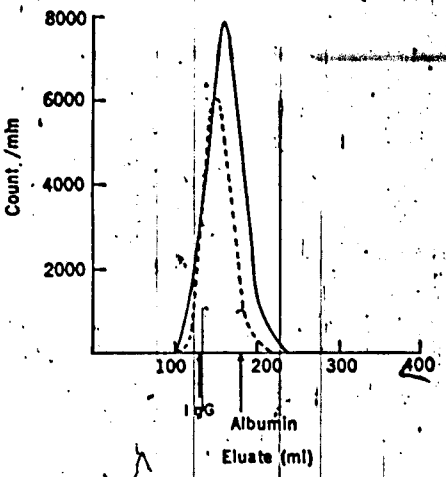

Fig. 1. Cochromatography of the substances which bind vitamin B_{12}. The dashed line is the serum binder labeled with [^{60}Co]-B_{12}. The solid line is the proventriculus binder labeled with [^{57}Co]B_{12}.

and in a supernatant made from a crude homogenate of proventriculus (1:5, weight to volume in saline; ground in the Sorvall Omni-Mixer at full speed for 5 minutes; centrifuged 3000*g* for 15 minutes; 4°C) was estimated by the zirconyl phosphate gel (z-gel) technique (*3*). The homogenate was purified by gel filtration on Sephadex G-100 columns (2.5 by 100 cm) eluted with *p*H 7.0 tris-HCl buffer (0.1*M* tris) made in 0.9 percent saline, with 0.02 percent azide added as preservative.

Antibody was elicited in rats by injection of a crude perchloric acid extract of binder saturated with vitamin B_{12} prepared in the following manner. Supernatant from five proventriculi was prepared as above. An equal volume of 1*M* perchloric acid was added. The supernatant, after centrifugation, was neutralized with NH_4OH, reduced to 5 ml in a Diaflo ultrafiltration cell (No. 50) fitted with an XM 10 membrane and placed on a Sepharose 6B column. The peak eluted with the above buffer was pooled and dialyzed to 5 ml against Carbowax; 90 percent of the binder was recovered.

The partially purified complex of proventriculus binder and B_{12} (saturated binder) was incorporated in an equal volume of Freund's complete adjuvant, and 0.1 ml of this was injected in each rear foot pad of two rats. Two weeks later, these rats were again injected in this manner, and 3 weeks after this, there was a third injection of 0.25 ml in the foot pads. Blood was collected by heart puncture 1 week after the last injection. Antibody that reacts with the B_{12}-binding site of the binders (CSAB) and antibody which reacts with B_{12} saturated binder (CAB) were assayed as described (*4*). Titers were determined at 50 percent of total binder neutralization.

Cobalt-labeled vitamin B_{12} was obtained in two forms: (i) a very high specific activity [^{57}Co]B_{12} (71.4 to 173 mc/mg; Philips-Duphar, Holland) and (ii) [^{60}Co]B_{12} (1 μc/1.484 μg; Squibb, New York). Radioactivity was counted in a Packard (gamma) scintillation spectrometer.

Figure 1 is the elution pattern of the binders in serum and proventriculus, with human serum marker. The serum binder was labeled with [^{60}Co]B_{12} and the proventriculus binder with [^{57}Co]B_{12}. The size of these binders is approximately 113,000 and 96,000, respectively. Extraction with perchloric acid did not alter the size of either binder. This has been further confirmed by chromatography of these binders with R binder from human gastric juice. As estimated by the gel filtration method, R binder has a molecular weight of 110,000. On chromatography the chicken serum binder moves with or slightly ahead of R binder, while the proventriculus binder migrates slightly behind R binder.

The CSAB elicited against proventriculus binder reacted equally with both binders [907 and 903 neutralized units (1 unit = 1 ng of B_{12} bound)], whereas CAB cross-reacts to a slightly lesser extent with serum binder as compared with proventriculus binder (853 and 1047 neutralized units per milliliter). The binding sites are probably identical. The different CAB titers might be expected since the difference in size of the binders indicates some structural difference. The R binder did not cross-react with this antiserum.

