Initiation of Embryo Implantation and Maintenance of Early Pregnancy in the Rat by Chlordecone (Kepone) (43116)

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Abstract. **The effect of chlordecone (Kepone), an insecticide/fungicide with reproductive toxicity, on the early stages of pregnancy in the rat was studied. Intraperitoneal injection of chlordecone into adult virgin female Holtzman strain rats before mating, in doses as high as 80 mg/kg, did not prevent fertilization, early development of the embryo to the blastocyst stage, transport of the embryo through the oviduct, or its implantation into the uterus. However, a single dose of 60 or 80 mg/kg, but not 20 or 40 mg/kg, before mating significantly reduced the concentration of progesterone in the serum of rats undergoing normal embryo implantation 5 days later. A dose of 80 mg/kg of chlordecone reduced progesterone levels in the serum by more than 50% within 48 hr in ovariectomized rats with Silastic tubing implants containing crystalline progesterone. This dose of chlordecone induced deciduomata formation in progesterone-primed ovariectomized rats to the same extent as 1 μg of estradiol benzoate. The minimal effective single dose of chlordecone to initiate implantation of blastocysts in the uteri of hypophysectomized progesterone-primed rats, and to maintain embryo development for at least 5 days, was 50 mg/kg. Daily doses of 20 mg/kg for 3 or 5 days were effective at initiating implantation but did not maintain pregnancy. The latter treatment, however, did not prevent initiation of implantation or embryo development induced by subsequent administration of estrone. The results are consistent with the view that chlordecone is a weak estrogen that has both nongenomic and genomic estrogenic actions.** [P.S.E.B.M. 1990, Vol 195]

Disturbances in reproductive performance of animals exposed to chlordecone (Kepone) have been recognized for many years (1–3). This polychlorinated pesticide appears to have multiple sites of action which include the hypothalamic-pituitary axis (2, 4), uterus (5, 6), and ovary (7). All of these effects have been associated, directly or indirectly, with the estrogenicity of the compound. Indeed, chlordecone has been shown to bind to the intracellular estrogen receptor in birds (2) and mammals (5, 6).

Chernoff and Rogers (8) reported that chlordecone was detrimental to normal fetal development in the rat and mouse. They began treatment on the seventh day of pregnancy, which is early in the organogenesis phase of development. However, the estrogenicity of chlordecone would also be expected to alter the course of early pregnancy. Therefore, a study was undertaken to determine the effects of chlordecone on normal implantation and initiation of implantation in the hypophysectomized rat with delayed implantation. The results indicate that the insecticide can initiate implantation and maintain early pregnancy in the rat, although the amounts needed are rather large. Furthermore, chlordecone lowered serum concentrations of progesterone without apparent disturbances in preimplantation embryo development or implantation in the rat.

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Materials and Methods

Chemicals. Chlordecone (Kepone; decachlorooctahydro-1,3,4-metheno-2H-cyclobuta[cd]pentalen-2-one) (Aliquot 943310) was obtained from Radian Corp. (Austin, TX). Estradiol benzoate, estrone, and progesterone were purchased from Sigma (St. Louis, MO). 1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2,2-trichloroethane (*o,p'*-DDT) was obtained from Aldrich. These compounds were dissolved in benzyl benzoate and then diluted with sesame seed oil (Sigma) to produce a 40:60 (v/v) ratio.

Animals. Virgin female rats (250–275 g body weight) (Holtzman strain; Holtzman Laboratory Animals, Madison, WI) were maintained in temperature (22 ± 1°C)- and light (lights on 0600–2000 hr)-controlled quarters and given free access to Purina Laboratory Chow (5001) and tap water. Estrous cyclicity was determined by examination of the vaginal exfoliative cytology. Four experimental designs were used.

