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Estrogenic activity of the insecticide chlordecone (Kepone) and interaction with uterine estrogen receptors

(reproductive tissues/insecticide toxicology/uterine growth/progesterone receptor)

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ABSTRACT **The chlorinated insecticide chlordecone (Kepone) interacts with the estrogen receptor system in the rat uterus *in vitro* and *in vivo*. It competes with estradiol for binding to the cytoplasmic receptor *in vitro* and also induces nuclear accumulation of estrogen receptor sites in uteri *in vitro*. When injected into immature rats, chlordecone translocates estrogen receptor sites to the uterine nucleus, increases uterine weight, and stimulates the synthesis of the progesterone receptor, an estrogen receptor-mediated process. Its slow onset of action but prolonged duration of interaction with estrogen receptor and stimulation of uterine weight gain and progesterone receptor synthesis indicates that, although it has an affinity for receptor only 0.01–0.04% that of estradiol, its considerable estrogenic activity may likely be derived from its long half-life and bioaccumulative character.**

Reproductive disorders (namely, sterility as judged by oligospermia and hypomotile sperm) have been observed in male employees at a chlordecone (Kepone) manufacturing plant and have been associated with exposure to high levels of this chlorinated insecticide (1). Previous examination of the toxicology of chlordecone in animals suggested that it might possess estrogenic properties: it induced constant estrus in mice (2), suppressed spermatogenesis in male quail (3), and caused hypertrophy of the oviduct in female quail (4). Similar toxicological effects have been reported for another chlorinated insecticide, 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane (*o,p'*-DDT), a contaminant in technical DDT (5, 6). Hence, in these studies our aim was to determine whether chlordecone, a caged ring compound with a structure very different from that of both steroidal and nonsteroidal estrogens (see Fig. 1), was capable of interacting with the estrogen receptor system in a manner that might explain its estrogenic and growth-promoting activity. To study these interactions, we have used the uterus of the immature rat, which is a well-studied model tissue for estrogen action (7, 8). We have quantitated and characterized the interaction of chlordecone with the uterine estrogen receptor system *in vitro* and *in vivo* and have found that, although chlordecone has a low but clearly detectable affinity for the estrogen receptor, its persistent character results in long-term interaction with the estrogen receptor that produces a pronounced stimulation of uterine growth. Chlordecone is shown to be considerably more potent as an estrogen than is *o,p'*-DDT in this rat uterine system.

MATERIALS AND METHODS

Materials. Immature female rats (20–24 days old; Holtzman, Madison, WI) were used in this study. [6,7-^{3}H]Estradiol (49.3 or 54.3 Ci/mmol, New England Nuclear; 1 Ci = 3.7×10^{10} becquerels) was checked for purity by silica-gel thin-layer chromatography (9) and found to be greater than 98% pure. The synthetic progestin R5020 (17,21-[17α-*methyl*-^{3}H]dimethyl-19-norpregna-4,9-diene-3,20-dione; 86 Ci/mmol; radiochemical purity verified by thin-layer chromatography) was from New England Nuclear. Chlordecone (Kepone; decachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalen-2-one) was kindly provided by the Quality Assurance Section, Environmental Toxicology Division, Environmental Protection Agency; *o,p'*-DDT was obtained from Aldrich, and Mirex (dodecachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalene) from Allied Chemical (see Fig. 1). Other radioinert compounds were estradiol-17β (Schwarz/Mann) and diethylstilbestrol (Sigma). Unlabeled R5020 was kindly provided by J. P. Raynaud (Roussel-UCLAF, Romainville, France). The charcoal/dextran slurry contained 5% acid-washed Norit A (Sigma) and 0.5% dextran C (Schwarz/Mann) in 10 mM Tris·HCl/1.5 mM EDTA/0.02% sodium azide, pH 7.4 at 25°C.

