Influence of High Levels of DDT in the Diet on Liver Microsomal Estrogen Metabolism in the Laying Hen

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The chlorinated hydrocarbon insecticides have received considerable attention since they were shown to be correlated with egg shell thickness in birds showing a population decline (Ratcliffe, 1967). Hickey and Anderson (1968) suggested that the eggshell thinning caused egg breakage. They proposed that the chlorinated hydrocarbons induced the formation of hepatic microsomal enzymes, thereby, causing an excessive steroid breakdown which altered calcium metabolism and resulted in a thinning of the egg shell.

Hart et al. (1963) showed that chlorinated hydrocarbons stimulated the "drug-metabolizing" enzymes of the liver microsomal fraction. Kuntzman et al. (1964) and Conney et al. (1965) demonstrated the relationship between hepatic microsomal NADPH and oxygen dependent enzymes which metabolize foreign substances (drug-metabolizing enzymes) and steroid hydroxylases. Kuntzman et al. (1965) suggested that steroids are the preferred substrates, but the same enzyme system metabolizes drugs. Insecticides are potent inducers of drug-metabolizing enzymes (Remmer, 1964).

Tests to study the effect of DDT on the drug-metabolizing enzymes have produced results indicating species differences (Kupfer, 1968). In birds Peakall (1967) showed that pigeons receiving DDT had an increased activity of liver enzymes which metabolize testosterone and progesterone. The ringdove fed DDT had increased hepatic estrogen metabolism and a decrease in circulating estrogen (Peakall, 1970). In the chicken various methods had been inconclusive as to the effect of DDT on liver microsomal enzymes (Stephens et al., 1971; Sell et al., 1971), but estrogen metabolism was not measured directly.

This study was conducted to determine the effect of high levels of dietary DDT on the metabolism of estrogen by liver microsomes isolated from laying hens.

PROCEDURES

Two experiments were conducted with Single Comb White Leghorn laying hens. In Experiment 1, 32 hens eight months of age were divided into two equal groups and housed in individual cages. One group was fed a commercial laying diet and the other

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group the same diet containing 500 ppm of technical grade DDT (75% p,p'-DDT, 25% o,p'-DDT). The DDT was dissolved in 500 ml of corn oil for mixing in the diet. After 0, 10, 18 and 51 days of feeding the diet, four hens from each group were killed and their livers removed.

In Experiment II, four groups of 10 birds each were housed in individual cages and fed a commercial laying ration containing 0, 300, 600 or 1200 ppm DDT, respectively. Two hens from each group were killed after 0, 7, 14, 21 and 35 days and their livers removed.

The assay for estrogen metabolism by the liver microsomal fraction was based on a modification of the method described by Conney and Klutch (1963). A 5.0 gm sample from each liver was homogenized in 0.25 M sucrose, centrifuged at 10,000 x g for 20 minutes to remove nuclei, cell debris and mitochondria. The supernatant was then centrifuged at 105,000 x g for one hour and the microsomal pellet collected. The pellet was washed three times with 0.25 M sucrose and then covered with 3.0 ml of 0.1 M phosphate buffer (pH 7.4) and frozen until used for the enzyme assay. Each liver sample was assayed in duplicate.

To conduct the enzyme assay the microsomal pellet was thawed and diluted to 7.0 ml with phosphate buffer (0.1 M, pH 7.4) and mixed to form a microsomal suspension. The assay mixture contained 0.75 ml of NADPH solution (4.0 mg/ml), 0.5 ml of glucose-6-phosphate solution (28 mg/ml), 0.25 ml of $MgCl_2$ solution (0.05M), 0.25 ml of TRIS buffer (0.05 M, pH 7.4), 0.5 ml of glucose-6-phosphate dehydrogenase (~5 units), 2.0 ml of the microsomal suspension and 0.5 ml of estradiol-17β-4-^{14}C (0.5 uC/ml). The mixture was incubated for 15 minutes at 38°C in a 50 ml Erlenmeyer flask in a shaking water bath. The reaction was stopped by the addition of 30 ml of dichloromethane, transferred to a 125 ml flask, stoppered and vigorously shaken for 20 minutes. The mixture was transferred to a 50 ml centrifuge tube, centrifuged for 10 minutes at 10,000 x g and 25 ml of the dichloromethane extract was removed. The dichloromethane was evaporated under N_2 and the residue dissolved in 1.0 ml of methanol.

A 0.1 ml aliquot of the methanol solution was spotted on a Silica Gel G thin layer plate and chromatographed for 30 minutes using benzene:ethyl acetate (1:1 v/v) in a lined tank. The thin layer plate coating was divided into 2.0 x 2.0 cm sections, scraped into a liquid scintillation counting vial and counted. Estradiol was distinguished from its metabolite(s) by the use of proper standards. Any radioactive material that chromatographed differently from the estradiol-17β-4-^{14}C was considered a metabolite produced by the assay system since estradiol gave a single peak when chromatographed.

