Estrogenic Activity of the Insecticide Kepone on the Chicken Oviduct

Abstract. *Kepone induces ovalbumin and conalbumin synthesis in explants of chick oviduct in vitro by acting as a weak estrogen. It binds to the nuclear estrogen receptor and is antagonized by the antiestrogen tamoxifen. Kepone also induces egg white protein synthesis in vivo by direct interaction with estrogen receptors and by indirectly increasing the concentration of progesterone in the serum.*

Several insecticides have estrogen-like activity in birds (*1*). For example, one of the isomers of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane (DDT) produces substantial weight gain in the avian oviduct and the rat uterus (*2*), and its effects on rat fetal development are reversed by the antiestrogen chlomiphene (*3*). Kepone induces oviduct morphology in immature quail similar to that of laying birds (*4*). There are several ways in which estrogenic activity might be elicited by insecticides. They might (i) alter the normal synthetic or degradative pathways in steroid metabolism leading to altered serum concentrations of the active steroids, (ii) perturb the normal regulatory controls in target tissues to increase the sensitivity of these tissues to endogenous steroids, (iii) mimic estrogenic steroids by binding to and activating the receptor molecules that normally mediate estrogenic activity, or (iv) bind to other molecules that become capable of eliciting estrogenic responses. In this report we show that Kepone impinges directly on the chicken oviduct by mechanism (iii) and also increases the concentration of other active steroids by mechanism (i).

Table 1. Induction of ovalbumin and conalbumin synthesis by Kepone, estrogen, or progesterone. Kepone was dissolved in ethanol (100 mg/ml) for intraperitoneal injections; or it was dissolved in acetone, mixed with chick feed (0.5 or 2 mg/g), dried, and fed ad libitum. Estradiol (17β-estradiol benzoate) and progesterone were dissolved in corn oil (5 mg/ml) and injected subcutaneously. Chicks aged 3 to 4 weeks old were used for primary stimulation. For secondary stimulation, hexestrol pellets (15 mg) were implanted subcutaneously between days 4 and 14 after hatching and then removed for 10 days prior to the administration of Kepone or steroids. For the measurements of ovalbumin and conalbumin in synthesis, explants from the magnum portion of the oviducts from two to four birds were pooled and incubated for 30 minutes in Hanks salts containing ^{3}H- or ^{14}C-labeled leucine. The tissue was then homogenized in a buffer containing detergents, centrifuged at 100,000*g* for 1 hour, and the supernatant was used to measure isotope incorporation into total protein (by acid precipitation) and ovalbumin or conalbumin (by immunoprecipitation). For details, see (*6*). The data are expressed as percentages of total protein synthesis.

Compound	Dose	Time	Ovalbumin synthesis (%)	Conalbumin synthesis (%)	Wet weight of oviduct magnum (mg)
		Primary stimulation			
None			0.0 to 0.02*	0.5 to 1.45*	11
Kepone†	12.5 mg/day	3 days	1.03	7.16	19
Estradiol‡	1 mg/day	3 days	7.30	7.40	138
Progesterone‡	1 mg/day	3 days	0.05	2.61	20
		Secondary stimulation			
None			0.0 to 0.3	0.7 to 1.9	25 to 40
Kepone§	500 ppm	7 days	0.92	5.5	55
Kepone§	500 ppm	10 days	1.63	6.4	72
Kepone§	500 ppm	16 days	4.7	8.45	101
Kepone§	2000 ppm	9 hours	0.14	1.4	30
Kepone§	2000 ppm	3 days	4.2 to 8.3	6.9 to 8.5	40
Kepone†	12.5 mg/day	1 day	2.07	5.3	35
Kepone†	12.5 mg/day	2 days	5.2	10.0	46
Kepone†	12.5 mg/day	3 days	12.1	12.5	60
Kepone†	50 mg	5.5 hours	2.4	3.8	50
Kepone†	50 mg	8 hours	6.4	5.2	51
Estradiol‡	1 mg	8 hours	1.5 to 4.5	5.0 to 8.0	40
Estradiol‡	1 mg/day	3 days	32.7	12.3	306
Progesterone‡	1 mg	8 hours	3.0 to 6.0	2.4 to 4.4	55
Progesterone‡	1 mg/day	3 days	37.6	10.6	130

*Values are the range observed in many experiments of this type [see (*9*)]. The variation is largely between experiments with different groups of birds. †Intraperitoneal injection. ‡Subcutaneous injection. §Administered with food.