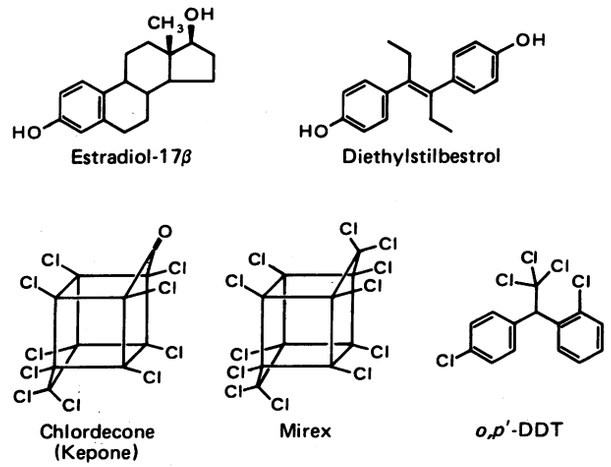

FIG. 1. Structures of the steroidal estrogen, estradiol-17β, the nonsteroidal estrogen, diethylstilbestrol, and the three chlorinated insecticides investigated in this study.

Determination of Uterotrophic Activity. Groups of five rats each received subcutaneous injections of various amounts of compound or estradiol-17β in 0.1 ml of sesame oil daily for 3 days and were killed 24 hr after the last administration. The uteri were excised and weighed. The increase in uterine weight was taken as a measure of uterotrophic activity.

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Abbreviations: *o,p'*-DDT, 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane; TE buffer, 10 mM Tris·HCl/1.5 mM EDTA, pH 7.4.

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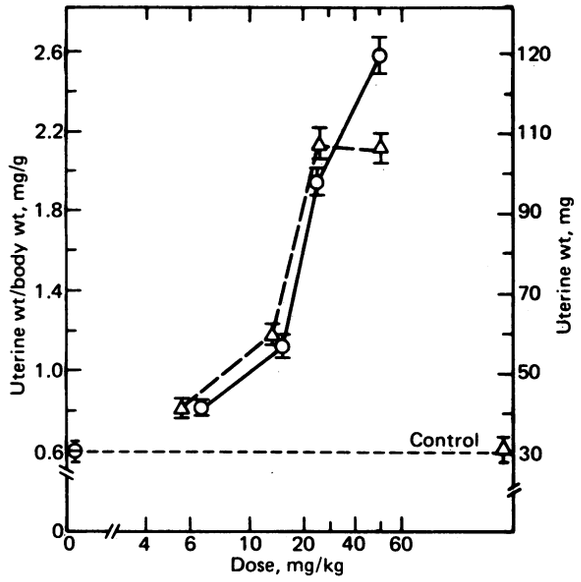

FIG. 2. Dose–response curves showing the uterotrophic activity of chlordecone. Rats (21 days old) were injected subcutaneously with the indicated daily dose of chlordecone in 0.1 ml of sesame oil once daily at 24-hr intervals on 3 successive days and uterine wet weights were determined 24 hr after the last injection. Control animals received oil alone. Each value is the mean of determinations from at least five individual animals ± SEM. o — o, Uterine weight/body weight; Δ - - - Δ, uterine weight.

Determination of Nuclear or Cytosol Binding Sites by [^{3}H]Estradiol Exchange. At indicated times after exposure to compound or vehicle control *in vivo* or *in vitro*, uteri (three per group) were thoroughly rinsed with 20 ml of iced 10 mM Tris·HCl/1.5 mM EDTA, pH 7.4 at 25°C (TE buffer) and then homogenized with Kontes all-glass tissue grinders in 1.3 ml of iced TE buffer containing 0.02% sodium azide. Nuclear binding sites were determined in the washed 800 × 20-min nuclear pellet by the exchange method of Anderson *et al.* (10) with slight modification (11), by using 10 nM [^{3}H]estradiol with and without 1000 nM unlabeled diethylstilbestrol in the exchange incubations. The cytosol binding and exchange assay of Katzenellenbogen *et al.* (12) was performed on the 180,000 × *g*, 60-min supernatant (cytosol) and was used to determine specific binding sites, both empty sites and sites previously filled with ligand.

