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INTRACELLULAR DISTRIBUTION AND ROLE OF CARBONIC ANHYDRASE IN THE AVIAN *(GALLUS DOMESTICUS)* SHELL GLAND MUCOSA

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SUMMARY

1. The enzyme, carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1), has been studied in the mucosal cells of the avian *(Gallus domesticus)* shell gland and has been found to be a soluble constituent of these cells.

2. Mitochondrial and microsomal fractions showed very low and non-fluctuating levels of carbonic anhydrase activity. These findings support a non-membrane-attached, intracellular role for the enzyme.

3. Intramuscular injections of the specific inhibitor, acetazolamide, were correlated with decreases of enzymatic activity in the mucosal cells and with decreases in egg shell weight. These results have been interpreted to indicate an intimate association of the enzyme in the shell gland mucosal cells with $CaCO_3$ deposition.

4. The fine structure of the shell gland mucosa in normal and acetazolamide-treated laying hens did not reveal ultrastructural changes in conjunction with carbonic anhydrase inhibition.

5. A model of the mode of action of carbonic anhydrase in the shell gland mucosa is discussed.

INTRODUCTION

The mucosa of the shell gland of the avian oviduct provides a model system for investigating the mobilization of relatively large amounts of Ca^{2+} and CO_3^{2-} which are ultimately deposited as a $CaCO_3$ shell on the egg. The deposition of the $CaCO_3$ occurs mainly in the shell gland, and the source of the CO_3^{2-} was thought to be the HCO_3^- of the blood[1]. Recently, it has been demonstrated that the source of the CO_3^{2-} is not circulatory HCO_3^- (see ref. 2). This finding seems to support the theory that the shell gland derives the CO_3^{2-} from its own metabolic CO_2 production[3]. Previous investigations have indicated that in any case the production of CO_3^{2-} may be facilitated by the enzyme, carbonic anhydrase[2,4].

The histology of the hen's shell gland at the light microscope level has been reported by numerous authors[5-8] and the electron microscopic appearance of the

mucosal cells has also been described in the laying hen[9,10]. In spite of extensive studies, the sites of CO_2 and HCO_3^- mobilization and Ca^{2+} movement in the shell gland mucosa remain undefined.

MELDRUM AND ROUGHTON[11] were the first investigators to suggest that carbonic anhydrase might play an important role in the egg shell formation of birds. The activity of this enzyme in the hen's oviduct was investigated by COMMON[12] who reported that activity was higher in the epithelial cells of the shell gland than in any other section of the oviduct. GUTOWSKA AND MITCHELL[1] proposed an essential role for carbonic anhydrase in the calcification of the egg shell on the basis of the inhibitory effect of sulfanilamide injections on the activity of the enzyme in the blood of hens that were observed to lay chalky or soft-shelled eggs. The latter workers proposed that carbonic anhydrase could act in the secreting cells of the shell gland by catalyzing the dehydration of H_2CO_3, a component in the equilibrium reaction: $2HCO_3^- \rightleftarrows H_2CO_3 + CO_3^{2-}$; and thus the enzyme could increase the amount of available CO_3^{2-}. Recent work, however, suggests that this theory is no longer tenable[2,13]. DIAMANTSTEIN's theory[3], in which intracellular carbonic anhydrase catalyzes the hydration of metabolic CO_2 to produce the carbon source for the egg shell, seems to have considerable support[2].

Recently, attempts have been made at the light microscope level to locate the site of action of the enzyme in the avian shell gland using HAUSLER's[14] histochemical staining technique. The use of this technique has produced contradictory results in several investigations[13,15,16]. BARKA AND ANDERSON[17] reported that the histochemical technique for detection of carbonic anhydrase gave uninterpretable results.

The techniques of tissue fractionation and analytical centrifugation have been employed previously to determine the intracellular distribution of carbonic anhydrase in rat tissues[18,19] and in chick embryos[20]. The results have indicated that the majority of enzymatic activity is associated with the soluble constituents of the cells. In general, all particulate enzyme concentrations were relatively low, although KARLER AND WOODBURY[19] emphasized that the small amount of activity found in rat mitochondrial preparations could not be eluted.