The serum binder may be a proventriculus binder with a secretory piece added or these binders may be made on similar genes in different organs. If B_{12} binding proteins evolved from a common ancestral protein, as amino acid sequence analysis has suggested for a variety of other proteins (*5*), then chickens with immunologically similar B_{12} binders in the serum and gastrointestinal tract may represent an intermediate evolutionary stage between an organism with a single binder and mammals which have evolved immunologically distinct binders in the serum and gastrointestinal tract.

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17 December 1969

p,p′-DDT: Effect on Calcium Metabolism and Concentration of Estradiol in the Blood

Abstract. *Ringdoves given 10 parts per million p,p′-DDT showed a decrease of estradiol in the blood early in the breeding cycle and egg-laying was delayed. There was also a decrease in deposition of medullary calcium and in eggshell weight. Injection of p,p′-DDE (150 milligrams per kilogram of body weight) caused reduction of eggshell weight and inhibition of carbonic anhydrase in the oviduct.*

The decline of several species of raptorial birds in Europe and North America has been linked to the use of chlorinated hydrocarbon pesticides (*1*). The symptoms noted during the decline include some or all of the following: (i) abnormally late breeding; (ii) failure to lay eggs (in some cases, laying was followed by egg-eating); (iii) reduced clutch size; (iv) failure to lay again after early loss of eggs; (v) thinner eggshells and increased egg breakage; and (vi) increased embryonic mortality. The total picture can be termed the "raptor-pesticide syndrome" because it was first observed in the falconiformes, although it has now been found in a number of fish-eating birds. I examined the possible underlying pathophysiologic changes.

All experiments were performed on paired ringdoves (*Streptopelia risoria*)

Table 1. All birds were given feed containing 10 ppm of *p,p'*-DDT for 3 weeks before the experiment was begun at the time of the second mating. Groups were killed 8 days after the second mating or after completion of a clutch of two birds. All birds were given ^{45}Ca (2 μc) 1 day before the second mating. The results are expressed as the average ± standard deviation. The numbers in parentheses are the sample sizes. N.D., none detected; cpm, counts per minute.

Estradiol in blood (ng/ml)	Hepatic enzyme activity (nmole) metabolized	Time to first egg (days)	Eggshell: Weight (mg)		Eggshell: Specific activity of calcium (cpm/g)		Specific activity of bone calcium (cpm/g)		Pesticide in brain (μg/g)	
			Egg 1	Egg 2	Egg 1	Egg 2	Femur	Tibia	*p,p'*-DDT	*p,p'*-DDE
Control (8 day)										
18.2 ± 1.2 (6)	26.1 ± 3.1 (4)						7084 ± 1100 (6)	6508 ± 961 (6)	N.D.	0.15 ± 0.07 (6)
Experimental (8 day)										
12.1 ± 1.9* (6)	71.1 ± 10.7* (4)						2654 ± 467* (6)	2731 ± 419* (6)	0.51 ± 0.31 (6)	0.40 ± 0.06* (6)
Control clutch (complete)										
1.4 ± 1.0 (6)	21.2 ± 3.3 (4)	16.5 ± 1.6 (9)	250 ± 10 (10)	25[illegible] ± 17 (9)	5412 ± 1182 (8)	4817 ± 1282 (8)	270 ± 31 (6)	289 ± 43 (6)	N.D.	0.13 ± 0.08 (7)
Experimental clutch (complete)										
1.18 ± 0.3 (6)	64.3 ± 3.0* (4)	21.5 ± 5.5† (10)	219 ± 14* (10)	225 ± 5* (9)	3154 ± 876* (10)	2829 ± 627* (10)	364 ± 101* (7)	321 ± 68 (7)	0.64 ± 0.18 (10)	0.39 ± 0.12* (10)

* $P > .01$. † $.02 < P > .05$.

Table 2. Effect of injected *p,p'*-DDE (150 mg/kg, intraperitoneally) and dieldrin (30 mg/kg, intraperitoneally) on eggshell weight and carbonic anhydrase activity in the oviduct. Results are expressed as the average ± standard deviation. The numbers in parentheses are the same sizes. N.D., none detected.