Competition of Chlordecone and *o,p'*-DDT with [^{3}H]-Estradiol for Binding to Cytosol. The binding ability of chlordecone and *o,p'*-DDT relative to estradiol was determined by competitive protein binding to cell-free uterine cytosol *in vitro* as described (13) with minor modification. Aliquots of uterine cytosol (50 μl; two uteri per ml in TE buffer; 180,000 × *g* for 60 min) were added to a mixture of 10 μl of 70 nM [^{3}H]estradiol in TE buffer and 10 μl of various concentrations of unlabeled competitor (in dimethylformamide/TE buffer, 1:1, vol/vol) to give final incubations that were 10 nM in [^{3}H]estradiol, 1 nM–0.1 mM (12 concentrations in duplicate) in competitor, and 1.4 uteri per ml. After 16 hr at 0°C, the free steroids were adsorbed by dextran/charcoal (10 μl of 0.5% Dextran C/5% Norit A in TE buffer) for 15 min at 0°C; radioactivity of the aliquots was measured after centrifugation at 800 × *g* for 15 min. The relative binding ability of the competitors is taken as the ratio of concentrations of unlabeled estradiol/competitor required to inhibit 50% of specific [^{3}H]-estradiol binding (14).

Cytosol Binding of [^{3}H]R5020. Uterine progesterone receptor content was measured by the binding of the synthetic progestin [^{3}H]R5020 (15). Uteri were homogenized in iced 10 mM Tris·HCl/1.5 mM EDTA/12 mM thioglycerol/20% (vol/vol) glycerol, pH 7.4 at 25°C (two uteri per 0.8 ml of buffer) with motor-driven Kontes all-glass tissue grinders. The homogenates were centrifuged at 180,000 × *g* for 60 min at 4°C in a Beckman L2-65B ultracentrifuge (type 65 rotor, 46,000 rpm) to yield the high-speed supernatant (cytosol). The cytosols were pretreated with 1 μM cortisol at 0°C for 30 min to exclude corticosteroid-binding globulin and glucocorticoid receptor binding and were then incubated with 30 nM [^{3}H]R5020 or with [^{3}H]R5020 plus 100-fold concentration radioinert R5020 for 3 hr at 0°C to determine total and nonspecific binding sites, respectively. After the 3-hr equilibration period, aliquots were assayed by adding charcoal/dextran slurry (slurry/cytosol, 1:4) to adsorb unbound steroid for 2 min at 0°C. The supernatant was obtained by centrifugation at 15,000 rpm (15,600 × *g*) for 2 min; radioactivity was measured in Triton/xylene scintillation fluid (0.3% 2,5-diphenyloxazole, 0.02% 1,4-bis[2(5-phenyloxazolyl)]benzene, and 25% Triton X-114 in xylene) at 40% efficiency.

RESULTS

Chlordecone evoked a dose-dependent increase in uterine weight (Fig. 2). A 33% increase in uterine weight was seen at 6 mg of chlordecone per kg of body weight, and a maximal uterine weight increase was obtained with 25 mg of chlordecone per kg of body weight. Whereas absolute uterine weight was not further increased at a chlordecone dose of 50 mg/kg, uterine weight/body weight was increased, since body weight was decreased by these high doses of chlordecone.

The uterotrophic activities of estradiol (50 μg/kg), chlordecone, and *o,p'*-DDT and Mirex (two other chlorinated insecticides), all at 50 mg/kg, are compared in Table 1. The uterotrophic potency of chlordecone was considerably greater than that of *o,p'*-DDT, which has been reported (16, 17) to interact with estrogen receptors (Table 1). The uterotrophic activity of chlordecone must depend on the presence of a carbonyl group because Mirex, a close structural analog, was devoid of activity at this dose (Table 1). However, despite the pronounced activity of chlordecone, its potency was much less than that of the natural estrogen estradiol-17β, which elicits near-maximal uterine growth at 1/1000th the dose.

