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SHORT COMMUNICATIONS

Effects of dichlorodiphenyltrichloroethane (DDT) analogs and polychlorinated biphenyl (PCB) mixtures on 17β-[^{3}H]estradiol binding to rat uterine receptor*

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THE ESTROGENIC activity of several dichlorodiphenyltrichloroethane (DDT) analogs,[1–5] as well as some polychlorinated biphenyl (PCB) mixtures,[2] is well established. Some structural similarities exist between these compounds and the potent, nonsteroidal estrogen, diethylstilbestrol.[2] On the bases of structural considerations and the observation that carbon tetrachloride pretreatment prevents the increase in uterine weight produced by *o,p'*-DDT† in the rat, it has been postulated that phenolic metabolites might account for the estrogenic activity.[2,4–6] Since interference with normal hormonal processes by the chlorinated hydrocarbons may be related to some of the detrimental effects of these substances in the environment,[6] it is desirable to learn which structural features and metabolites are related to estrogenic or antiestrogenic activity.

One of the initial events for estrogen action is the binding of the estrogenic substance with a specific cytoplasmic receptor.[7] Indirect evidence that the active DDT analogs or their metabolites also produce estrogen-like effects via interaction with the estrogen receptor *in vivo* has been obtained.[3–5] This communication describes the inhibition by some of these compounds of ^{3}H-estradiol binding *in vitro* to the cytoplasmic receptor from rat uterus. The results indicate a positive correlation between inhibition of binding *in vitro* and estrogenic action *in vivo*. With the reservation that affinity for the estrogen receptor may not be associated with agonist properties, the correlation suggests that some of these compounds may be estrogenic *per se*.

The sources of materials used were: 17β-[6,7-^{3}H]estradiol (48 Ci/m-mole), New England Nuclear Corp.; diethylstilbestrol, Eli Lilly & Co.; Dextran T40, Pharmacia Fine Chemicals, Inc.; *p,p'*-DDT and *p,p'*-DDE, Montrose Chemical Corp. of California; *p,p'*-DDD, Analabs, Inc.; *o,p'*-DDT, *o,p'*-DDE and *m,p'*-DDD, Aldrich Chemical Co.; *o,p'*-DDD and technical grade methoxychlor (technical methoxychlor), Sigma Chemical Co.; purified methoxychlor (methoxychlor), E. I. duPont de Nemours & Co.; Arochlor 1221 and Arochlor 1254 (commercial polychlorinated biphenyl mixtures containing 21 and 54 per cent chlorine), Monsanto Co. All other chemicals were obtained from Sigma Chemical Co.

Rat uterus supernatant was prepared by homogenizing the uteri from three or four Sprague–Dawley rats (2–5-months-old) in 10 ml of 10 mM Tris HCl and 1·5 mM EDTA, pH 7·4. The supernatant obtained after centrifugation at 105,000 *g* for 60 min was strained through four layers of cheesecloth and diluted to contain approximately 2 mg protein/ml. The binding assay was performed by adding 400 μg of the supernatant protein to 0·5 pmole of ^{3}H-estradiol (48 Ci/m-mole) in a final volume of 0·25 ml. The ^{3}H-estradiol concentration was, therefore, 2 nM. After incubating for 1 hr at 4°, 0·5 ml of a suspension containing 0·5 per cent activated charcoal and 0·05 per cent Dextran T40 in 10 mM Tris-HCl (pH 7·4) was added. The tubes were then allowed to stand for 15 min at 4°. After centrifugation at 2000 *g* for 5 min, the radioactivity remaining in the supernatant was determined by liquid scintillation counting. This radioactivity represents a measure of bound ^{3}H-estradiol.[7–9] Under these conditions, the bound radioactivity was reduced to 10 per cent or less of control values in the presence of excess diethylstilbestrol. On the other hand, testosterone, progesterone, aldosterone or ouabain were without effect on binding when present at concentrations as high as 10 μM. The chlorinated hydrocarbons were added to the binding