Up to the time of the present study, work reported in the literature has yielded very limited information on the intracellular biochemical role of carbonic anhydrase in the deposition of $CaCO_3$ and its intracellular locus in avian shell gland tissue. The present study was designed to contribute to the elucidation of the role of carbonic anhydrase in the mucosal cells of the avian shell gland. Evidence was also sought concerning the mode of enzyme synthesis and action within the specialized tissue of the shell gland. Acetazolamide (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide), which is a potent carbonic anhydrase inhibitor (I_{50} equals approx. 10^{-7} M)[21], was used. Appropriate comparisons of morphology and enzymatic activity could then be made between the normal and acetazolamide-treated birds in an attempt to understand better the relationships which exist *in vivo*.

MATERIALS AND METHODS

Experimental hens

1-year-old, regularly laying, single-comb, white Leghorn hens weighing 2 to 2.5 kg were used in the investigation. All hens were maintained on the same regular schedule in wire cages under artificial light, and were fed water and a standard laying

ration *ad libitum*. Egg position in a particular one of the three major sections of the oviduct: the magnum, the isthmus, or the shell gland, was the criterion for selection of experimental animal tissue donors.

Biochemical methods

The hens were sacrificed by decapitation and bled. The shell gland was quickly removed and cooled to 0°. All further manipulations were carried out at 0–4°. When an egg was present in the oviduct, its position was noted. If the shell membrane was scheduled to be analyzed for carbonic anhydrase activity, the egg was also immediately cooled. 2 g of shell gland mucosal tissue were then scraped away from the muscle layers, suspended in enough 0.44 M sucrose[22,23] to make a 1:10 (w/v) preparation, and homogenized in a glass Potter–Elvehjem homogenizer with a motor-driven Teflon pestle (clearance: 0.015–0.023 cm) for 4 min. A sample of the homogenate, equivalent to 0.4 g of wet tissue, was taken to represent the total unfractionated material while the particulate cellular components were isolated from the remainder of the homogenate according to the differential centrifugation procedure previously worked out for this tissue by HOHMAN AND SCHRAER[23].

Representative pellets of particulate matter, isolated from several hens by the above homogenization and centrifugation techniques were observed in the electron microscope and proved to be in excellent agreement with the results of the latter workers. All fractions, including the homogenate were analyzed for carbonic anhydrase activity according to the manometric technique of KREBS AND ROUGHTON[21] using a Gilson Medical Electronics Warburg apparatus which was cooled to 0° with a Neslab portable bath cooler. 2 ml of a cold 0.1 M phosphate buffer (pH 6.9) were pipetted into the main compartments of the Warburg vessels in an ice bath and were followed by 0.2 ml of a suspension in 0.44 M sucrose of the subcellular fraction to be assayed. Experimental control blanks of 0.2 ml of 0.44 M sucrose, which also served as thermobarometers, were assayed as well. Finally, 1.0 ml of 0.1 M $NaHCO_3$ was pipetted into each side arm and the flasks were attached to the manometers, placed in the 0° Warburg bath, and allowed to equilibrate for 10 to 15 min while being shaken at 75 oscillations per min. At time zero, after equilibration, the solutions were mixed, shaken at 145 oscillations per min, and the pressure changes recorded at intervals of 30 sec over a period of about 5 min. The pressure changes in each flask were converted to μl CO_2 by the appropriate flask constants, and the increase in μl CO_2 evolved in the first 30 sec (*i.e.*, the initial velocity) above the blank was used as a measure of the activity of the enzyme[24]. Subcellular fractions were assayed for protein according to OYAMA AND EAGLE's modification[25] of the colorimetric method of LOWRY *et al.*[26].

The possibility of whole or lysed red blood cell carbonic anhydrase contamination of the subcellular fractions has been excluded in a fashion similar to the method used by DATTA AND SHEPARD[18]. 10 ml of venous blood were withdrawn from a hen whose shell gland was to be assayed for enzymatic activity. The red blood cells were spun down in a heparinized centrifuge tube, the serum was discarded, and the red blood cells were resuspended in enough 0.44 M sucrose to make a total of 10 ml. This suspension of red blood cells was then diluted 1:125 (v/v) with 0.44 M sucrose. 10 ml of the diluted red blood cell suspension were homogenized with 1.0 g of the shell gland mucosal cells while 10 ml of 0.44 M sucrose (without red blood cells) was used to homogenize another g of the mucosal cells from the same hen. In this way, the usual 1:10 (w/v) concentration

of homogenate was maintained while both homogenates were then carried through the rest of the fractionation scheme. In terms of carbonic anhydrase activity, there was complete sedimentation of red blood cells in the nuclear fraction without any measurable destruction during the homogenizing procedure[18]. This finding is most important because of the extremely high concentration of carbonic anhydrase in red blood cells[11].