Doses of chlordecone that evoked marked uterine growth also resulted in a movement of estrogen receptor sites into the nuclear fraction of uterine cells. The details of the time course of chlordecone effects on estrogen receptor distribution (Fig. 3) differed in certain respects from that seen after administration of estradiol (Fig. 3). The accumulation of nuclear receptor sites after administration of chlordecone was gradual, increasing steadily until 36–48 hr, whereas the movement induced by estradiol reached maximal levels by 1 hr and decreased there-

Table 1. Comparison of uterotrophic activities*

Treatment group	Uterine weight, mg
Control	27.7 ± 1.8
Estradiol	85.7 ± 6.5†
o,p'-DDT	28.6 ± 0.9
Mirex	28.1 ± 2.8
Chlordecone	106.1 ± 2.5†

* Rats (21 days old) were injected subcutaneously daily for 3 days with 50 mg of compound per kg (except for estradiol, which was 50 μg/kg) in 0.1 ml of sesame oil and uterine wet weight was determined 24 hr after the last injection. Values are the mean ± SEM, with seven animals per group.

† Significantly different from control, $P < 0.001$.

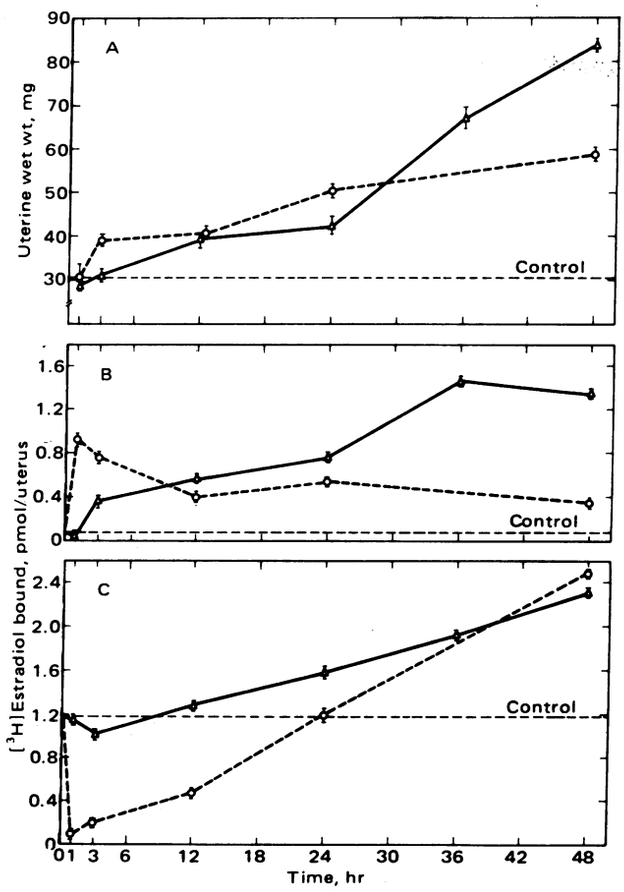

FIG. 3. Time course of the effects of a single injection of chlordecone or estradiol on uterine wet weight (*A*) and distribution of specific estrogen-binding sites between nuclear (*B*) and cytosol (*C*) fractions of the immature rat uterus. Rats (21 days old) were injected subcutaneously with 2.5 mg of chlordecone (50 mg/kg of body weight) or 5 μg of estradiol (0.1 mg/kg of body weight) in 0.1 ml of sesame oil. At the indicated times, high-affinity binding sites in uterine nuclear and cytosol fractions were determined by exchange assays. Each point represents the mean ± SEM of three determinations with three uteri per determination and is corrected for nonspecific binding. Δ—Δ, Chlordecone; O---O, estradiol.

