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 dried 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 (18.3 m) containing 2 percent QF-1 on Anakron ABS at 200°C, direct injection with inlet 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 6*N* HCl, evaporated to dryness, and redissolved in water. A portion was taken for determination of radioactivity in a scintillation counter.
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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

to determine whether decreased activity could account for the defect in eggshell formation.

The Japanese quail were housed in individual cages on a schedule of 14 hours of light and 10 hours of dark. They were fed diets containing 100 ppm of *p,p'*-DDT or 100 ppm of *p,p'*-DDE for 3 months. Diets of both adequate (2.5 percent) and low (0.6 percent) calcium content were used. Activity of CA was assayed electrometrically by the Wilbur and Anderson procedure (*7*) as modified by Woodford *et al.* (*8*), in which substrate is supplied in the form of gaseous carbon dioxide. The saturated KCl solution of the combination electrode of the Corning model 12 *p*H meter was replaced with 4*M* KCl to prevent freezing out of KCl in the asbestos fiber salt bridge and in the body of the electrode. All *p*H standardizations and reactions were conducted at 0°C. The CA standard was a purified preparation from beef blood (Worthington Biochemical Corp.).

Birds were killed by decapitation 6 to 8 hours before estimated oviposition, at which time a calcifying egg was present in the shell gland. Whole blood was collected in oxalated tubes. The samples of blood (0.5 to 1.0 ml) and weighed samples of whole shell gland (300 to 400 mg) were homogenized in ice-cold water for 3 minutes. The homogenates were centrifuged at 2500*g* for 15 minutes at 0°C. The supernatant solutions were then centrifuged again at 9500*g* for 20 minutes at 0°C. The opalescent supernatants (or a dilution) were then assayed immediately for CA activity. Calcium was determined by atomic absorption spectrophotometric analysis of solutions obtained by wet-ashing eggshell in concentrated HCl. Pesticide residues were determined in body fat and eggs by gas-liquid chromatography with an electron capture detector (*9*).

Carbonic anhydrase activity was lower in both the shell gland and blood of the treated Japanese quail (Table 1). Decreases of 16 to 19 percent occurred in CA from the shell gland of Japanese quail fed *p,p'*-DDT or *p,p'*-DDE. There were no differences in the weights of the shell glands among the groups. It was not possible to determine whether DDT and DDE caused a decrease in the total amount of enzyme or whether they partially inhibited the enzymatic activity in these extracts. The activity of CA in the blood of the quail treated with DDT or DDE exhibited larger declines—22 and 44 percent, respectively (Table 1).

Table 1. Carbonic anhydrase activity in the shell gland and blood of control quail and quail treated with DDT or DDE. Results are expressed as the number ± standard error.

Group	*N*	Shell gland weight (g)	CA activity		
			Shell gland		Blood (unit/ml)
			Unit/g	Total units	
Control	20	1.59 ± .09	186 ± 7	298 ± 22	1184 ± 96
p,p'-DDT	18	1.54 ± .08	156 ± 12*	242 ± 18†	924 ± 82†
p,p'-DDE	11	1.57 ± .07	150 ± 10*	235 ± 18†	663 ± 56‡

* $P < .01$. † $P < .05$. ‡ $P < .001$.

Table 2. Eggshell calcium and pesticide concentration in lipid and eggs of quail treated with DDE or DDT. Eggshell calcium is expressed as a percentage of egg weight.

Group	Eggshell calcium (%)	Eggs		Lipid	
		DDE (μg/g)	DDT (μg/g)	DDE (μg/g)	DDT (μg/g)
Control	2.58 ± .06	0.20	0.40	1.47	3.70
p,p'-DDT	2.37 ± .03*	48	196	483	1373
p,p'-DDE	2.38 ± .07†	196		1610	

* $P < .005$. † $P < .05$.

The concentrations of pesticides in the lipid and eggs and the percent of calcium in the eggshells were determined (Table 2). Pesticide concentration in the eggs was approximately one-eighth of the body lipid concentration. Eggshell calcium was significantly lower in eggs from the quail treated with DDT or DDE.

Carbonic anhydrase is inhibited by DDT in human blood (*10*) and a sensitive method for DDT detection has been based on inhibition of bovine erythrocyte CA by DDT (*11*). In contrast, Anderson and March (*12*) were unable to demonstrate an effect of DDT on insect CA either in vivo or in vitro.

In the formation of the avian eggshell, CA is believed to be necessary to supply the carbonate ions required for calcium carbonate deposition. Several investigations have supported an active role for CA in eggshell formation (*13*), showing that CA was lower in shell glands producing soft-shelled eggs or no eggs than in glands producing normal eggs. Bernstein *et al.* (*14*) have provided additional evidence for an obligatory role for CA in eggshell formation.

Mueller (*15*) has questioned this role for CA because he did not find significant differences in CA activity in the shell gland at different stages of egg formation, suggesting that active shell formation was not accompanied by increased CA activity. Heald *et al.* (*16*) also did not find a significant correlation between CA activity and shell strength.

Treatment with DDT results in decreased CA activity in the avian shell gland. This demonstration in vitro does not preclude normal functioning of the CA enzymatic machinery in the intact tissue in vivo. Under the conditions of our experiments, however, the percentage declines in shell gland CA activity were 16 to 19 percent, amounts which could account for observed decreases in eggshell thickness of 10 to 15 percent in birds treated with DDT or DDE (*2–4*). The limitation by carbonic anhydrase of carbonate ions needed for the deposition of the calcium carbonate of the shell could provide the mechanism by which chlorinated hydrocarbons affect eggshell thickness.