Electron microscopy

In order to examine shell gland mucosa from tissue treated *in vivo* with acetazolamide, 3 hens were injected intramuscularly with 50 mg of acetazolamide in 0.9% saline once a day for 5 days and sacrificed 4 h after the last injection. Two other hens were treated with a single injection of 100 mg of acetazolamide in 0.9% saline and sacrified 2 h later. The inhibitory effects of the drug were judged by the production of soft-shelled eggs and the results of enzymatic assays of the shell gland mucosae of all treated animals.

In preparing tissues for electron microscopy, the birds were decapitated and small pieces (1 mm^3) from the shell gland mucosa were fixed in 1% OsO_4 buffered with 0.12 M phosphate buffer (pH 7.3) at room temperature for 1 h (ref. 27). After dehydration in increasing concentrations of ethanol the tissue pieces were embedded in Epon 812 (ref. 28). Thin sections were prepared in a Porter-Blum MT-2 ultramicrotome (Sorvall) using glass knives and were then picked up on uncoated copper grids. The sections were stained with uranyl acetate[29] and lead citrate[30] and studied in an RCA EMU-3H electron microscope. Thick sections (1–2 μ) were prepared from the same OsO_4-fixed and Epon-embedded blocks, and stained with basic fuchsin[31] for light microscopy.

The shell gland mucosa remaining after the sampling of tissue for electron microscopy was assayed for carbonic anhydrase activity as above.

RESULTS AND DISCUSSION

Biochemical

In Table I the distribution of carbonic anhydrase activity in mitochondrial, microsomal, and soluble cell fractions are compared. The data illustrate that carbonic anhydrase is essentially a soluble constituent of the mucosal cells of the shell gland. Since Table I indicates that the enzymatic activity in the homogenate might not account for the combined activity observed in the particulate and soluble fractions on a wet weight basis, an attempt was made to recombine the soluble and particulate subcellular fractions into a "recombined homogenate" in order to see if the activity of these combined fractions would thus be depressed. The result (Table II) was that the activity in the "recombined homogenate" was depressed to approximately the same level as the original homogenate. That is, it was lower on the basis of wet weight than the sum of the activities in the total particulate and soluble fractions from which it had been prepared. It is assumed that the enzyme does not exhibit full activity in the homogenate because of the presence in this suspension of inhibitors.

The data shown in Table III were obtained by eliminating washing procedures during the centrifugation of cell fractions. A comparison of Table I with Table III is sufficient to demonstrate that the minimal activity present in doubly washed par-

TABLE I

INTRACELLULAR DISTRIBUTION OF CARBONIC ANHYDRASE IN THE MUCOSAL CELLS OF THE AVIAN SHELL GLAND

See METHODS for details of assay procedure.

*Fraction**	*Initial velocity in µl CO_2/30 sec ± standard deviation (7 hens)*	
	per mg wet wt.	*per mg protein*
Homogenate	0.42 ± 0.09	3.5 ± 0.5
Nuclear**	0.10	1.9
Mitochondrial	0.005 ± 0.005	0.5 ± 0.5
Microsomal	0.015 ± 0.010	1.2 ± 1.0
Soluble	0.48 ± 0.08	11.0 ± 2.5

* Subcellular fractions were isolated by the differential centrifugation procedure of HOHMAN AND SCHRAER[23].