Effect of chlordecone on early pregnancy. Proestrus females were injected intraperitoneally with various doses of chlordecone (20, 40, 60, 80 mg/kg) or its vehicle just before being placed with males. One female was placed at 1700 hr in the home cage of two males of proven fertility. The finding of a copulatory plug or spermatozoa in the vaginal lavage the following morning confirmed the establishment of pregnancy (Day 1). Animals were autopsied on Days 3, 4, 5, or 6. After brief anesthetization with ether animals were decapitated, and the blood from the trunk was collected in 12- × 75-mm culture tubes. The blood was allowed to clot at room temperature and was stored overnight at 4°C. The serum was separated by centrifugation and stored at −20°C until used for assay of progesterone, which is described below. Oviducts and uteri were flushed with normal saline, and the embryos were counted with the aid of a dissecting microscope. Animals that were killed on Day 6 were injected, under light ether anesthesia, with a macromolecular dye (0.7 ml of a 1% solution of Chicago Blue B in normal saline) a few minutes before autopsy. The extravasation of the protein-bound dye, due to increased capillary permeability, indicated the implantation site of the embryo (9).

Uterine decidualization. Animals were ovariectomized without regard to the stage of the estrous cycle, under ether anesthesia, using the dorsolateral approach; the skin incisions were closed with 11-mm wound clips. Three days later progesterone priming was initiated: 2 mg of progesterone, dissolved in 0.1 ml of oil, was injected subcutaneously daily for 5 days. On the sixth day one uterine horn was traumatized by injecting 0.1 ml of sesame seed oil into the lumen. The uterus was exteriorized by a dorsolateral incision in the skin and peritoneum. At the time of this operation the animals were injected subcutaneously with progesterone (2 mg) and intraperitoneally with either 1 μg of estradiol benzoate, 200 mg/kg of *o,p'*-DDT, or 80 mg/kg chlordecone. Progesterone (2 mg/rat) was injected subcutaneously for 4 days with autopsy 24 hr after the last dose of this hormone. The traumatized, oil-injected, and noninjected control uterine horns of each animal were weighed separately using a torsion balance.

Initiation and maintenance of pregnancy by chlordecone. The delayed implanting hypophysectomized model was used for this study (10). On the morning of Day 3 of pregnancy the pituitary was removed from ether-anesthetized animals using the parapharyngeal approach. A 5% solution of glucose was used for drinking water after this operation. At the time of hypophysectomy and daily for the next 5 days, i.e., until Day 8 after mating, each animal received 2 mg of progesterone subcutaneously dissolved in 0.1 ml of sesame seed oil. Under these conditions implantation did not occur, and the blastocysts remained unattached in the uterine lumen (9). However, a small amount of estrogen will promptly initiate implantation of these embryos. Estrone (1 μg) or chlordecone (20, 40, 60, 80 mg/kg) was injected intraperitoneally into these progesterone-primed animals, and their uteri were examined for implantation sites 24, 48, or 72 hr later using Chicago Blue B as described above. Progesterone (4 mg) was injected subcutaneously at the time of estrone or chlordecone administration and daily until autopsy. If no implantation sites were found, the uterine horns were flushed with saline to determine the number of embryos present; absence of embryos indicated that the animal was not pregnant and it was discarded. On the other hand, if the hypophysectomy was incomplete, implantation occurred at the expected time and the embryos were older and larger than those in animals with delayed implantation; such animals were not included in the results.

For maintenance of pregnancy a combination of estrogen and progesterone is required. Each animal received 4 mg of progesterone daily, but the schedule of treatment with estrone or chlordecone was varied and is detailed along with the results. At the time of autopsy, 5 days after initial exposure to estrone or chlordecone, each uterine swelling, which contains the embryo, decidual tissue, and uterine muscle, was weighed on a torsion balance. Representative swellings were placed in Bouin's fixative, dehydrated through alcohols, embedded in paraffin, sectioned at 10 μm, and stained with hematoxylin and eosin. Histologic examination of the sections confirmed the presence of decidual tissue and developing embryo.