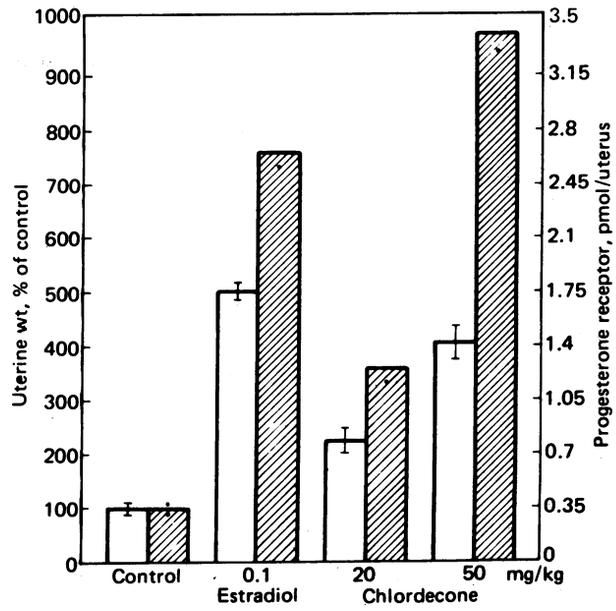

FIG. 4. Stimulation of uterine progesterone receptor content by estradiol and chlordecone. Rats (20 days old, two per group) were injected subcutaneously with 5 μg of estradiol (0.1 mg/kg of body weight), with 1 mg (20 mg/kg) or 2.5 mg (50 mg/kg) of chlordecone in 0.1 ml of sesame oil, or with sesame oil alone (controls) once daily on 3 successive days. Twenty-four hours after the last injection, uteri were excised and individually weighed, and progesterone receptor was then assayed in the uterine cytosol fraction. The control (100%) uterine wet weight was 24.5 ± 1.3 mg (n = 4). Progesterone receptor values represent the mean and actual determinations in duplicate, with two separate groups. □, Uterine weight; ▨, progesterone receptor.

after. Whereas the rapid nuclear receptor accumulation after administration of estradiol was accompanied by a rapid depletion of cytoplasmic receptor sites, the more gradual nuclear accumulation of receptor after administration of chlordecone never resulted in marked cytoplasmic depletion. However, in both cases, cytoplasmic receptor levels began to rise after 3 hr, presumably due to receptor replenishment. The receptor distribution pattern seen with chlordecone was similar to that seen with weak, but long-acting, estrogens (18).

As is characteristic of estrogens, chlordecone was also a potent stimulator of uterine progesterone receptor synthesis (Fig. 4). At 20 mg/kg, chlordecone increased both weight and progesterone receptor content of the uterus. At 50 mg/kg, uterine weight was increased 4-fold and progesterone receptor production nearly 10-fold.

In an attempt to ascertain whether chlordecone might be exerting its estrogen-like action *in vivo* by direct interaction with estrogen receptors in the uterus, we studied its interaction with cytoplasmic and nuclear estrogen receptor *in vitro*. The relative binding affinity of chlordecone for the cytoplasmic estrogen receptor was determined by a competitive binding assay (Fig. 5). A comparison of the relative concentrations required to achieve 50% inhibition of the binding of [^{3}H]estradiol to receptor indicated the relative binding affinity of chlordecone to be 0.04% that of estradiol. Approximately 10-fold higher concentrations of *o,p'*-DDT were required for equal suppression of tritiated estradiol binding to receptor.

In *in vitro* organ cultures of whole uteri, chlordecone showed a dose-related depletion of cytoplasmic receptor and uptake of estrogen receptor sites into the nuclear fraction (Fig. 6).

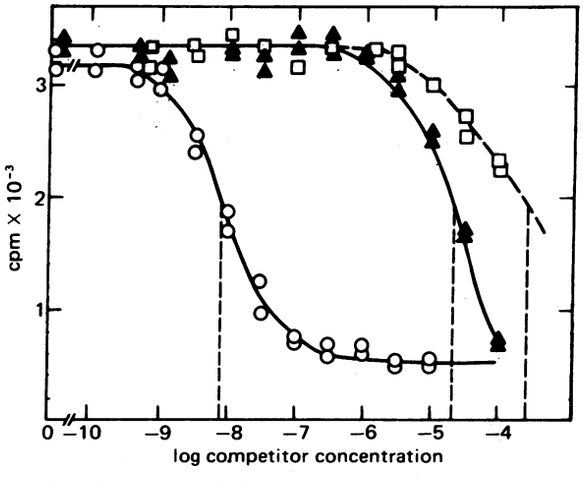

FIG. 5. Competitive binding assay of chlordecone and *o,p'*-DDT with rat uterine cytosol. Cytosol was incubated for 16 hr at 0°C with 10 nM [^{3}H]estradiol and the indicated concentrations of unlabeled competitor (O, estradiol; ▲, chlordecone; □, *o,p'*-DDT). Bound [^{3}H]estradiol was then determined by charcoal/dextran adsorption. Dashed vertical lines indicate the concentration of competitor inhibiting 50% of [^{3}H]estradiol binding to receptor.