** A representative value for the nuclear fraction is included here for comparison. Routinely, such fractions were only monitored for morphological components and were demonstrated to contain the few red blood cells remaining in the tissue[18].

ticulate fractions (Table I) is decreased even further when the fractions are not washed. On the basis of these results, it was concluded that carbonic anhydrase is primarily a soluble constituent of the shell gland mucosal cells of the laying hen. The small and inconsistent amount of carbonic anhydrase activity associated with the mitochondrial and/or microsomal fractions might also have indicated that the enzyme is associated in a labile fashion with these subcellular components. Therefore, an attempt to dislodge further enzymatic activity from the mitochondrial or microsomal fractions was deemed necessary. When the particulate and soluble fractions were subjected to freeze-drying for 16 h and then reassayed for enzymatic activity and protein, there was no enhancement of the activity in particulate fractions. The activity of soluble fractions remained

TABLE II

THE EFFECTS OF RECOMBINATION ON PARTICULATE AND SOLUBLE CARBONIC ANHYDRASE ACTIVITY

*Treatment**	*Fraction*	*Initial velocity in µl CO_2 per 30 sec*	
		per mg wet wt.	*per mg protein*
A	Homogenate	0.50	4.0
	Total particulate	0.15	2.2
	Soluble	0.50	13
B	"Recombined homogenate"	0.49	3.2

* A, The homogenate was centrifuged once at 106 000 × *g* for 56 min in the Spinco Model L Ultracentrifuge (30 rotor, R_{max} = 10.5 cm) to obtain a "Total Particulate" fraction (including nuclei, cellular debris, and any red blood cells present) and a soluble fraction. B, The latter two fractions (*i.e.*, "Total particulate" and "Soluble") were then recombined and assayed again for enzymatic activity and protein. Of the protein concentration originally present in the homogenate, 95% was recovered in the "Total particulate" and "Soluble" fractions.

TABLE III

THE CARBONIC ANHYDRASE ACTIVITY IN UNWASHED FRACTIONS*

Fraction	*Initial velocity in μl CO_2 per 30 sec ± standard deviation (4 hens)*	
	per mg wet wt.	*per mg protein*
Homogenate	0.45 ± 0.04	3.6 ± 0.5
Mitochondrial	0.0	0.0
Microsomal	0.0	0.0
Soluble	0.39 ± 0.05	9.0 ± 2.4

* The differential centrifugation schedule employed was identical to that previously described except that fractions were not subjected to the washing procedures.

at approximately the same level. It is assumed that cellular substructure such as endoplasmic reticulum and mitochondria was disrupted by the freeze-drying process. In light of this evidence, it would seem reasonable that the very low and inconsistent carbonic anhydrase activity sometimes observed in the mitochondrial and/or microsomal fractions was in fact due to an artifact of adsorption of the soluble enzyme to the components of these fractions during homogenization and washing[32,33] rather than to a functional and/or structural role of the enzyme in the architecture of the cells.

When assays of the homogenized shell membranes removed from isthmus and shell gland eggs were performed, it was found that in all cases there was no measurable carbonic anhydrase activity. This finding is contrary to the report by ROBINSON AND KING[15] of extracellular histochemical localization of the enzyme in the mammillae of the shell membrane. It was concluded that the histochemical method used by these workers was not specific for the enzyme, possibly because it did not include a modification suggested by BLEYL[34]. BLEYL reported that the specific demonstration of carbonic anhydrase activity by the HAUSLER technique "requires the use of chelating agents to eliminate unspecific reactions with bivalent metals (especially Zn)". The value and meaning of the histochemical work can only be viewed in light of contradictory results and oversights in methodology[16,17].

GUTOWSKA AND MITCHELL[1] observed that it was possible to make the same hens lay either thick-shelled eggs, soft-shelled eggs, or rough-shelled eggs by manipulation of doses of subcutaneously injected sulfanilamide. MUELLER[35] demonstrated the diuretic action of acetazolamide in hens laying soft-shelled eggs during drug treatment and suggested that the interference of carbonic anhydrase inhibitors with shell formation might be due to their diuretic effect at the kidney level and not to interference with the enzyme's function at the level of the shell gland. This hypothesis has been refuted by MAREN[36]. However, neither of these investigators has assayed the shell gland tissue for enzymatic activity during treatment with sulfanilamides.

Carbonic anhydrase activity in all subcellular fractions and in the homogenate decreased to zero in the case of 4 hens sacrificed 2 h after a single dose of 100 mg of acetazolamide. When 3 hens were sacrificed 4 h after the last of 5 doses of 50 mg each per day, the carbonic anhydrase activity was present only in the homogenate and the soluble fraction at extremely low levels. Acetazolamide thus reduced the level of

carbonic anhydrase activity in the shell gland mucosa while the average shell weight of eggs laid by these birds decreased from 5.1 ± 0.4 g (S.D. for 6 eggs) before treatment began to 3.0 ± 0.1 g (S.D. for 7 eggs) during treatment.