Effect of chlordecone on serum progesterone concentration. As indicated above, serum was obtained from intact animals treated with chlordecone at the time of mating. In addition, females were ovariectomized without regard to stage of the estrous cycle and were implanted with Silastic tubes containing progesterone. Crystalline steroid was packed into Silastic tubes (312.5 mm o.d., 155 mm i.d. × 400 mm length) both ends of which were closed with Silastic adhesive (Dow-Corning, Midland, MI); each animal received three tubes. Four days after implantation a blood sample (500 μl) was obtained from the ventral tail artery of animals lightly anesthetized with ether. At the time of bleeding chlordecone (80 mg/kg) or vehicle was injected intraperitoneally. Blood samples were obtained (tail artery) at four other times and at autopsy, but no animal was bled more than once in 48 hr.

Progesterone concentrations in the serum were determined using radioimmunoassay kits purchased from Diagnostic Products Inc. (Los Angeles, CA). The minimal detectable concentration of progesterone was 100 pg/ml using 100 μl of serum; the intrassay coefficient of variation did not exceed 3% and the interassay variation was less than 10%.

In all of the studies, the data were subjected to one-way analysis of variance, followed by Student's t test. Differences between means with a P value less than 0.05 were considered statistically significant.

Results

The first experiments examined the effect of chlordecone on early pregnancy. The compound did not prevent mating even though doses of 40 mg/kg and above produced a pronounced tremor within a few hours. Doses as high as 80 mg/kg had no detectable effect upon embryo development or transport; blastocysts developed normally and were present in the uterus on Day 5 (Table I). Furthermore, when examined on Day 6, all animals had the normal number of implantation sites and the intensity of blue dye staining, after injection of Chicago Blue B, of the sites was indistinguishable from that in control animals. Not shown in the table are results obtained by injection of either 60 or 80 mg/kg of chlordecone on Day 4 of pregnancy with autopsy on Day 6; all animals had the normal complement of implantation sites.

The results of using chlordecone for initiating implantation in the hypophysectomized rat with delayed implantation are shown in Table II. In confirmation of previous experience (11), all control animals treated with 1 μg of estrone showed implantation. Although these animals were examined 48 hr after exposure to estrogen, implantation is initiated within 24 hr (11). With chlordecone treatment, on the other hand, we found only 1 of 5 animals had a few, barely detectable, implantation sites 24 hr after receiving a dose of 60 mg/kg. Five of six animals, however, had intensely staining sites after 48 hr. Dividing the 60 mg/kg dose into three daily injections (20 mg/kg × 3) reduced the degree of tremor in the animals, and this treatment was just as effective for initiating implantation as the single dose. With a single dose, however, 50 mg/kg was the minimal amount that consistently produced the normal number of implantation sites (Table II).

Clearly, chlordecone's estrogenicity could initiate implantation in the rat, but was it also sufficient to maintain pregnancy? Animals were autopsied 5 days after the initial implantation-inducing treatment, i.e., equivalent to Day 10 of a normal pregnancy. A single dose of 1 μg of estrone (Group 1, Table III) produced uterine swellings that weighed 32.8% less ($P < 0.005$) than those obtained with three doses on alternate days (Group 2). However, daily injections of estrone (Group 3) did not significantly increase the weights further. Replacing two of the doses of estrone with chlordecone (20 mg/kg each) (Group 4) did not significantly alter the weights of the swellings over that obtained with a single dose of estrone. Although three doses of chlordecone (20 mg/kg each) initiated implantation in all animals (Table II), this amount was inadequate for the maintenance of pregnancy (Group 5, Table III). The swellings were distinct but small in the latter group. Increasing the amount of chlordecone to five doses (total dose = 100 mg/kg) (Group 6) did not enhance the degree of pregnancy maintenance. In one of the rats given five doses of chlordecone, there were only three implantation sites in one horn, whereas the other horn contained normal appearing blastocysts; i.e., implantation had not occurred in this horn. With a single dose of 50 mg/kg of chlordecone (Group 7, Table III), the weights of the swellings were not significantly different