In response to estrogen treatment, the oviduct of immature birds shows extensive cell proliferation and evidence of egg white protein synthesis. After a week of estrogen treatment, the oviduct may increase 100-fold in weight and use up to 70 percent of its total protein synthetic effort for egg white proteins, with ovalbumin and conalbumin accounting for 50 and 10 percent, respectively. Withdrawal of estrogen results in a rapid cessation of egg white protein synthesis. Synthesis of these proteins resumes upon secondary stimulation with estrogens, progestins, or glucocorticoids (*5–7*). This system is being actively exploited to help elucidate the mechanism of steroid hormone action (*8, 9*).

Kepone is an organochlorine cage compound (*10*) which, in addition to its estrogen-like activity on birds (*4*), induces mixed-function oxygenase in the liver (*11*), inhibits mitochondrial adenosinetriphosphatase (*12*), and produces severe neurological symptoms in experimental animals and man.

Table 1 shows the effects of administering Kepone as a primary stimulation to immature chicks or as a secondary stimulation to immature chicks subjected previously to 10 days of primary stimulation with estrogen followed by an interval of several weeks with no stimulation. As a primary stimulation, Kepone induced conalbumin synthesis as well as did an optimal dose of estrogen, but with Kepone the induction of ovalbumin and the stimulation of oviduct growth were meager. Most of our studies concern the effects of secondary stimulation because this response with other hormones has been examined in detail (*6–9*). Kepone was not very effective when injected subcutaneously, but 50 mg given intraperitoneally induced ovalbumin and conalbumin synthesis to an extent comparable to that observed with an optimal dose of estrogen or progesterone. This dose of Kepone is lethal within 6 to 10 hours, but at a dose of 12.5 mg/day the rate of induction is lower but the birds will survive for several days, thus allowing a higher rate of egg white protein synthesis to be attained (Table 1). Kepone is also effective when injested with food. In chicks fed for 3 days on a diet supplemented with Kepone [2000 parts per million (ppm)] rates of ovalbumin and conalbumin synthesis were comparable to the rate achieved in 8 hours with 50 mg of Kepone injected intraperitoneally or an optimal dose of estrogen or progesterone. The effects of a single injection of Kepone (15 mg) are long-lasting; after a week the rate of ovalbumin and

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conalbumin synthesis were still elevated above control levels. These observations suggest that prolonged exposure to low levels of Kepone could have a cumulative effect.

The induction of specific protein synthesis by steroid hormones is brought about by the accumulation of their respective messenger RNA's (mRNA's) (*8, 9*); this is also true with Kepone induction of ovalbumin and conalbumin synthesis. Eight hours after chicks received an intraperitoneal injection of Kepone (30 mg per bird), the concentration of ovalbumin mRNA in the oviduct showed a 25-fold increase to 3200 molecules per tubular gland cell assayed by either hybridization with complementary DNA (cDNA) or translation of the mRNA in a reticulocyte lysate (*9*); conalbumin mRNA showed a 3.6-fold increase.