The present investigation has therefore served to demonstrate an intimate relationship between the carbonic anhydrase activity of the shell gland mucosal cells and the specific function of this tissue with regard to $CaCO_3$ deposition.

EHRENSPECK, SCHRAER AND SCHRAER[37] have established that the movement of ^{45}Ca *in vitro* from the shell gland serosa to the mucosa (*i.e.*, toward the lumen) is in the ratio of 2.4:1 (movement toward mucosa: movement toward serosa) when an egg shell is calcifying in the shell gland, whereas the unidirectional fluxes are in a 1:1 ratio when the egg is in any other section of the oviduct or absent from the oviduct. Recent, as yet unpublished, work by the latter investigators indicates that acetazolamide also affects this ratio.

Morphological

The fine structure of the shell gland mucosa of the control birds was largely in agreement with that published by JOHNSTON, AITKEN AND WYBURN[9]. However, the vacuolation of the tubular gland cells described by these authors was not evident in

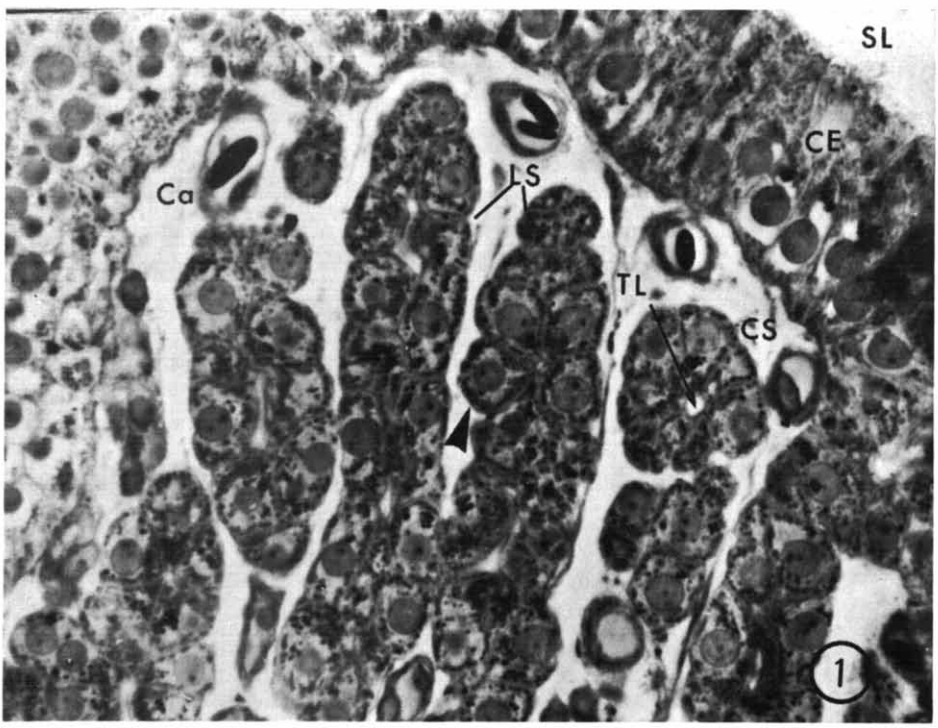

Fig. 1. A light micrograph of the shell gland mucosa (SL: shell gland lumen) from a hen 2 h after intramuscular acetazolamide injection (100 mg). No carbonic anhydrase activity was observed in the tissue. Normal columnar epithelium (CE) and the tubular glands are shown with tubular glands in longitudinal section (LS) and in cross section (CS) showing the tubular gland lumen (TL). The large arrow points to a tubular gland cell similar to the cell seen in Fig. 2. Capillaries (Ca) with red blood cells are also visible. Magnification 950 ×.