Table I. Effect of Chlordecone on Early Pregnancy in the Rat

Dose of chlordecone (mg/kg)	No. pregnant/ total no.	Day examined	No. of embryos/ animal
0	9/9	5	11.0 ± 0.8[a]
0	6/6	6	14.0 ± 1.0
20	7/7	5	10.4 ± 0.7
40	7/7	5	9.6 ± 1.2
	8/8	6	13.4 ± 1.5
60	6/6	6	10.2 ± 2.1
80	4/4	5	10.2 ± 0.8
	5/5	6	9.8 ± 2.7

[a] Mean ± SE. Chlordecone, or vehicle, was injected intraperitoneally just before mating. Day 1 = morning of vaginal spermatozoa. Day 6 represents implantation sites, determined 15 min after intravenous injection of 0.5 ml of a 1% solution of Chicago Blue B.

Table II. Initiation of Implantation by Chlordecone in Hypophysectomized Rats with Delayed Implantation

Dose of chlordecone (mg/kg)	No. of animals with sites/total (%)	No. of sites/animal	No. of blastocysts recovered/animal
Control	9/9 (100)	14.7 ± 1.0[a]	—
20	1/6 (16.6)	3	7.4 ± 2.0
20 × 3[b]	4/4 (100)	14.0 ± 2.5	—
40	2/8 (25)	1, 13	7.10 ± 1.0
50	7/9 (77.8)	11.2 ± 1.0	9, 10
60	5/6 (83.3)	14.2 ± 0.5	9
60[c]	1/5 (20)	3	8.1 ± 0.9

[a] Mean ± SE. Animals were hypophysectomized on Day 3 and injected subcutaneously daily with progesterone (2 mg). After 5 days of this treatment, they were injected with chlordecone or 1 μg of estrone (control). Implantation sites were visualized by injection of 0.5 ml of a 1% solution of Chicago Blue B 15 min before autopsy.
[b] Animals injected once daily and examined 72 hr after the first dose; all other animals were examined 48 hr after injection of the test compound.
[c] Animals examined 24 hr after injection of chlordecone.

Table III. Maintenance of Pregnancy in Hypophysectomized Rats Treated with Chlordecone and Progesterone

Treatment	Group	No. of animals	No. of embryos/ animal	Weight[a] (mg)
Estrone (1 μg), Day 8	1	11	14.3 ± 0.4[b]	43.3 ± 2.5c
Estrone (1 μg), Days 8, 10, and 12	2	5	16.0 ± 1.0	64.5 ± 5.7d
Estrone (1 μg), Days 8–13	3	4	13.2 ± 0.2	68.7 ± 8.8d
Estrone (1 μg) Day 8 + chlordecone (20 mg/ kg), Days 10 and 12	4	4	13.7 ± 0.8	50.6 ± 3.6c,d
Chlordecone (20 mg/kg), Days 8, 10, and 12	5	8	12.0 ± 1.2	16.4 ± 1.3e
Chlordecone (20 mg/kg), Days 8–13	6	11	11.2 ± 1.2	21.2 ± 3.2f
Chlordecone (50 mg/kg), Day 8	7	4	14.7 ± 1.1	41.8 ± 4.1c

[a] Weight of the uterine swellings containing embryo, decidual tissue, and myometrium. All animals were autopsied 5 days after initial injection of estrone or chlordecone. Means with the same letters are not statistically different ($P > 0.05$).
[b] Mean ± SE.

from those found in animals treated with a single dose of estrone. Animals treated with 50 mg/kg of chlordecone had a considerable tremor for the first 48 hr after treatment, but it was reduced distinctly within another 48 hr. Histologic examination of the embryonic swellings indicated that the decidual tissue as well as the embryos were not grossly different between estrone- and chlordecone-treated animals.

Estrogens augment the actions of progesterone for uterine decidualization (12). The effect of chlordecone (80 mg/kg) was not different from that produced by 1 μg of estradiol benzoate, which equates to about 4 μg/ kg body weight (Fig. 1). The benzoate was used to avoid the need for multiple injections. For comparison, the effect of another implantation-inducing xenobiotic, *o,p'*-DDT, was included. Even with 200 mg/kg, which induces implantation in 67% of animals and can maintain pregnancy (13), the response was not greater than with progesterone alone.