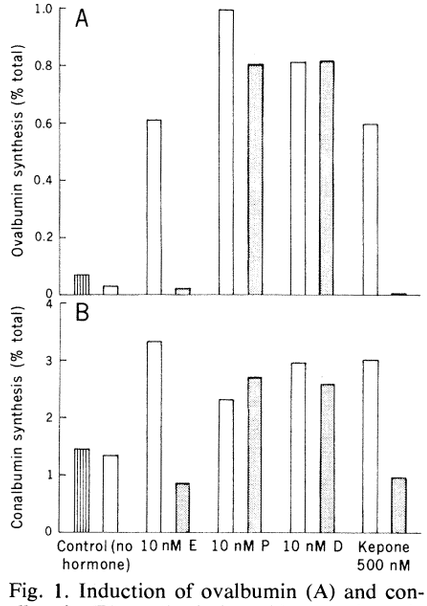

Fig. 1. Induction of ovalbumin (A) and conalbumin (B) synthesis in oviduct explants in vitro by addition of 17β-estradiol (*E*), progesterone (*P*), dexamethasone (*D*), or Kepone to the medium. Oviduct magnum tissue from eight birds that had received 10 days of primary stimulation followed by several weeks of no stimulation was minced finely and distributed into ten flasks containing 5 ml of Ham's F-10 medium with 12 percent fetal calf serum that had been extracted with charcoal-dextran. Cultures were gassed with air and 5 percent CO_2 and incubated for 8 hours at 41°C. Each of the compounds to be tested was dissolved in ethanol and diluted 200-fold into the culture medium to give the concentration indicated. At the end of the 8-hour incubation period, the tissue was washed in phosphate-buffered saline; a portion was suspended in 2 ml of Hanks salts and labeled for 30 minutes with [^{3}H]leucine to measure the rate of ovalbumin and conalbumin synthesis (see Table 1); another portion of the tissue was used to isolate total nucleic acid for quantitation of specific mRNA sequences by hybridization with cDNA (see text). Open histograms represent cultures incubated for 8 hours with the compounds indicated; histograms with vertical lines represent controls that were labeled during the first 1/2 hour of culture; stippled histograms represent cultures that contained $10^{-5}M$ tamoxifen in addition to the compounds indicated and were incubated for 8 hours.

To ascertain whether Kepone acts directly on the oviduct, we tested it with oviduct explants in culture as described by McKnight (*13*). Figure 1 shows that 500 n*M* Kepone induced ovalbumin and conalbumin synthesis to an extent comparable to that obtained with 10 n*M* 17β-estradiol [an optimal concentration (*13*)]. This induction of protein synthesis by Kepone was completely blocked by 10 μ*M* tamoxifen, an antiestrogen that binds competitively to the estrogen receptor (see Fig. 2B) (*14*), suggesting that Kepone interacts directly with estrogen receptors. Tamoxifen had little effect on progesterone or dexamethasone induction of egg white protein synthesis in culture (Fig. 1), indicating that it is specific for estrogen receptors and is not toxic at the concentration used. Kepone gave maximal induction between 100 and 1000 n*M*; higher concentrations were inhibitory and 10 n*M* Kepone was ineffective. Kepone caused about a tenfold increase in ovalbumin mRNA levels in culture.

To investigate the estrogenic activity of Kepone further, we measured its ability to compete with 17β[^{3}H]estradiol uptake into the nucleus of oviduct explants in culture. Figure 1A shows that Kepone is about 1/200th as effective as unlabeled 17β-estradiol at competing for labeled 17β-estradiol uptake into oviduct nuclei. This could be due to either different affinities of the two compounds for estrogen receptors or to differences in their uptake into the cells. A more direct experiment favors the former alternative. Oviduct nuclei from estrogen-treated chicks were isolated and incubated with 17β-[^{3}H]estradiol plus varying concentrations of unlabeled 17β-estradiol or Kepone (Fig. 2B). Again, using this cell-free system we observed a 200-fold difference in the ability of Kepone and 17β-estradiol to compete for nuclear estrogen receptors. Kepone did not compete with [^{3}H]progesterone uptake into oviduct nuclei, nor did it compete with [^{3}H]progesterone in an exchange assay with nuclear progesterone receptors; in the former assay up to a 200-fold molar excess of Kepone was examined, while in the latter assay up to a 700-fold excess was tried.

We interpret these data to indicate that Kepone acts in vitro by binding to estrogen receptors, albeit with a weak affinity (K_d estimated to be about 1 μ*M*), thereby causing the migration of these receptors into the nucleus and promoting specific mRNA production. The complete inhibition of its biological effectiveness in vitro by tamoxifen indicates that it binds predominantly to the estrogen receptors and not to the other steroid receptors in these oviduct cells.