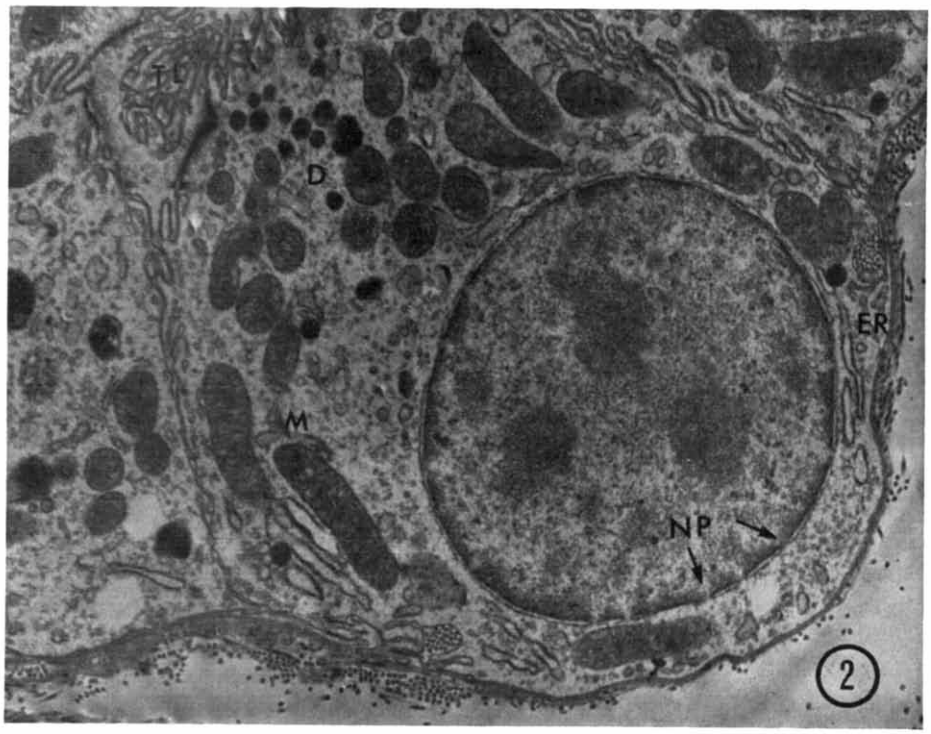

Fig. 2. An electron micrograph of a tubular gland cell from the same bird as in Fig. 1. The appearance of the cell is normal. Microvilli project into the tubular gland lumen (TL). The nucleus is large and round with nuclear pores (NP) in the envelope. A few flattened cisternae of the endoplasmic reticulum (ER) are scattered in the cytoplasm. Besides mitochondria (M), there are dense osmiophilic bodies (D) in the cytoplasm. Magnification 15 000 ×.

the present material fixed in MILLONIG's OsO_4[27] having an osmolarity of approx. 360 mosM. On the other hand, the vacuolation could be consistently produced in the acetazolamide-treated birds as well as in the control birds by fixation in 5% glutaraldehyde[38]. This phenomenon may be an artifact due to the high osmolarity of the latter fixative (approx. 1000 mosM).

The ultrastructure of the cells in the shell gland mucosa of the short-term (100 mg × 2 h) acetazolamide-treated birds (Figs. 1 and 2), where the carbonic anhydrase activity of the tissue was observed manometrically to be completely inhibited, did not differ from that observed in the control birds. Neither were there any noticeable fine structural changes in birds treated for 5 days. The swelling of the microvilli in the tubular gland cells reported by JOHNSTON, AITKEN AND WYBURN[9] was not observed in the acetazolamide-treated birds. This phenomenon is not found consistently in the normal bird either; and its occurrence may vary from block to block sampled from the same shell gland. In the pecten oculi and the ciliary body in the eye of the domestic fowl, marked ultrastructural changes 60–90 min after acetazolamide administration have been reported[39]. Dog tissues studied by MAREN, MAYER AND WADSWORTH[40] did not show light microscopical changes after prolonged acetazolamide treatment. Ultrastructural changes were also absent in acetazolamide-treated rat kidney[41].

GENERAL CONCLUSIONS

From the preceding data and discussion, it can be seen that an evaluation of the mode of action of carbonic anhydrase in the shell gland mucosa is a complex and multifaceted one. MUELLER's[35] hypothesis that acetazolamide treatment leads to decreased shell deposition by interfering with the movement of ions at the level of the kidney is apparently only part of the biochemical action of the drug. The repeated and consistent demonstration in the present investigation that carbonic anhydrase activity of the shell gland mucosa and $CaCO_3$ deposition were both drastically affected by the drug strengthens the hypothesis of a role for carbonic anhydrase in the shell deposition. Furthermore, the authors feel that the soluble enzyme may thus function as an intracellular mediator to promote the production of HCO_3^- from the metabolic CO_2 produced in the cells of the epithelium as suggested by DIAMANTSTEIN[3]. Investigations[42] of other types of cells which secrete extracellularly acting enzymes have demonstrated fluctuating levels of enzyme activity associated with the endoplasmic reticulum (the microsomes); such fluctuations have not been observed in this study.