Because chlordecone induces cytochrome P-450 enzymes in the liver (14), it could be expected to alter the disposition of steroids in the blood. To examine this, the effect of chlordecone upon the concentration of progesterone in the serum was examined in pregnant animals autopsied at about the time of implantation (Table IV). Females were injected intraperitoneally with oil or oil-containing chlordecone at the time they were placed with males; mating would be expected to occur within 6–8 hr. Only animals that had confirmed matings were used. On Day 4, when the late morula are just entering the uterus, animals treated with 60 mg/kg chlordecone (Group 2, Table IV) had 26% ($P < 0.05$) less progesterone than did the control animals. A larger dose (80 mg/kg, Group 3) resulted in a further 25%

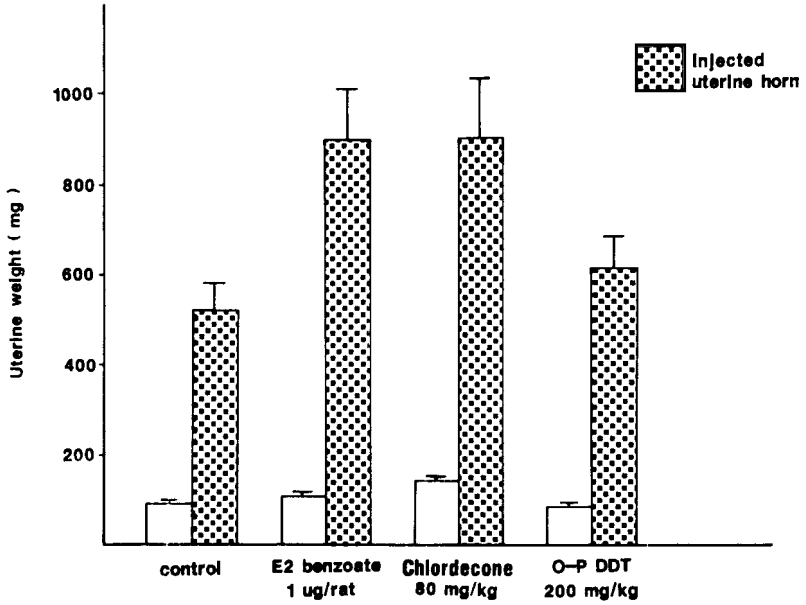

Figure 1. Weight of the traumatized (oil-injected) and control uterine horns of ovariectomized rats 5 days after intraperitoneal injection of estradiol benzoate (E2 benzoate), chlordecone, or *o,p'*-DDT, or only progesterone (control). Vertical lines indicate mean ± SE for groups of six rats.

Table IV. Concentration of Progesterone in the Serum of Pregnant Rats Treated with Chlordecone[a]

Treatment	Group	Autopsy day	No. of animals	Progesterone (ng/ml)
Control	1	4	4	59.7 ± 5.7a
Chlordecone (60 mg/kg)	2	4	12	44.0 ± 3.5b
Chlordecone (80 mg/kg)	3	4	10	27.3 ± 2.4c
Control	4	5	7	57.2 ± 5.8A
Chlordecone (20 mg/kg)	5	5	6	62.3 ± 6.1A
Chlordecone (40 mg/kg)	6	5	6	43.6 ± 3.7A,B
Chlordecone (80 mg/kg)	7	5	6	37.8 ± 3.5B
Control	8	6	3	80.0 ± 11.6@
Chlordecone (80 mg/kg)	9	6	7	44.7 ± 7.0

[a] Mean ± SE. Chlordecone or the oil vehicle was injected intraperitoneally a few hours before mating. Day 1 = day of vaginal spermatozoa. Implantation occurs on Day 5. Means with the same letters are not different from each other.

reduction ($P < 0.001$). On day 5 (the day of implantation) animals treated with 80 mg/kg chlordecone had higher levels of progesterone than those killed on Day 4 ($P < 0.02$), but it was still 34% below the level found in control animals. Neither 20 nor 40 mg/kg chlordecone had a significant effect upon serum progesterone levels on Day 5. Animals that received 80 mg/kg chlordecone also had reduced serum progesterone levels on Day 6, a time when implantation was nearly complete.