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†Abbreviations used are: *p,p'*-DDT, 1,1,1-trichloro-2, 2-bis(*p*-chlorophenyl)ethane; *o,p'*-DDT, 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane; *p,p'*-DDD, 1,1-dichloro-2, 2-bis(*p*-chlorophenyl) ethane; *m,p'*-DDD, 1,1-dichloro-2-(*p*-chlorophenyl)-2-(*m*-chlorophenyl)ethane; *o,p'*-DDD, 1,1-dichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane; *o,p'*-DDE, 1,1-dichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl) ethylene; *p,p'*-DDE, 1,1-dichloro-2, 2-bis(*p*-chlorophenyl)ethylene; *p,p'*-methoxychlor, 1,1,1-trichloro-2, 2-bis(*p*-methoxyphenyl)ethane; *o,p'*-methoxychlor, 1,1,1-trichloro-2-(*o*-methoxyphenyl)-2-(*p*-methoxyphenyl)ethane; and ^{3}H-estradiol, 17β-[6,7-^{3}H]estradiol.

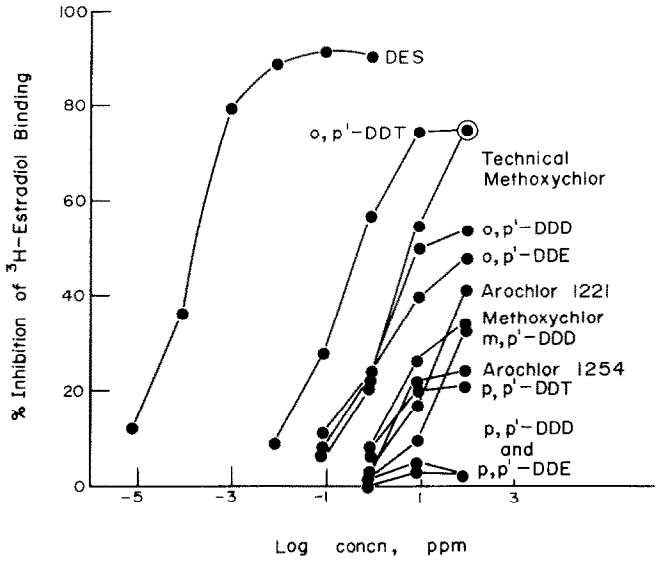

FIG. 1. Inhibition by chlorinated hydrocarbons of ^{3}H-estradiol binding to rat uterus cytosol *in vitro*. The values shown are means for determinations using three separate uterine and drug preparations. Standard errors of the means were ±10 per cent or less of the values shown. DES = diethylstilbestrol.

assay as solutions in absolute ethanol to give a final ethanol concentration of 4 per cent. To insure solubility of these compounds in the dose-response determinations (see Fig. 1), propylene glycol was also added to give a final concentration of 16 per cent. The ethanol and propylene glycol at these concentrations were without effect on ^{3}H-estradiol binding.

Uterotropic activity *in vivo* was determined by measuring the increase in uterine wet weight of 20 25-day-old rats exactly as described by Welch *et al.*[5]

Some of the DDT analogs, particularly the *o,p'*-isomers, are effective inhibitors of specific ^{3}H-estradiol binding to the rat uterine receptor (Fig. 1). This observation confirms experiments *in vivo* of others which suggest that these compounds interact with the receptor.[3 5]

