Effect of DDT on Induction of Microsomal Enzymes and Deposition of Calcium in the Domestic Chicken

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The decline in the reproductive success of certain birds has been attributed to the magnification of low concentrations of chlorinated hydrocarbon insecticides through the food chain (1). Following exposure to these insecticides, the appearance of thin-shelled eggs (2), as well as egg-breakage and egg-eating as a result of abnormal behavior (3), have been suspected as mechanisms for a decline in the breeding potential of birds, especially predatory species. The induction of hydroxylating enzymes which alter the metabolism of steroids governing deposition of eggshell calcium in birds has been suggested as an explanation of decreased eggshell thickness (4). Adverse effects of DDT on calcium deposition have been reported in the Japanese quail (5), the zebra finch and ring dove (6). Decreased eggshell thickness and reduced reproductive success as a result of DDT or DDE treatment have also been demonstrated experimentally in mallards (7) and sparrow hawks (3). On the other hand, an increase in eggshell thickness following administration of DDT has been reported in the Bengalese finch (8).

Studies on the in vitro aspects of induction in birds have been less extensive. Increases in microsomal epoxidase activity in Japanese quail following treatment with certain DDT analogues and dieldrin have been reported (9). N,N-dimethylaniline demethylase activity was shown to be induced by DDT and its analogs in the chicken embryo (10). Phenobarbital (hereinafter called PB) was found to induce cytochrome P450 content and O- and N-demethylase activity in the chick embryo and young chick (11). The estrogenic effect of o,p-DDT has been reported in the Japanese quail (12), but p,p-DDT was found to have a negligible estrogenic action.

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[1] The results described herein were from the M.S. thesis of the senior author.

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The present study was undertaken to examine the effect of DDT and PB on several aspects of the mixed function oxidase system of microsomes from the early and adult stages of Gallus domesticus. Results were confirmed, and extended with other insecticides, with in vivo tests in which PB sedation time was determined following administration of the appropriate stressor. A study of the effect of DDT and DDE on eggshell calcium was also conducted.

METHODS

All insecticides were of 98% or greater purity except toxaphene and chlordane which were technical grade. PB was U.S.P. grade of the sodium salt. The NADPH was obtained from Sigma Chemicals Company, and all chemicals used for enzyme assays were of analytical reagent grade.

For enzyme assays, a single intraperitoneal injection of DDT (10 mg/kg in 50-100 ul acetone for young chicks and 20 mg/kg in 200 ul acetone for mature hens) was administered three days before sacrifice, whereas PB (100 mg/kg in 100-400 ul water depending on weight) was administered each day for 3 days prior to sacrifice. Food provided during the holding period contained 0.022ppm of DDT. At the appropriate intervals (as indicated in the accompanying tables), livers from the treated organisms were removed to 1.15% ice cold KCl, minced, and homogenized in an all-glass homogenizer or Sorvall Omni-mixer. Results with the two types of homogenizers were identical. All steps prior to final enzyme assay were conducted at 0-4°C. The homogenate was centrifuged for 20 minutes at 10,000x g and the supernatant fraction further centrifuged at 100,000x g for one hour. The resultant microsomal pellet was suspended in 0.1 M phosphate buffer (pH 7.4) and used for assay. In the cytochrome P_{450} determinations the microsomes were re-centrifuged to remove hemoglobin. Each value reported represents 3-6 (generally 5) pooled livers.

NADPH oxidase activity was measured by the method of Gillette et al. (13). N-demethylase activity was measured by the procedure of Cochin and Axelrod (14) as modified by Hansen and Hodgson (15), and O-demethylase activity was measured by a modification of the method of Nash (16). The content of cytochrome P_{450} in the microsomes was determined with washed microsomes by the method of Omura and Sato (17). Determination of protein content in all microsomal preparations was according to the Lowry method (18).