The mechanism of Kepone action in vivo is more complex. The most obvious discrepancy from a pure estrogen response is the relatively small effect of Kepone on oviduct growth (Table 1). This response resembled that previously observed with combinations of estrogen and progesterone (*15*). Since experiments performed in vitro ruled out direct effects of Kepone on progesterone receptors, we examined the possibility that Kepone raises the concentration of cir-

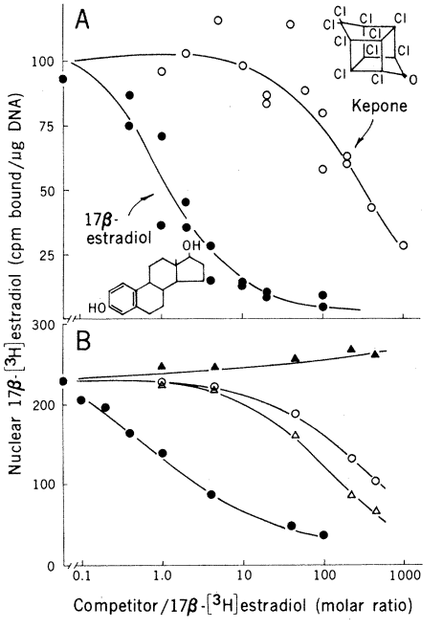

Fig. 2. Competition of Kepone for 17β-estradiol uptake into oviduct nuclei and exchange into estrogen receptors in isolated nuclei. (A) Oviduct explants were incubated under the culture conditions described in Fig. 1. for 60 minutes with 10 n*M* 17β-[^{3}H]estradiol (100 Ci/mmole) plus the indicated molar excess of unlabeled 17β-estradiol (•) or Kepone (○). The tissue was homogenized, nuclei were isolated by centrifugation through 1.3*M* sucrose (10 minutes at 13,000*g*), the steroid extracted into ethanol, and the DNA content of the residue assayed by the diphenylamine reaction. (B) Oviduct nuclei were prepared from estrogen-treated chicks and then portions (25 μg of DNA) were incubated with 10 n*M* 17β-[^{3}H]estradiol plus the indicated molar excess of unlabeled 17β-estradiol (•), tamoxifen (△), Kepone (○), or progesterone (▲) for 1 hour at 37°C. The binding of labeled estradiol to nuclear receptors was measured by charcoal-dextran absorption of unbound steroid (*16*).

culating progesterone. The concentrations of progesterone and 17β-estradiol in the serum after Kepone administration were measured by radioimmunoassay (*16*) and compared to the concentration achieved by injecting doses of progesterone or 17β-estradiol that give intermediate to full induction of ovalbumin and conalbumin. The serum concentration of 17β-estradiol was unaffected by Kepone, but the concentration of progesterone increased from 0.7 n*M* to about 2 n*M* with 12.5 mg of Kepone and to about 3.5 n*M* with 50 mg of Kepone (injected intraperitoneally). A 3.5 n*M* concentration of total serum progesterone is sufficient to achieve about 70 percent of maximum conalbumin induction and 25 percent of maximum ovalbumin induction. This effect of Kepone on serum progesterone is similar to that of ethionine which also induces egg white protein synthesis in the chicken oviduct (*17*). These drugs may raise serum progesterone either by stimulating the synthesis and secretion of progesterone from the adrenals or ovaries or by inhibiting the breakdown and excretion of progesterone. The end result is that Kepone behaves in vivo like a combination of estrogen plus progesterone. Consistent with this interpretation is our observation that tamoxifen only partially inhibits Kepone induction of egg white protein synthesis in vivo, whereas it is completely effective in vitro. Doses of tamoxifen (30 mg) were used that completely prevented the induction of ovalbumin by 1 mg of estradiol-benzoate but had no effect on the induction of ovalbumin by 1 mg of progesterone. The effect of Kepone on oviduct growth is entirely consistent with this dual effect of Kepone in vivo (*5, 6*).