Of the chlorinated hydrocarbons studied, *o,p'*-DDT was the most potent inhibitor of ^{3}H-estradiol binding *in vitro* (Fig. 1). This substance produced 50 per cent inhibition of binding at a concentration of about 0·6 ppm (2 μM), whereas only approximately 0·2 ppb (1 nM) of diethylstilbestrol was required to produce the same effect. Therefore, the order of activity in this assay for *o,p'*-DDT is about 1/2000 that of diethylstilbestrol. The other chlorinated hydrocarbons are less active than *o,p'*-DDT by orders of magnitude, i.e. concentrations of about 10–100 ppm or greater are required to inhibit binding by 50 per cent. Concentrations as high as 100 ppm may have approached limits of solubility of these agents in the solvent system used, since some curves appeared to flatten between concentrations of 10 and 100 ppm. Consequently, higher concentrations were not tested. Some structure activity relationships can be surmized from examination of Fig. 1. In the series, *o,p'*-DDD, *m,p'*-DDD and *p,p'*-DDD, activity decreased in that order. For the series of *o,p'*-isomers of DDT, DDD and DDE, activity decreases in the order DDT > DDD ~ DDE. For the *p,p'*-isomers, a similar relationship appears to exist. The *o,p'*-isomer of methoxychlor is likely to be more active than the *p,p'*-isomer since technical methoxychlor (*p,p'*- + *o,p'*-isomers) was more active than the purified methoxychlor (*p,p'*-isomer). Arochlor 1221 and Arochlor 1254 produced similar effects at 1 and 10 ppm, but Arochlor 1221 was more active at 100 ppm.

When the chlorinated hydrocarbons shown in Fig. 1 were administered to immature rats at a dose of 50 mg/kg, i.p., the increase in uterine wet weight observed 6 hr later was statistically significant ($P < 0.05$) for all the compounds which produced greater than 30 per cent inhibition of binding at the highest dose level, 100 ppm. The change in uterine weight was not significant ($P > 0.05$) for the four compounds which produced less than 30 per cent inhibition of binding at 100 ppm. These data are in general agreement with the results obtained by others.[2,5] There was a significant, positive correlation between inhibition of binding *in vitro* and the increase in uterine wet weight observed *in vivo* for all the compounds studied (Fig. 2). This observation suggests that the agents are active estrogens as such; however, inhibition of ^{3}H-estradiol binding does not distinguish between estrogenic and antiestrogenic properties. Since a phenolic hydroxyl group appears to be essential for estrogenic activity among steroidal[10] as well as

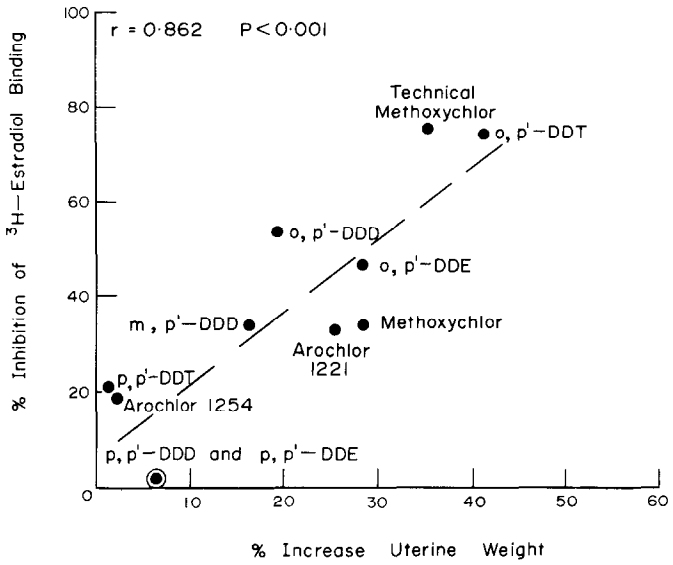

FIG. 2. Correlation between inhibition by chlorinated hydrocarbons of ^{3}H-estradiol binding *in vitro* and their uterotropic effect *in vivo*. Inhibition of binding was measured at 100 ppm (Fig. 1), and uterine weights were determined 6 hr after administration of 50 mg/kg, i.p., to immature rats. Mean values for three binding assays and uterine weights from groups of five mice are given.

nonsteroidal[11] compounds, hydroxylation of the chlorinated hydrocarbons to phenolic metabolites may be required for estrogenic activity. Such a requirement would be consistent with the observation that carbon tetrachloride pretreatment prevents the uterotropic action of *o,p'*-DDT in the immature rat.[5] Carbon tetrachloride pretreatment (0·67 ml/kg, 24 hr) of immature rats did not alter the amount of ^{3}H-estradiol bound to the uterine supernatant *in vitro*, nor did it alter the inhibitory effect of *o,p'*-DDT on the ^{3}H-estradiol binding.* Therefore, the effect of carbon tetrachloride reported by Welch *et al.*[5] may be