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Human Glomerular Basement Membrane: Chemical Alteration in Diabetes Mellitus

Abstract. *The human glomerular basement membrane belongs to the collagen family of proteins. It contains about 7 percent carbohydrate, half of which occurs as glucosylgalactose disaccharide units linked to hydroxylysine. Glomeruli from diabetics contain increased amounts of basement membrane material. In addition, these membranes show a distinct chemical alteration characterized by a significant decrease in lysine, accompanied by an equivalent increase in hydroxylysine and hydroxylysine-linked disaccharide units.*

Thickening of the capillary basement membrane, as observed with the electron microscope, is a characteristic pathological alteration in many tissues of human diabetics (*1*). Under the light microscope, this may be seen as an increase in material which reacts intensely with the periodic acid–Schiff stain. In the renal glomerulus, alterations are particularly prominent and involve, in addition to the thickening of the basement membranes of the capillary loops, the accumulation of material in the mesangial region similar to that of basement membranes; this thickening eventually results in the characteristic Kimmelstiel-Wilson nodular lesions (*2*).

The glomerular basement membrane is believed to function as the major filtration barrier between the blood and the urine (*3*). In diabetes, where it undergoes marked thickening, it becomes defective in this function, and patients with diabetic nephropathy often lose large quantities of protein in the urine. This suggests that diabetes leads not only to an increased amount of basement membrane material but also to alterations in its structure.

Using methods previously described for the bovine membrane (*4*), we have extensively studied the composition of human glomerular basement membrane. Basement membranes were isolated from normal and diabetic human kidneys obtained at autopsy by a modification of the method of Krakower and Greenspon (*5*). In this procedure steel sieves are used to disrupt the renal cortex and to separate the glomeruli from other tissue elements (*4*). Basement membranes were obtained from the isolated glomeruli after ultrasonic treatment. The purity of the preparations was evaluated under the electron microscope and by chemical analysis. Electron microscopic examination showed that the preparations of basement membrane had an amorphous appearance, similar to that seen in the intact glomeruli; only negligible amounts of fibrillar collagen could be seen. The average content of DNA was 0.36 percent of the weight of the dry membrane, and the average total phosphorus was 0.14 percent. The total lipid content of the basement membranes was less than 1 percent, and no significant amounts of covalently bound fatty acids could be detected. Normal and diabetic basement membranes were not significantly different in these analyses.

Table 1. Composition of normal human glomerular basement membrane. Values represent the average of analyses performed on six pools each of which contained the glomerular basement membranes from 15 to 20 pairs of kidneys. The components were analyzed by the methods previously used (*4*, *9*).

Component	Residue weight (g/100 g dry wt. ± S.D.M.)	Residues per 1000 total amino acid residues
Hydroxyproline	7.51 ± 0.35	84.1
Aspartic acid	5.87 ± 0.37	64.6
Threonine	2.72 ± 0.23	34.1
Serine	3.49 ± 0.23	50.8
Glutamic acid	8.83 ± 0.87	86.7
Proline	6.09 ± 0.69	79.4
Glycine	9.95 ± 0.37	220.9
Alanine	3.87 ± 0.18	68.9
Valine	2.93 ± 0.24	37.4
Methionine	1.27 ± 0.08	12.7
Isoleucine	2.53 ± 0.13	28.3
Leucine	4.88 ± 0.23	54.8
Tyrosine	1.97 ± 0.21	15.4
Phenylalanine	2.90 ± 0.34	26.3
Hydroxylysine	2.39 ± 0.19	21.3
Lysine	2.91 ± 0.21	28.8
Histidine	1.66 ± 0.08	15.4
Arginine	5.79 ± 0.36	47.1
Half-cystine	1.71 ± 0.09	21.0
Tryptophan	0.25	2.0
Amide nitrogen	0.87	(64.9)
Glucose	2.03 ± 0.07	15.8
Galactose	2.34 ± 0.13	18.3
Mannose	0.55 ± 0.02	4.32
Fucose	0.14 ± 0.007	0.98
N-Acetylglucosamine	1.21 ± 0.02	7.40
N-Acetylgalactosamine	0.16 ± 0.004	0.99
N-Acetylneuraminic acid	0.62 ± 0.025	2.67
Glucosylgalactosylhydroxylysine		16.13 ± 0.76

Compositional studies on the basement membrane of normal human kidney indicated that it belongs to the collagen family of proteins (Table 1). More than one-fifth of the total amino acid residues was glycine; substantial amounts of hydroxyproline and hydroxylysine were also present. The total carbohydrate content made up 7.05 percent of the dry weight of the membrane, and the sugars were identified as glucose, galactose, mannose, fucose, glucosamine, galactosamine, and *N*-acetylneuraminic acid. The presence of this large amount of carbohydrate, as well as the occurrence of half-cystine and a high content of hydroxylysine, clearly differentiates the analyses of this material from those of vertebrate fibrillar collagen (*6*).

Digestion of the membrane with collagenase and Pronase, followed by separation of the glycopeptides by gel filtration on Sephadex G-25 and Sephadex G-50, was performed as previously described (*7*). Analyses of the glycopeptides obtained in this manner indicated that the carbohydrate is distributed in two distinct types of units.

One unit is a heteropolysaccharide made up of galactose, mannose, hexosamine, sialic acid, and fucose residues; it accounts for 45 percent of the carbohydrate in the membrane. If we assume that this unit contains three mannose residues, as in the heteropolysaccharide units of the bovine glomerular basement membrane and many other glycoproteins (*8*), then its average composition would consist, in addition to the mannose, of 3.7 galactose, 5.9 hexosamine, 2.7 sialic acid, and 1.2 fucose residues. From the amino acid content of the glycopeptides contained in this unit, it is likely that it is linked to the protein through a glycosylamine type of bond to asparagine.

The other carbohydrate unit was a disaccharide consisting of glucose and galactose linked by a β-glycosidic bond