{max} at 4° for 90 min using either a 30 or 50 rotor in a Beckman L-2 ultracentrifuge. Portions of the cytosol were incubated at 4° with chlorinated hydrocarbons, o,p'-DDT, p,p'-DDT, m,p'-DDD, p,p'-DDD, o,p'-DDD, o,p'-DDE, p,p'-DDE or DDA (Aldrich Chemicals), for 1 hr and for an additional 30 min with ^{3}H-6,7-estradiol-17β (New England Nuclear, 55 Ci/m-mole, purified by celite and paper chromatography). The chlorinated hydrocarbons were solubilized in ethanol. The concentration of ethanol in the incubated cytosol was 1%. The incubated cytosol (0·2 ml) was layered onto 6 ml linear 5-20% sucrose density gradients and centrifuged at 151,000 g_{max} at 4° for 15 hr using a SW-41 rotor. Sucrose solutions were prepared in 0·04 M Tris-HCl, 1·5 mM EDTA, pH 7·4, at 4°. Gradients were fractionated by drops. The radioactivity in each 20-drop fraction was determined in a Packard Tri-Carb model 526 scintillation counter using 3 ml ethanol and 10 ml scintillation fluid (0·5% PPO,* 0·03% POPOP in toluene). Counting efficiency of 19–21 per cent was determined using internal standards. Sedimentation coefficients were determined by the method of Martin and Ames [19] using yeast alcohol dehydrogenase (Worthington) as a standard, and protein concentrations by the method of Lowry *et al.* [20].

Nuclear binding *in vitro* was examined using homogenates prepared as described above and incubating the homogenate for 30 min at 25° with ^{3}H-estradiol-17β with or without chlorinated hydrocarbons or unlabeled estradiol-17β. The homogenate was centrifuged at 900 g at 4° for 20 min and the pellet washed five times with 5 ml of the buffer. The final sediment was suspended in 0·8 M KCl, 0·04 M Tris-HCl, pH 8·5 at 4°, homogenized gently and mixed vigorously once every 5 min for 1 hr, and centrifuged at 18,000 g at 4° for 1 hr. The radioactivity in the supernatant (nuclear extract) was determined either on 5–20% sucrose density gradients made with the 0·8 M KCl, 0·04 M Tris-HCl buffer or by quantitating a portion of the radioactive extracts directly in the scintillation counter.

Binding studies *in vivo* utilized 24-day-old ovariectomized female rats injected intraperitoneally with 1 μg estradiol-17β (Searle, 3 × crystallized) in 0·1 ml dimethylsulfoxide (DMSO, Aldrich), 1·5 mg o,p'-DDT in 0·1 ml DMSO, 1·5 mg p,p'-DDT in 0·1 ml DMSO, or the vehicle alone. Two hr after injection, animals were killed by cervical dislocation, uteri were immediately removed, and stripped of adhering fat. The uteri were homogenized in the 0·04 M Tris-HCl, 1·5 mM EDTA buffer (5:1, v/w), and the cytosol was obtained as previously described. The cytosol was incubated with 1×10^{-9} M ^{3}H-estradiol-17β for 30 min at 4°; 0·2 ml was then layered onto 5-20% sucrose density gradients which were processed as for the cytoplasmic binding studies *in vitro*.

The concentration of chlorinated hydrocarbons bound by the receptor could not be measured directly, because isotopes of these compounds with sufficiently high specific activities were unavailable.

* PPO, 2,5-diphenyloxazole; and POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene.

RESULTS

The competition of the different hydrocarbons studied with the binding of ^{3}H-estradiol-17β to the uterine cytoplasmic estrogen receptors is shown in Table 1. Under the conditions described in the legend, o,p'-DDT decreased ^{3}H-estradiol-17β binding by 40 per cent ($P < 0·001$), and o,p'-DDE slightly decreased the ability of ^{3}H-estradiol-17β to bind to uterine cytoplasm, while m,p'-DDD, p,p'-DDT, p,p'-DDE, p,p'-DDD, o,p'-DDD and DDA had no significant effect on estradiol binding. Similar results were obtained when the estradiol-17β cytoplasmic binding capacity was assayed using the method described by Clark and Gorski [21] that depends upon the adherence of the estrogen-receptor complex to glass or Alumina particles. Progesterone (1×10^{-5} M) did not compete with ^{3}H-estradiol-17β ($8·7 \times 10^{-9}$ M) binding. Unlabeled estradiol-17β, however, at $8·7 \times 10^{-8}$ M completely inhibited ^{3}H-estradiol-17β binding, while an equimolar concentration of unlabeled estradiol-17β inhibited the labeled estradiol-17β binding by 50–55 per cent.