Chlordecone given to intact animals would be expected to have multiple sites of action and could alter serum steroid levels by several mechanisms. In order to rule out the pituitary and/or ovary as targets, the effect of chlordecone on elevated serum progesterone levels, which were maintained by Silastic implants in ovariectomized animals, was studied (Fig. 2). Over a period of 1 week the serum progesterone level of control animals decreased by about 10%. In those treated with chlordecone, on the other hand, there was a dramatic decrease in serum progesterone within 24 hr with a further decline during the next 24 hr. Between the second and seventh days there was no further change in the progesterone level indicating that there was no recovery during this interval.

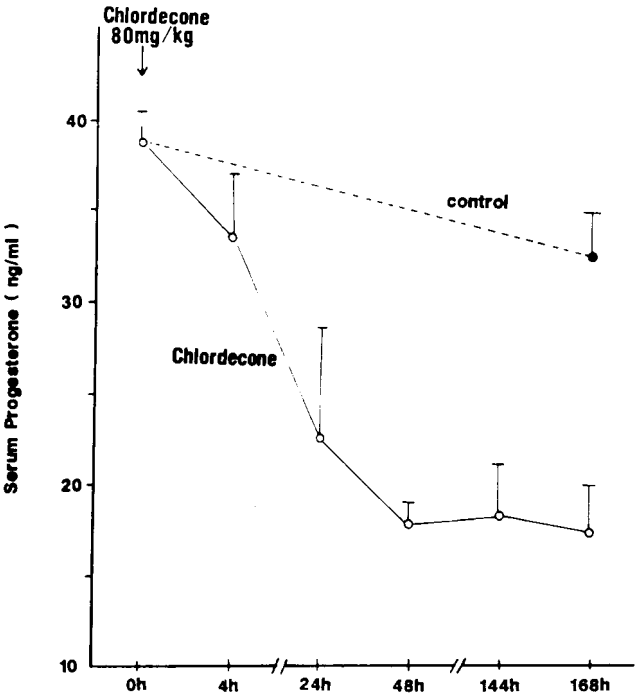

Figure 2. Concentration of progesterone, determined by radioimmunoassay, in the serum of ovariectomized rats carrying Silastic tubing implants which contained crystalline progesterone. Four days after receiving the implants a blood sample (500 μl) was obtained from the tail artery of each rat (time 0). Chlordecone (80 mg/kg), or vehicle, was injected intraperitoneally at this time. The final value was obtained for blood obtained at autopsy. Vertical lines indicate the mean ± SE for groups of at least four rats at each bleeding and six rats at autopsy.

Discussion

Implantation of the embryo into the uterus of the rat or mouse requires interactions between progesterone and estrogen (9). The progesterone-dominated uterus is exquisitely sensitive to a small amount of estrogen as it relates to implantation. The mechanisms involved have not been resolved, but include release of histamine, activation of phospholipase A_2, and increased synthesis of prostaglandins (10). These changes have been considered a part of the early, or Phase I, responses to estrogen (15). How Phase I responses are related to the classical cytoplasmic/nuclear estrogen receptor is not clear, but because inhibitors of RNA or protein synthesis do not inhibit initiation of implantation, typical genomic mechanisms do not appear to be involved (16). Furthermore, metabolic alteration of the natural estrogens appears to be important for implantation because those that do not readily form catechol estrogens do not initiate implantation (17). The late, or Phase II, responses are those associated with the uterotropic responses to estrogen and clearly involve the intracellular receptor. Included in these responses is the synthesis of uterine progesterone receptors, a function that may be an important factor for maintenance of pregnancy (18).