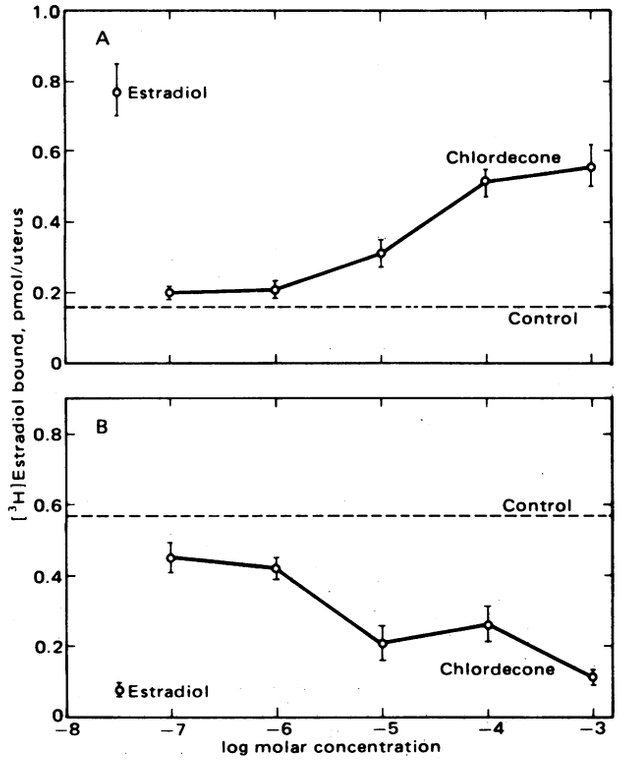

FIG. 6. Distribution of specific estrogen-binding sites between nuclear (*A*) and cytosol (*B*) fractions of the immature rat uterus after exposure of uteri to chlordecone or estrogen *in vitro*, as determined by nuclear and cytosol exchange assays. Uteri (three per group) were incubated in 3 ml of Eagle's HeLa medium with 0.1 μM–1 mM chlordecone, 30 nM estradiol, or control vehicle (1% ethanol) for 2 hr at 37°C. Each value represents the mean ± SEM of three determinations in duplicate, with three separate groups (three uteri per group), and is corrected for nonspecific binding.

Chlordecone, at 0.1–1 mM, showed nuclear receptor movement and cytoplasmic receptor depletion of almost the same magnitude as that evoked by 30 nM estradiol (which is the lowest maximally effective dose; ref. 19). A comparison of the dose levels of chlordecone and estradiol for maximal receptor translocation or for half-maximal receptor translocation (for estradiol, 50% of maximal translocation is achieved with 2 nM; ref. 19) indicated that 10,000 times as much chlordecone as estradiol was required. This estimate of effectiveness of chlordecone (0.01% that of estradiol) is consistent with its affinity for the estrogen receptor in cell-free cytosol *in vitro* (Fig. 5).

DISCUSSION

These studies indicate that chlordecone interacts with the estrogen receptor system in the uterus and, *in vivo*, behaves like a weak estrogen with prolonged activity. Hence, chlordecone competes with estradiol for binding to the cytoplasmic receptor, translocates receptor sites to the nucleus *in vitro* and *in vivo*, increases uterine weight, and stimulates synthesis of the progesterone receptor. The long-acting character of chlordecone is reflected in the slow but progressive increase in uterine weight that accompanies its action and by its ability to continue to increase uterine weight for time periods beyond that evoked by a single, high dose of estradiol (Fig. 3). Its effects on receptor distribution profiles, in which nuclear receptor levels increase gradually with time and cytoplasmic receptor levels never appear markedly depleted, are also consistent with that seen with other weak estrogens having a prolonged biological half-life (18).