Treatment	Eggshell weight (mg)	Carbonic activity (unit/g)	Oviduct: *p,p'*-DDE (ppm)	Oviduct: Dieldrin (ppm)	Egg: *p,p'*-DDE (ppm)	Egg: Dieldrin (ppm)
Control	231 ± 11 (7)	17.3 ± 3.1 (5)	0.40 ± 0.11 (3)	N.D.	0.21 ± 0.09 (3)	N.D.
p,p'-DDE	177 ± 27 (12)*	7.1 ± 1.1 (5)*	76.9 ± 34.8 (5)*		16.6 ± 5.8 (6)*	
Dieldrin	232 ± 11 (7)	16.8 ± 1.3 (4)		4.6 ± 1.7 (4)		5.8 ± 1.3 (4)

* $P > .01$.

which had bred successfully at least once (*2*). Pairs were kept in cages (45.7 by 53.3 by 91.4 cm) and maintained on a 16-hour light and 8-hour dark schedule at a temperature of 25°C. At the start of the experiment the female of each pair was removed from the cage and maintained in isolation from the male on an 8-hour light and 16-hour dark schedule for 3 weeks. The *p,p'*-DDT (10 ppm) (*3*) was fed to both sexes in turkey grower pellets (*4*); the feed was started at the time the pair was separated and continued until they were killed. Radioactive ^{45}Ca (2 μc) was given orally to each female 1 day before being put back with her original mate. Females were killed by direct cardiac puncture either 8 days after the second mating or at completion of the clutch of two eggs. Blood was used for determination of the concentration of circulating estradiol. Liver was removed for enzyme metabolism studies, brain for pesticide analysis (*5*), and the tibia and femur for ^{45}Ca analysis (*6*). Also *p,p'*-DDE or dieldrin was injected intraperitoneally into the female within a day of the laying of the first egg. In these experiments the females were killed immediately after the completion of the clutch. Oviducts were removed for pesticide analysis and determination of carbonic anhydrase activity.

The initial chemical extraction and thin-layer chromatographic purification of estradiol were carried out by the method of Attal *et al.* (*7*). To identify estradiol, [^{14}C]estradiol was added and the constancy of the ratio of ^{3}H to ^{14}C was checked by thin-layer chromatography (*8*). The final gas-phase chromatographic determination was performed after the estradiol was esterified with heptafluorobutyric anhydride (*9*) by the method of Exley (*10*). The metabolic activity of hepatic enzymes on [6,7-^{3}H]estradiol was measured in fresh livers in vitro (*11*). The oviduct was dissected out and washed carefully with physiological saline to remove blood. The uterus was then removed from the rest of the oviduct. A small portion of the magnum (about 10 percent) was removed for pesticide analysis and the remainder homogenized with two volumes of ice-cold water and centrifuged for 15 minutes at 5000 rev/min in order to remove cell debris. The carbonic anhydrase concentration in the supernatant was determined by the technique of Miyake and Finons (*12*).

Of the birds killed 8 days after the second mating, those fed DDT showed a significant reduction in the concentration of estradiol in the blood and in the specific activity of ^{45}Ca in the leg bones (Table 1). The metabolic activity of the hepatic microsomal enzyme system on estradiol increased significantly. In the birds killed after completion of the clutch, there was no difference in amounts of estradiol in the blood nor in the specific activity of ^{45}Ca in the leg bones. However, the eggshell weight and specific activity of the ^{45}Ca in the eggshell were significantly reduced for the group fed DDT, and the time from the second mating to laying was significantly increased. The activity of the hepatic microsomal system was elevated for the birds fed DDT.

The decreased amount of ^{45}Ca in the tibia and femur can be related to reduced concentrations of estradiol in the blood, since the deposition of medullary bone calcium is largely under the control of this hormone (*13*). These results confirm the observation (*14*) that *p,p'*-DDE counteracts the effects of injected estradiol to stimulate the deposition of medullary bone. The decreased concentrations of estradiol also explain the increase in the period before egg-laying commences, because this hormone is involved in the stimu-

lation and maintenance of the sex organs and breeding behavior (*15*). A similar increase in the time between the second mating and egg-laying was found by Jefferies (*16*) in the Bengalese finch (*Lonchura striata*), although he suggested a different mechanism than that proposed here.