Sedation time assays with PB consisted of one pretreatment with the appropriate insecticide (10 mg/kg for young birds and 20 mg/kg for mature birds) 3 days before final PB injection or 3 daily treatments with PB (50 mg/kg). Three days following pretreatment, each bird was treated with 100 mg/kg of PB and the sleeping times recorded. The length of sleeping time was determined by use of the righting reflex modified from that described by Hansen and Fouts (19). Sleeping birds were placed back

down and those not immediately righting themselves were judged to be under sedation. Eight to 15 birds were used for each treatment schedule at the onset of the sedation time experiments in the tests with the young birds while 15 birds were used for each treatment in the experiment involving the mature hens.

The experiment on eggshell calcification by laying hens was conducted with 13 White Leghorn hens 18 months of age. They were maintained in individual cages in a temperature controlled room. The basal diet was composed of corn, soybean meal, methionine, soybean oil, vitamins and minerals. The diet was complete with respect to all known nutrients. The experimental diets contained 3.20% calcium by analysis. The DDT and DDE were dissolved in soybean oil and added to the diets at the level of 20 ppm of each. The added oil comprised 1% of the diet. The diet without DDT and DDE received only the 1% additional oil. The additional vitamin D_3 was added in the form of a concentrated premix in place of 0.1% of corn. Individual food consumption was recorded. All eggs were collected and weighed. The eggshell was separated, dissolved in hydrochloric acid and total eggshell calcium was determined by atomic absorption spectrometry. Eggshell calcification was expressed as the percent of total egg weight which was attributable to shell calcium.

RESULTS AND DISCUSSION

As noted in Table 1, PB markedly increased the activity of N-demethylase activity and content of cytochrome P_{450}, in general

TABLE 1

Effect of insecticides and PB on induction of microsomal enzymes (increase over solvent check shown in parenthesis.)

Age	N-demethylase activity[a]		O-demethylase activity[b]		Cytochrome P_{450} content[c]	
	p,p-DDT	PB	p,p-DDT	PB	p,p-DDT	PB
1 week	1.49 (-1%)	4.47 (306%)	.159 (17%)	.246 (25%)	.0096 (1%)	.0421 (207%)
2 weeks	2.93 (73%)	6.24 (231%)	.073 (-28%)	.142 (18%)	.0156 (16%)	.0183 (146%)
13 months	1.04 (-8%)	4.82 (194%)				

[a] µg HCHO/mg protein/20 min.

[b] µmoles p-nitro phenol/mg protein/20 min.

[c] ΔOD cytochrome P_{450}/mg protein

TABLE 2

Effect of daily accumulative treatments with PB on the induction of microsomal enzymes in nine-day-old chicks

Assay	Day of PB Administration 0	1	2	3	4	5
NADPH oxidase nM/min/mg protein (% increase over 0 days)	.961	1.397 (45)	1.397 (45)	1.532 (59)	1.197 (25)	1.371 (43)
P450 content ΔOD cytochrome P450/mg protein (% increase over 0 days)	.0076	.0260 (244)	.0464 (513)	.0382 (405)	.0371 (390)	.0437 (477)
O-demethylation µmoles p-nitrophenol/ mg protein/20 min (% increase over 0 days)	.087	.103 (18)		.151 (74)	.169 (94)	.171 (97)
Sleeping time[a] hours after terminal PB treatment	11.3	7.4	1.3	1.8	1.6	

[a] 2-day old chicks

agreement with the results of others (11). The activity of O-demethylase, was increased at a much lower level of induction. PB induction was further investigated by assaying the enzyme effects following daily treatment for 5 days. The activity of all components (O-demethylase, NADPH oxidase, cytochrome P_{450} content, and sedation time) rose after one day of PB administration and reached a plateau by the second or third day (Table 2).

DDT did not consistently induce the activity of the enzymes assayed in the chick nor adult hen. (Table 1). In preliminary experiments with 16-20 day egg embryos, pretreatment with DDT also failed to induce either N- or O-demethylase activity. Except for the increase of N-demethylase activity in the 2-week chick, the inductive effects seem of doubtful physiological significance due to the lack of consistency among groups and assays. These results, and the results of sedation experiments which follows, are in disagreement with the conclusions reached by others (10).