The biological potency of chlordecone, as measured by uterotrophic activity *in vivo*, is consistent with its affinity for cytosol and nuclear estrogen receptor monitored *in vitro*. About 10,000 times as much chlordecone as estradiol is required to elicit an equal stimulation of uterine growth; chlordecone, likewise, has an affinity for cytoplasmic receptor that is 0.04% that of estradiol and an ability to translocate cytoplasmic receptor to the nuclear compartment of the uterus *in vitro* that is 0.01% that of estradiol. *o,p'*-DDT has an even lower affinity for the uterine estrogen receptor and did not increase uterine weight at dose levels that were maximally stimulatory with chlordecone. At higher dose levels, and in particular with the (−)-enantiomer, *o,p'*-DDT has been reported (5, 6, 20) to show estrogen-like uterotrophic activity *in vivo*. *o,p'*-DDT bears some structural relationship to the potent estrogens diethylstilbestrol and hexestrol, so that its ability to compete with estradiol for binding to estrogen receptors in the uterus (5) might be expected. However, chlordecone is a caged ring compound with a structure very different from that of either the natural steroidal estrogens or the synthetic stilbestrol or hexestrol estrogens, and its significant affinity for estrogen receptor might not have been predicted.

The effects of chlordecone in the rat uterus appear to be explainable by its direct interaction with the estrogen receptor. Chlordecone action in the rat is not associated with any increase in the levels of estrogen in the blood (21), and the finding that the level of cytoplasmic progesterone receptor is high in the uteri of animals exposed to chlordecone suggests minimal, if any, increases in the levels of circulating progesterone. In the chicken, however, chlordecone has been reported (22) to increase modestly the plasma level of progesterone and to act in the chicken oviduct *in vivo* as a combination of an estrogen and progestin, although it acts purely as an estrogen *in vitro*.

Although the affinity of chlordecone for estrogen receptors is admittedly weak, it may nevertheless be capable of inducing severe impairment of reproduction in chronically exposed individuals. Although the *in vitro* studies give strong evidence that chlordecone is itself estrogenic and can act directly on the uterus, more important is the observation of its prolonged interaction with estrogen receptors and protracted stimulation of uterine weight gain *in vivo*. Related findings of chlordecone interaction with the rat uterus have been reported recently (23). Chlordecone has a very long half-life in the body due to its unusual bioaccumulative properties (1, 24, 25), and considerable amounts of this compound are sequestered in the liver and fat. We have found that after chronic exposure of mature female rats to 30 ppm of chlordecone in the feed for 7 weeks, all animals enter constant estrus and stop cycling. When killed at 10 weeks, the uteri of these animals were very enlarged and swollen with fluid. Hence, our data suggest that chronic exposure to this insecticide might impair reproductive function by acting, at least in part, as a low-affinity but very long-acting estrogen.

A point of particular interest is the marked quantitative differences in estrogenic potency and estrogen receptor affinity of chlordecone in different species. Chlordecone has a 100-fold higher affinity for estrogen receptor in the chicken oviduct (1% that of estradiol; ref. 22) than is seen here for estrogen receptor in the rat uterus, and only 50- to 100-fold higher concentrations of chlordecone than estradiol are needed for optimal induction of ovalbumin and conalbumin, two estrogen-stimulated proteins, in the chicken oviduct (22).

Although the reasons for these marked differences between species are unclear at present, they highlight potential difficulties involved in trying to make quantitative extrapolations of the effects of this compound in humans and other species.

Note Added in Proof. Recently, Eroschenko and Palmiter (26) reported the affinity of chlordecone for chicken oviduct estrogen receptor to be 1/1000th–1/5000th that of estradiol, correcting a higher value that had been reported earlier (22). The affinity and potency of chlordecone now reported in chicken oviduct is similar to that reported here for the rat uterus. We thank Richard Palmiter for bringing this report to our attention prior to its publication.

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