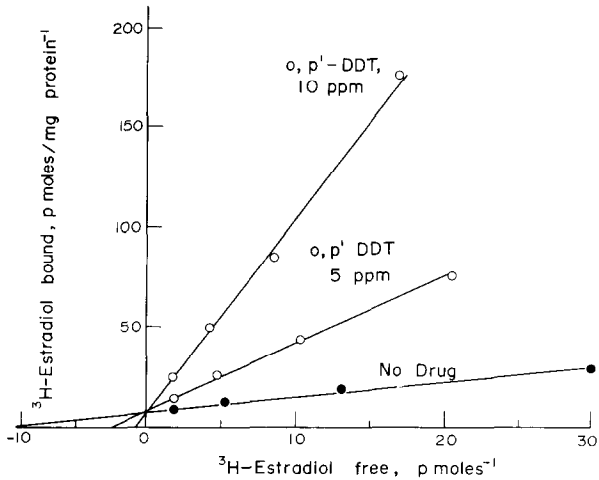

FIG. 3. Competitive inhibition by *o,p'*-DDT of ^{3}H-estradiol binding to rat uterine receptor. The supernatant was incubated under the conditions described above in the presence of various concentrations of ^{3}H-estradiol. The free ^{3}H-estradiol was computed as the difference between the total and bound ^{3}H-estradiol.

* Unpublished observation.

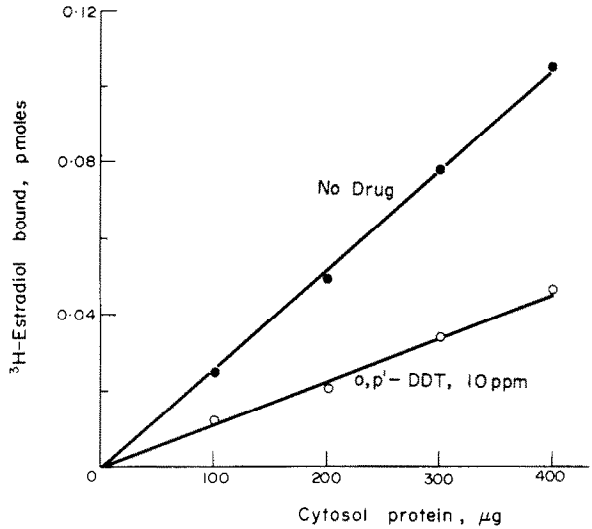

FIG. 4. Inhibition by *o,p'*-DDT of ^{3}H-estradiol binding to rat uterine receptor in the presence of various amounts of cytoplasmic protein. Incubation of ^{3}H-estradiol with the indicated amounts of rat uterine supernatant was performed as described above.

due to the inhibition of *o,p'*-DDT hydroxylation by a mixed function oxidase of the liver. Several phenolic metabolites of *o,p'*-DDT have recently been reported in the rat.[12]

The inhibition of ^{3}H-estradiol binding produced by *o,p'*-DDT appears to be competitive by a double reciprocal plot analysis (Fig. 3). This is consistent with an interaction of *o,p'*-DDT at the specific binding site on the cytoplasmic receptor. The apparent dissociation constant for estradiol determined by this method is 4×10^{-10} M, which agrees well with previously reported values.[7] When the amount of cytoplasmic protein was varied from 100 to 400 μg in the binding assay, no effect on the degree of inhibition produced by *o,p'*-DDT was observed (Fig. 4). This finding suggests that under these conditions, binding of *o,p'*-DDT to protein molecules other than the estrogen receptor was not sufficient to alter the availability of the drug to the estrogen-binding site. The constant degree of inhibition at various levels of receptor protein also suggests that the inhibition is reversible.[13]