In *in vivo* experiments (Table 3), PB was found to induce its own metabolism (as determined by decreased sedation time), and dieldrin pretreatment caused decreased sleeping time in the immature but not in the mature bird. The results with the mature

hens are in agreement with N-demethylase activities reported in Table 1 (as well as 0% N-demethylase increase by dieldrin in a test not shown). On the other hand, Table 3 shows that DDT pretreatment increases sleeping time in both young and mature chicks. This latter observation is in agreement with the results of Bitman (20) in the Japanese quail who suggested that liver microsomal enzymes which metabolize PB were inhibited by prior DDT treatment. In companion tests with smaller numbers of birds two and four weeks of age (results not shown in Table), o,p-DDT and p,p-DDE were found to increase sleeping time, and lindane, chlordane, and toxaphene were found to decrease sleeping time. It is interesting to note that the sleeping time decreased from ages 1-4 weeks but was highest in the adult birds.

No consistent increase in liver weight or protein content of liver was noted for either PB or DDT.

The results of the laying hen experiment (Table 4) indicate a slight decrease in eggshell calcium when DDT plus DDE were dietary constituents. The presence of 65 times the requirement for vitamin D_3 did not improve eggshell calcification. The metabolism of vitamin D_3 is known to occur by hydroxylation in the liver (21). Therefore, the high level of vitamin D_3 was investigated in order to gain some insight into what effect, if any, DDT and DDE might have on the known vitamin D_3 dependent calcium transport in the intestine and eggshell-forming gland of the hen (22). A comparison of the differences in shell calcification with the differences in feed and hence average daily calcium intake suggests that decreased calcium consumption rather than the experimental additives were responsible for a lesser shell calification in the domestic chicken.

TABLE 3

Effect of insecticide and PB pretreatment on drug sedation time in young and mature chickens.

	Average PB sleeping time (hours) after indicated treatment			
Age at assay	Untreated	PB	p,p-DDT	Dieldrin
1 week	13.3	7.4[a]	17.2[a]	
2 weeks	7.9	7.5	11.7[a]	2.0[a]
4 weeks	5.6	3.2[a]	9.1[a]	3.7[a]
13 months	23.2	16.4[b]	35.6[b]	24.0

[a]Statistically different from the control at the 95% confidence level as determined by Student's t test.

[b]Statistically different from the control at the 99% confidence level as determined by Student's t test.

TABLE 4

Effect of DDT-DDE on eggshell calcification by chickens

Pre-experiment period[a]			Experimental periods[b]				
						Differences	
Diet	Shell Calcium[c]	Calcium eaten g/hen/day	Diet	Shell Calcium[c]	Calcium eaten g/hen/day	Shell Calcium[c]	Calcium eaten
Control	2.60	3.29	Control	2.68	3.31	+0.08	+0.02
Control	2.70	3.94	DDT-DDE[e]	2.62	3.54	-0.08	-0.40
Vit D[d]	2.70	3.91	Vit D	2.53	3.32	-0.17	-0.59
Vit D	2.92	3.56	Vit D +DDT-DDE	2.68	2.92	-0.24	-0.64

[a] Hens were fed these diets for 21 days. Data shown are the average of the last 5 days of the period, and the number of eggs per treatment was 13±3

[b] Experimental period was 18 days. Data shown are the average of the last 10 days of the period, and the number of eggs per treatment was 22±3.

[c] % of egg weight

[d] 38,400 units of Vit D_3 were added per kg. of feed. Control diet contained 600 units/kg.

[e] p,p DDT and DDE were added to the diet at 20 ppm each of food.

SUMMARY

These results show that PB pretreatment induced microsomal enzyme activity in all stages studied (as measured directly or by decreased sedation time). Induction of microsomal enzymes by DDT was inconsistent in the enzyme assays and pretreatment with DDT and its analogs increased sedation time of PB in in vivo tests, an apparent inhibition of microsomal enzymes. Dieldrin and insecticides of a similar mode of action were found to decrease the sedation time of PB in young chicks, but dieldrin did not alter the sedation time of PB in mature birds. Addition of DDT-DDE to the food of laying hens had no effect on eggshell calcium. These experiments lend additional support to the hypothesis that alteration of oxidative enzyme activity and/or calcium deposition by chlorinated hydrocarbons does not appear to be a general phenomenon among the class Aves.

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