The sedimentation rate of the uterine cytoplasmic estrogen receptor on linear sucrose density gradients can be varied according to the ionic conditions under which the cytosol is prepared and maintained. This fact suggests that the receptors are either composed of subunits or have a structure such that their conformation can be changed drastically but still retain the ability to bind estradiol-17β [22–25]. When chlorinated hydrocarbons are incubated with uterine cytosol at 4°, the sedimentation rate of the estradiol receptor complex is not altered, as is shown in Fig. 1. The profiles of radioactivity on the sucrose density gradients are similar whether or not the cytosol is exposed to chlorinated hydrocarbons, with the peak of radioactivity sedimenting at about 8·5 S. The chlorinated hydrocarbons, listed in Table 1, apparently did not alter the conformation or subunit structure of the receptor as seen on the gradients.

Table 1. Competition of chlorinated hydrocarbons with ^{3}H-estradiol-17β binding in uterine cytosol*

Competitor	^{3}H-estradiol bound (pmoles/mg protein ± S.E.)
Control (8)	0·802 ± 0·056
o,p'-DDT (6)	0·472 ± 0·009†
o,p'-DDE (4)	0·700 ± 0·021
o,p'-DDD (3)	0·835 ± 0·035
m,p'-DDD (3)	0·870 ± 0·031
p,p'-DDT (5)	0·857 ± 0·014
p,p'-DDE (3)	0·804 ± 0·043
p,p'-DDD (3)	0·810 ± 0·034
DDA (3)	0·832 ± 0·024

*Uteri from twenty-five 23-day-old rats were homogenized (5:1, v/w) in 0·04 M Tris-HCl, 1·5 mM EDTA, pH 7·4 and the cytosol was incubated with $1·4 \times 10^{-4}$ M chlorinated hydrocarbon (1% ethanol) for 1 hr followed by incubation with $8·7 \times 10^{-9}$ M ^{3}H-estradiol-17β for 30 min at 4°. ^{3}H-estradiol-17β binding was measured using sucrose density gradients. Numbers in parenthesis indicate the number of groups of twenty-five animals used for each hydrocarbon.

† Level of significant difference from control $P < 0·001$.

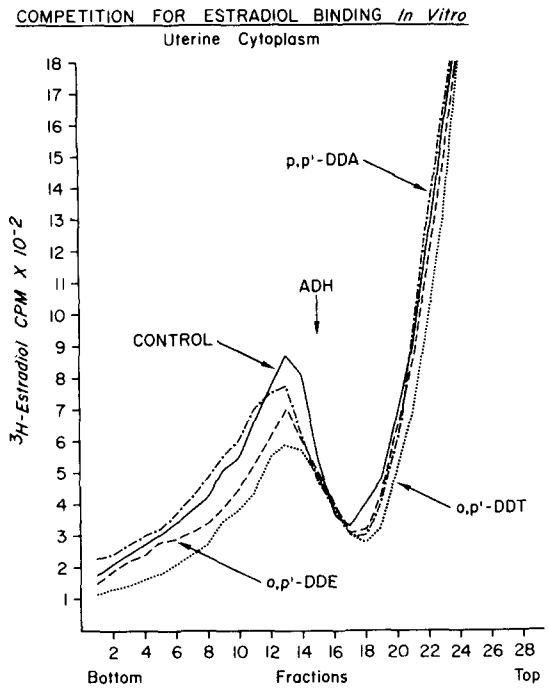

Fig. 1. Sucrose density gradients of inhibition of ^{3}H-estradiol-17β binding to uterine cytosol receptors. Cytosol preparation is the same as described in Table 1. The $1{\cdot}4 \times 10^{-4}$ M competitors, and control, both with 1% ethanol, were incubated with cytosol for 1 hr and subsequently with $8{\cdot}7 \times 10^{-9}$ M ^{3}H-estradiol-17β for 30 min. Yeast alcohol dehydrogenase (ADH) was used as a standard.