The fact that Kepone has estrogenic activity is surprising to us, considering its structure. Although it may be modified in vivo, this is less likely in vitro and only a remote possibility in the competition assay with nuclear estrogen receptors. The neurological symptoms appear in chicks at doses slightly lower than those required to induce egg white protein synthesis. These symptoms are not ameliorated by tamoxifen, suggesting that they are unrelated to the estrogenic activity of Kepone. It is likely that exposure of humans and other vertebrates to high levels of Kepone would have deleterious effects on normal reproductive physiology as well as the more obvious neurological manifestations.

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Brain Edema: Induction in Cortical Slices by Polyunsaturated Fatty Acids

Abstract. *The presence of polyunsaturated and saturated fatty acids in leukocytic membranes prompted study of their possible role in the induction of brain edema. Polyunsaturated fatty acids including sodium arachidonate, sodium linoleate, sodium linolenate, and docosahexaenoic acids induced edema in slices of rat brain cortex. This cellular edema was specific, since neither saturated fatty acids nor a fatty acid containing a single double bond had such effect.*

Granulocytes and their products (pus) are associated with the massive brain edema found in fatal cases of purulent meningitis and brain abscess. Experimental models of such cases of "granulocytic brain edema" have the features of vasogenic, cytotoxic, and interstitial (hydrocephalic) edema (*1*). Using single cortical slices of the rat brain as a bioassay system in vitro, we have demonstrated that metabolism was altered by incubation of the slices with crude preparations of membranes from granulocytic leukocytes (WBC) obtained from glycogen-induced rat peritoneal exudates. Such leukocytic preparations induced brain edema characterized by increases in water content, cellular swelling (decreases in inulin space), increased intracellular sodium, and decreased intra-

Table 1. Effects of leukocyte membrane fractions on brain swelling and lactate production in vitro. Single, first cortical brain slices 40 to 50 mg in weight and 0.35 mm in thickness, and leukocyte membranes from glycogen-induced rat peritoneal exudates were prepared as described (*1, 17*). Each slice was incubated in 5 ml of Krebs-Ringer medium or medium with lipid for 90 minutes at 37°C. The membranes obtained from 3.0×10^7 leukocytes were extracted with chloroform-methanol (2:1, by volume) and centrifuged at 12,000 rev/min for 10 minutes at 4°C. The pellet was dialyzed for 24 hours with at least one change of Krebs-Ringer buffer. The chloroform-methanol soluble fraction was dried under a stream of N_2 in the dark. A mixture of 0.5 ml of methanol and water (1:1, by volume) was added and mixed, and the two phases were then separated. The water-soluble fraction was dried in vacuo and then washed again with water and dried several times. The final water-soluble fraction was dissolved in Krebs-Ringer buffer. The methanol-soluble fraction was dried under N_2 in the dark, and the solvent was changed to methanol-ethanol (1:1, by volume) followed by ethanol-water (1:1, by volume) and finally to Krebs-Ringer buffer to obtain the experimental medium. Cortical slices were dried at 105°C for 16 hours to determine tissue water. The percentage of swelling was determined by subtracting the percentage of initial from the final water content and dividing by the initial water content. Lactate was determined enzymatically as described (*1, 17*). Values are means ± standard error of slices given in parentheses.

Experiment	Swelling (%)	Lactate production [mmole (kg dry wt)$^{-1}$ 90 min^{-1}]
Medium alone (control)	1.85 ± 0.09 (13)	213.8 ± 7.2 (13)
Medium plus lipid fraction	3.40 ± 0.27 (8)*	305.2 ± 35.2 (6)†
Medium plus water-soluble fraction	1.99 ± 0.17 (6)	276.5 ± 9.6 (6)*
Medium plus insoluble fraction	1.70 ± 0.35 (9)	220.2 ± 19.2 (6)

*$P < .001$. †$P < .05$, Student's *t*-test.

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