Data were evaluated by analysis of variance. Duncan's multiple range test was used to test mean differences ($P < 0.05$).

RESULTS AND DISCUSSION

The influence of feeding 500 ppm DDT in the diet of laying hens on liver microsome estrogen metabolism is shown in Table 1. The metabolite(s) of estradiol-17β-4-^{14}C appeared as a single peak in the chromatographic system used and were more polar than the estradiol. The metabolite(s) were tentatively identified as hydroxylated form(s) of estradiol similar to that identified in rats by Kuntzman et al. (1964). The percent of estradiol converted to polar metabolites was not changed significantly by 10 days of feeding DDT, but a significant increase was obtained after 18 days of feeding DDT. After 51 days of feeding 500 ppm DDT, liver microsomal conversion of estradiol to polar metabolite(s) was increased over two-fold. These results indicated that the metabolism of estrogen by the liver microsomes is increased in laying hens fed 500 ppm DDT and that estrogen metabolism response to DDT is time dependent.

TABLE 1

The Effect of Feeding DDT to the Laying Hen on the Metabolism of Estradiol-17β-4-^{14}C by Liver Microsomal Fraction

Dietary DDT (ppm)	Days fed DDT 0	10	18	51
	(% Estradiol Metabolized)[1,2]			
0	20.4[a]	19.6[a]	20.6[a]	21.8[a]
500	20.8[a]	23.6[ab]	29.2[b]	45.5[c]

[1]Each value is a mean of individual samples from four birds, each assayed in duplicate.

[2]Values not followed by the same superscript are significantly different ($P < 0.05$).

Table 2 shows the influence of DDT fed at 0, 300, 600 or 1200 ppm in the diet on liver microsome estrogen metabolism. The rate of increase in estrogen metabolism related to the DDT level of the diet. There was no significant difference in estrogen metabolism after 7 days of feeding the diets, but a numerical increase was seen in birds fed the 1200 ppm DDT diet. After 14 days of feeding the experimental diets, estrogen metabolism was significantly increased in hens fed 600 ppm DDT and hens fed 1200 ppm DDT showed an increase in estrogen metabolism which was significantly greater than obtained in the hens fed 600 ppm. By 21 days of feeding the diets, all levels of DDT caused a significant increase in estrogen metabolism.

TABLE 2

The Influence of Feeding High Levels of DDT to Laying Hens on Liver Microsome Metabolism of Estradiol-17β-4-^{14}C

Dietary DDT (ppm)	Days fed DDT 0	7	14	21	35
	(% Estradiol Metabolized)[1,2]				
0	20.5[a]	24.2[a]	17.2[a]	24.7[a]	22.4[a]
300	22.3[a]	18.7[a]	25.1[a]	44.2[cd]	48.8[de]
600	21.8[a]	23.1[a]	36.5[bc]	52.8[e]	53.9[e]
1200	20.1[a]	27.8[ab]	52.8[e]	52.9[e]	54.0[e]

[1]Each value is a mean of individual samples from two birds, each assayed in duplicate.

[2]Values not followed by the same superscript are significantly different ($P < 0.05$).

Estrogen metabolism in hens fed 1200 ppm DDT reached its highest level after 14 days and showed no further increase at 21 or 35 days. Hens fed 600 ppm DDT showed the same level of metabolism at 21 days and no further increase was seen at 35 days. The hens fed 300 ppm reached the same high level of estrogen metabolism by 35 days. The data indicate that approximately a two-fold increase in liver microsome estrogen metabolism can be obtained by feeding DDT. The time required to obtain this increase is dependent upon the amount of DDT in the diet.

The data would support the hypothesis that the laying hen is relatively insensitive to induction of estrogen metabolizing enzymes by DDT as compared to the several-fold increase seen in the rat with much lower levels of DDT (Kuntzman *et al.*, 1964).

SUMMARY

Two experiments were conducted with laying hens to evaluate the effect of technical grade DDT on estrogen metabolism by liver microsomes using an assay which measured the metabolism of estradiol-17β-4-^{14}C. In Experiment I feeding 500 ppm DDT in the diet had no significant effect on estrogen metabolism after 10 days. However, the metabolism was increased after 18 days and more than doubled after 51 days. In Experiment II hens were fed diets containing 0, 300, 600 or 1200 ppm DDT and estrogen metabolism by liver microsomes was examined at 0, 7, 14, 21 and 35 days. A significant increase in estrogen metabolism was seen at 14 days with the feeding of 600 or 1200 ppm DDT and at 21 days

with 300 ppm DDT. The ability to metabolize estrogen reached a maximum increase of approximately two-fold in all hens fed DDT. This required 14 days when the birds were fed 1200 ppm DDT, 21 days when fed 600 ppm DDT and 35 days when fed 300 ppm DDT. The increase in estrogen metabolism caused by DDT was found to be dose and time dependent.

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