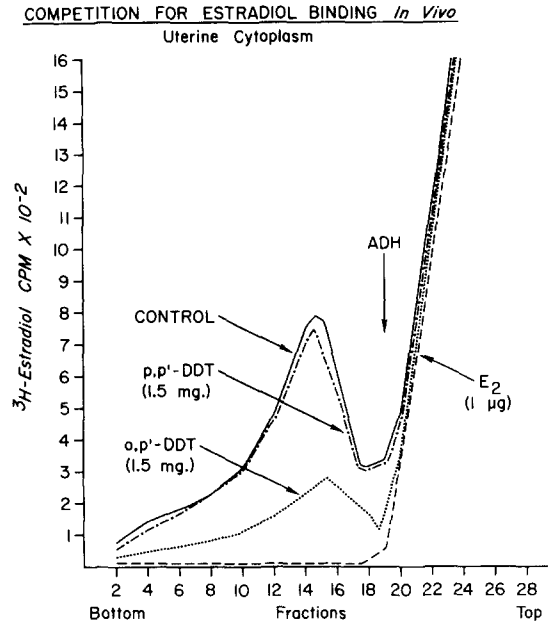

Fig. 3. Chlorinated hydrocarbon binding to immature rat uteri *in vivo*. Rats were ovariectomized on day 15 and when 24 days old were injected intraperitoneally (five rats/group) with either 1 µg estradiol-17β, 1·5 mg o,p'-DDT, 1·5 mg p,p'-DDT, or the vehicle, 0·1 ml DMSO. Two hr later uteri were removed and cytosol was prepared as described in the text, and binding was measured by sucrose density gradients. Yeast alcohol dehydrogenase (ADH) was used as a standard. E_2 denotes estradiol-17β.

The ability of the hydrocarbons to inhibit estradiol binding to the cytoplasmic receptor was examined further by varying the concentrations of hydrocarbon incubated with the cytosol. Figure 2 shows that $0{\cdot}175 \times 10^{-4}$ M o,p'-DDT produces no effect on ^{3}H-estradiol-17β binding, but a significant inhibition in binding appears at $1{\cdot}4 \times 10^{-4}$ M ($P < 0{\cdot}001$). o,p'-DDE, however, is even less inhibitory at these concentrations. Complete inhibition of estradiol binding was not achieved with either o,p'-DDT or o,p'-DDE, even at concentrations of maximum solubility ($1{\cdot}4 \times 10^{-4}$ M) [26].

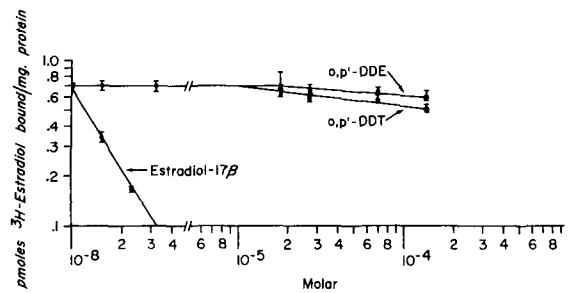

Fig. 2. Competition of ^{3}H-estradiol-17β binding with increasing concentrations of o,p'-DDT, o,p'-DDE and estradiol-17β. Cytosol fractions were prepared as in Table 1 and were incubated with the indicated concentrations of either o,p'-DDT, o,p'-DDE or estradiol-17β for 1 hr followed by incubation with $8{\cdot}7 \times 10^{-9}$ M ^{3}H-estradiol-17β for 30 min. Each point represents the mean ± S. E. M. of six determinations for estradiol 17-β and o,p'-DDT and of four determinations for o,p'-DDE.

The inhibition *in vitro* by o,p'-DDT of estradiol-17β binding to the 8·5 S cytoplasmic receptor was confirmed *in vivo* by injecting o,p'-DDT or p,p'-DDT into immature ovariectomized rats and examining the subsequent ability of the cytosol to bind ^{3}H-estradiol-17β. Figure 3 shows that the 8·5 S peak of ^{3}H-estradiol-17β binding subsequently is reduced by 50–60 per cent when animals were pretreated for 2 hr with o,p'-DDT. Unlabeled estradiol, however, at a 1000-fold lower dose completely eliminated binding of the labeled steroid. P,p'-DDT had no apparent effect on the binding of ^{3}H-estradiol to the 8·5 S receptor.