Since the breeding cycle proceeded to the stage of egg-laying, even though delayed, it is unlikely that severe depletion of stored calcium in the medullary bone occurs. Thus, although the phenomenon of hepatic enzyme induction has an effect on calcium balance, it does not explain the extremely thin eggshells found in the brown pelican (*Pelecanus occidentalis*) colonies along the California coast (*17*). In this case the average percentage thinning was 53 percent, and the extreme was 95 percent. These cases must result from inhibition of calcium availability near the site of eggshell formation. If calcium were unavailable in the body of the female, then inhibition of egg-laying would be expected (*13*). Further, acute hypocalcemia would be incompatible with flight since both muscle and the nervous system would be affected.

Eggshell thinning caused by DDT may result largely from inhibition of carbonic anhydrase by DDT and its metabolites (*18*). Carbonic anhydrase controls the hydration of carbon dioxide and is involved in the secretion of the calcareous eggshell; inhibition of this enzyme by sulfanilamide causes poorly calcified eggshells (*19*). If this idea is correct, then one would expect DDT and DDE to be more effective in causing thin eggshells than other chlorinated hydrocarbons because, within this group of materials, the inhibition of carbonic anhydrase is specific to DDT and its metabolites (*20*). Analysis of field results (*18*) suggests that DDE is more effective than either dieldrin or polychlorinated biphenyls in causing eggshell thinning.

Experiments were carried out on doves by injecting *p,p'*-DDE or dieldrin within a day of egg-laying. Any effect on eggshell thinning could not result from the hepatic enzyme system, because estradiol was decreasing in concentration at this stage of the breeding cycle. Further, the time involved was too short for this mechanism. Eggshell weights were significantly decreased by DDE but were unaffected by dieldrin (Table 2). Also, carbonic anhydrase activity was markedly reduced in the oviducts of birds receiving DDE but were unaffected by dieldrin. Thus, a relation of eggshell weight to carbonic anhydrase activity has been established. The inhibition of carbonic anhydrase could explain the extremely thin eggshells found in California (*17*). Inhibition of this enzyme would prevent utilization of calcium in the oviduct even though the bird was otherwise in normal calcium balance.

The symptoms of the "raptor-pesticide syndrome" can now be considered in terms of physiological mechanisms. The abnormally late breeding and failure to lay again after early loss of eggs can be readily explained in terms of altered hormone concentrations resulting from hepatic enzyme induction. The failure to lay eggs could be caused by depressed hormone concentrations, or apparent failure to lay could be caused by early breakage and eating of eggs. It is likely that reduced clutch size is also caused by breakage and eating of the eggs since reduced clutch size has been noted mainly in cases where the nests were not frequently checked. The phenomena of thin eggshells and egg-breakage are explained on the basis of inhibition of carbonic anhydrase. The cause of embryonic mortality remains to be investigated.

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4. Sampling showed that pesticide concentration was within 10 percent and calcium content within 3.2 percent.
5. For pesticide analysis, the sample was [illegible] for 48 hours at 40° to 45°C; ground with sodium sulfate; extracted for 8 hours with a mixture of diethyl ether and petroleum ether (1:3); and cleaned by passage through a Florisil column. Readings were made on a Varian Aerograph 2100 with ^{63}Ni electron-capture detector with a glass column ([illegible] m) containing 2 percent QF-1 on Anakrom ABS at 200°C, direct injection with [illegible] at 225°C, detector at 280°C.
6. Weights of bones and eggshells were measured after ashing overnight at 800°C. The ashed material was dissolved in 6N HCl, evaporated to dryness, and redissolved in water. A portion was taken for determination of radioactivity in a scintillation counter.
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5 January 1970; revised 9 March 1970

DDT-Induced Inhibition of Avian Shell Gland Carbonic Anhydrase: A Mechanism for Thin Eggshells

Abstract. *The shell-forming glands of Japanese quail fed p,p'-DDT or p,p'-DDE had carbonic anhydrase activity 16 to 19 percent lower than shell glands of quail on a diet free of pesticides.*

The pesticide DDT (*1*) produces a decrease in eggshell thickness in Japanese quail (*2*), sparrow hawks (*3*), and mallards (*4*). The content of calcium in the eggshell declined (*2*) and reproduction was impaired (*3, 4*) by the direct addition of DDT or DDE (*1*) to the diet, thus confirming correlative evidence (*5, 6*) that DDT and related organochlorine compounds decrease eggshell thickness. We investigated carbonic anhydrase (CA) (E.C.-4.2.1.1) in the shell-forming gland of Japanese quail fed DDT or DDE