The hypophysectomized rat with delayed implantation is an excellent model to test the estrogenic effect of compounds because of the sensitivity of a progesterone-primed uterus that has not been exposed to endogenous estrogen for several days. The increase in capillary permeability characteristic of initiation of implantation is induced by 4 μg/kg of estrone (either intraperitoneally or subcutaneously). With chlordecone the minimal single dose to initiate implantation rather consistently was 50 mg/kg, which indicates it is more than 12,000-fold less potent than estrone. On the other hand, it would appear to be at least four times more potent than *o,p'*-DDT for induction of implantation (13), which is consistent with its greater uterotropic action (6). Comparisons of potency are difficult, however, because of unknown metabolic changes to the compound that may be required for activity. That is, the Phase I action of these compounds may require the endogenous formation of a small amount of a metabolite, or it may be the consequence of repeated weak estrogenic action. The efficacy of three 20 mg/kg doses of chlordecone for initiating implantation is consistent with the latter idea. A similar result can be obtained with natural estrogen in the mouse (19). Several of the estrogenic responses to chlordecone, i.e., hypothalamic-pituitary changes in prolactin and luteinizing hormone output, persistent vaginal epithelium cornification (3), or maximal uterotropic response (6) require about 50 mg/kg. In the present study the same dose was required for the maintenance of pregnancy. All of these responses, as well as decidualization enhancement (Fig. 2), are associated with the genomic action (phase II) of estrogen, suggesting that in large doses chlordecone acts via the classical cytosolic/nuclear receptor.

We did not find chlordecone embryotoxic during early pregnancy, even at high doses. When given to intact animals at 80 mg/kg, there was no obvious effect on normal embryo transport or on implantation, even though such a dose altered endogenous progesterone concentrations (Table IV). In contrast, *o,p'*-DDT (13) and an analog, methoxychlor (20), caused embryo losses during the first few days of pregnancy, presumably by increasing the rate of transport of the embryo. When given beginning at Day 7 of pregnancy chlordecone was found to produce fetal abnormalities (8). However, it is difficult to determine how much of the abnormality was a consequence of altering the hormonal milieu of the mother and how much was a direct effect upon the fetus. We attempted to get some idea of this by combining chlordecone and estrone treatment. Animals exposed to 100 mg/kg of chlordecone over a period of 5 days (20 mg/kg/day) initiated implantation of embryos but there was little, if any, further development. When estrone was administered after the fifth dose of chlordecone, the embryos resumed development. Seven of 7 such animals had embryos (14.3 ± 1 embryos/rat) after 5 days of estrone + progesterone treatment, but the weights of the uterine swellings were quite variable. The average weight was 213.1 ± 30.8 mg/uterine swelling, with a range of 119.4 ± 10 to 338.8 ± 36 mg. These differences could be explained by assuming that some embryos implanted later than others and some may have undergone a small degree of development under the influence of chlordecone. Our results indicated that 20 mg/kg initiated implantation within 72 hr in all animals (Table II) and thus treatment with estrone + progesterone for 8 days (72 hr plus 5 days) should provide a control for comparison of development. Three of these controls had 16.0 ± 1.1 embryos/rat that weighed an average of 265 ± 3.0 mg each. Thus in most animals the chlordecone did not seriously cause a decrement in fetal development once a potent estrogen was present. Development of the fetus over the entire period of organogenesis will be required before we can have information relative to the direct toxic effect of the early exposure to chlordecone upon the fetus.

Chlordecone is known to increase expression of hepatic microsomal cytochrome P-450 in the rat (14). This could explain the decrease in progesterone in the serum of animals treated with larger doses of the pesticide (Table IV). The metabolites produced by the mixed function oxidases were not determined but they, nor the decrease in progesterone, were sufficient to alter establishment of pregnancy in the intact rat. Furthermore, the metabolites, which would be expected to persist in the animals used to test maintenance of pregnancy, did not appear to alter embryo survival.

In summary, the initiation of implantation, the induction of decidualization, and the maintenance of pregnancy must be added to the list of estrogenic actions of chlordecone. The mechanisms responsible for these actions remain unresolved but may involve more than interaction with the classical cytoplasmic-nuclear estrogen receptor.

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