According to the current concept of steroid hormone–receptor interaction, if o,p'-DDT acts intrinsically as an estrogen in the uterus, as the above data on cytosol binding indicate, it should also inhibit the transfer of ^{3}H-estradiol to the nuclei of uterine cells. Figure 4 illustrates that ^{3}H-estradiol-17β can be extracted from nuclei bound to a 5 S moiety under the conditions described in the legend. The quantity of estradiol-17β binding is decreased by 40 per cent in the presence of $1{\cdot}4 \times 10^{-4}$ M o,p'-DDT, and reduced slightly with $1{\cdot}4 \times 10^{-4}$ M o,p'-DDE, while p,p'-DDT and o,p'-DDD have no effect. Estradiol-17β at 100 times the concentration of the labeled steroid completely inhibited nuclear binding of the isotope.

DISCUSSION

We conclude from these observations that o,p'-DDT and to a lesser extent o,p'-DDE compete with estradiol binding to the uterine 8·5 S cytoplasmic and the 5 S nuclear estrogen receptors in a completely *in*

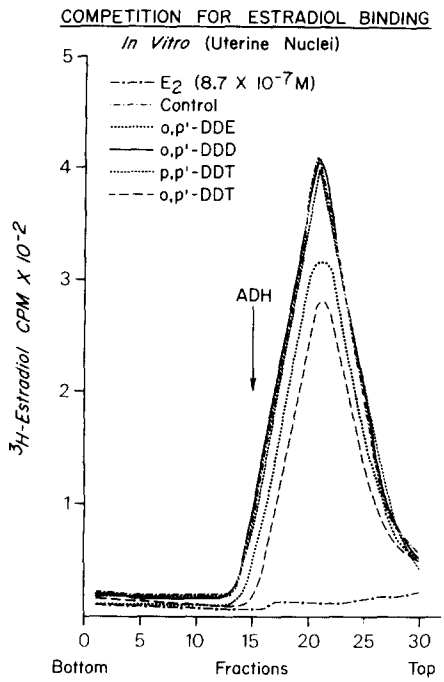

Fig. 4. Nuclear binding of ^{3}H-estradiol-17β after cell-free incubation of uterine homogenates *in vitro* with chlorinated hydrocarbons. Uteri were homogenized in 0·01 M Tris-HCl, 0·1 M KCl, 1 mM EDTA, pH 7·4 at 25° (5:1, v/w). Portions of the homogenate were incubated with $8{\cdot}7 \times 10^{-9}$ M ^{3}H-estradiol-17β and the indicated competitors, or nothing as control for 30 min at 25° and treated as described in the text. Sucrose density gradients were centrifuged at 151,000 g_{max} for 17 hr. Yeast alcohol dehydrogenase (ADH) was used as a standard. E_2 denotes estradiol-17β.

vitro system in which metabolism should be minimal. These findings generally support the results of Nelson [27] who demonstrated, using the charcoal binding assay, that o,p'-DDT competed with estradiol binding in rat uterine cytosol. These findings provide strong evidence that these compounds may themselves be estrogenic and that metabolism in the liver or elsewhere is unnecessary for them to compete with estradiol binding. Metabolic studies have indicated that o,p'-DDT is not isomerically converted to p,p'-DDT prior to its conversion to DDE in rat, sheep, chicken and quail [28], and that DDE is the principal metabolite of DDT stored in rats as well as humans [29].

The need for $10^3 - 10^4$ larger concentrations of the chlorinated hydrocarbons than of estradiol-17β to compete with ^{3}H-estradiol binding agrees with Cecil *et al.* [8] who report that 10^4 times more o,p'-DDT than estradiol is needed *in vivo* to yield similar estrogenic responses in rat uteri based on changes in wet weight, water content, RNA and glycogen. However, the sensitivity of tissues to estrogens is known to change with age, and exposure to estrogens at critical periods of development may result in permanent endocrine reproductive changes [13]. In many mammals, including humans, this critical period occurs during fetal development. Chlorinated hydrocarbons are known to pass through the placenta and DDT in the fetal environment has been implicated in relative sterility and oligo-ovulation of humans [30, 31]. Whether this relationship indeed exists and whether it exists because o,p'-DDT and other chlorinated hydrocarbons or perhaps their total amounts are slightly estrogenic and bind to cytoplasmic and nuclear receptors need to be established.

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