The finding that some of the chlorinated hydrocarbons are effective inhibitors of ^{3}H-estradiol binding at concentrations in the 1–10 ppm range (Fig. 1) suggests that interaction with the estrogen receptor *in vivo* may occur after environmental exposure to these agents. *p,p'*-DDT enhancement of estradiol metabolism through induction of liver enzymes may reduce the levels of this estrogen in the blood of treated doves.[14] The commercial DDT preparation contains both the *p,p'*- and *o,p'*-isomers.[1] A cooperative effect may occur in intact animals, therefore, since the interaction of *o,p'*-DDT with the estrogen receptor is competitive (Fig. 3). Reduced levels of estradiol may permit more of the *o,p'*-DDT to bind to the receptor, thereby magnifying the estrogenic or antiestrogenic effects of the exogenous substance.

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Biliary excretion of metabolities of bromobenzene

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THIS LABORATORY has previously reported that bromobenzene is converted by liver microsomes to an arene oxide intermediate which binds covalently with protein and perhaps other tissue macromolecules.[1,2] The degree of covalent binding of this intermediate to tissue macromolecules appears to be directly related to the hepatotoxic properties of bromobenzene.[1–4] The arene oxide, however, also reacts with glutathione (GSH) to form a bromobenzene–GSH conjugate,[1,5] which ultimately is converted to bromophenylmercapturic acid, one of the major urinary metabolities of bromobenzene.[6] Since it has been shown that drug conjugates having a molecular weight greater than 300 are often excreted in the bile of rats[7,8] and since the molecular weight of the bromobenzene–GSH conjugate is 480, it seemed possible that this conjugate might be excreted in the bile of rats. The present study not only confirms this view, but also shows that the biliary excretion of the bromobenzene–GSH conjugate is increased by prior treatment of rats with phenobarbital and is decreased by the prior administration of diethylaminoethyl[2,2]–diphenylvalerate HCl (SKF 525-A).

Male Sprague–Dawley rats (Hormone Assay) weighing 200–220 g were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), the bile ducts were cannulated with polyethylene No. 10 tubing, and bile was collected in preweighed vials. A heating lamp was placed over each animal to maintain normal body temperature. After an initial 30-min collection period, ^{14}C-bromobenzene dissolved in rat plasma was administered via the femoral vein at a dose of 20 mg/kg (sp. act., 2 μCi/μmole) and bile was collected in the preweighed vials at intervals up to 3 hr. This dose of bromobenzene was selected because of its limited solubility in rat plasma and because it produced no apparent histological changes in the liver at 3 hr. Three hr after the administration of bromobenzene, the animals were sacrificed and urine was collected from the bladder. Since heptane extraction of the bile or urine removed no radioactivity, it was assumed that no free bromobenzene was excreted in these fluids. Therefore, the total metabolites excreted were determined by liquid scintillation counting of aliquots of bile or urine. Aliquots of bile and urine were chromatographed on Whatman No. 3 MM paper, developed with butanol–acetic acid–water (4:1:2) and then scanned to reveal radiolabeled peaks corresponding to the various metabolites of bromobenzene.

Figure 1 indicates the cumulative excretion of ^{14}C-bromobenzene metabolites appearing in the bile. Each point represents the mean value for five to six animals and is expressed as the per cent of administered ^{14}C-bromobenzene. In these experiments, the effects of pretreatment with phenobarbital (80 mg/kg, i.p., daily for 3 days) or SKF 525-A (75 mg/kg, i.p., 2 hr before bromobenzene) on the biliary excretion of bromobenzene metabolites were also investigated. Within the first 30 min., 11 per cent of the administered ^{14}C-bromobenzene appeared in the bile of control rats as bromobenzene metabolites. Thirty-five per cent was present in the bile of phenobarbital-induced rats and only 3 per cent was present in the bile of rats pretreated with SKF 525-A. During the second 30-min collection period, 18 per cent of the administered dose was excreted in the control and phenobarbital-pretreated