It can be seen from Fig. 1 that the columnar epithelial cells, the tubular glands (which are highly convoluted but ultimately secrete into the shell gland lumen), and the blood capillaries are all in close proximity within the mucosa of the shell gland. The secretion of Ca into the lumen from the blood during the 20 h the egg spends in the shell gland may be *via* the tubular glands[6] or the ciliated columnar epithelial cells[43]. From the concentrations of Ca, CO_2, and HCO_3^- which have been reported in the uterine fluid surrounding the egg in the shell gland during these 20 h (refs. 44, 45) it is clear that mechanisms for rapidly replenishing these molecules must be available (otherwise volumes of fluid on the order of 600 to 2500 ml would be required to supply an average egg shell containing 5 g of $CaCO_3$). The function of carbonic anhydrase in the uterine mucosa is apparently to assure that the HCO_3^- supply is not limiting. The concentration of HCO_3^- expressed in mmoles/ml has been shown to be about 4 times that of Ca in the uterine fluid[44,45].

The addition of fluid to the albumin of the egg (*i.e.*, "plumping") occurs during the first 4 h of the 20-h period[4]. The shell gland tissue becomes distended after the egg enters the shell gland and plumping begins. The juxtaposition of capillaries, tubular glands, and epithelial cells is also affected by the distention. Also, during the first 4 h the rate of $CaCO_3$ deposition increases slowly and approaches a maximum. This maximum rate of deposition is then maintained for the next 16 h after which expulsion of the egg occurs[4]. The nature of the control system, providing for maximal $CaCO_3$ deposition only after plumping is completed, is undefined at this time[37].

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REFERENCES

1 M. S. GUTOWSKA AND C. A. MITCHELL, *Poultry Sci.*, 24 (1945) 159.
2 R. D. HODGES AND K. LÖRCHER, *Nature*, 216 (1967) 609.
3 V. T. DIAMANTSTEIN, *Arch. Geflügelk.*, 30 (1966) 310.
4 P. D. STURKIE, *Avian Physiology*, Comstock, Cornell Univ. Press, Ithaca, 2nd ed., 1965.
5 F. M. SURFACE, *Maine Agr. Expt. Sta. Bull.*, 206 (1912) 395.
6 H. GIERSBERG, *Z. Wiss. Zool.*, 120 (1923) 1.
7 K. C. RICHARDSON, *Phil. Trans. Roy. Soc. London Ser. B* 225 (1935) 149.
8 D. J. MCCALLION, *Can. J. Zool.*, 31 (1953) 577.
9 H. S. JOHNSTON, R. N. C. AITKEN AND G. M. WYBURN, *J. Anat.*, 97 (1963) 333.
10 P. C. BREEN, *Anat. Record*, 154 (1966) 321.
11 N. U. MELDRUM AND F. J. W. ROUGHTON, *J. Physiol. London*, 80 (1933) 113.
12 R. H. COMMON, *J. Agr. Sci.*, 31 (1941) 412.
13 V. T. DIAMANTSTEIN AND J. SCHLUNS, *Acta Histochem.*, 19 (1964) 296.
14 G. HAUSLER, *Histochemie*, 1 (1958) 29.
15 D. S. ROBINSON AND N. R. KING, *Nature*, 199 (1963) 497.
16 V. T. DIAMANTSTEIN, K. BRONSCH AND J. SCHLUNS, *Nature*, 203 (1964) 88.
17 T. BARKA AND P. J. ANDERSON, *Histochemistry*, Harper and Row, New York, 1965, p. 295.
18 P. K. DATTA AND T. H. SHEPARD, *Arch. Biochem. Biophys.*, 81 (1959) 124.
19 R. KARLER AND D. M. WOODBURY, *Biochem. J.*, 75 (1960) 538.
20 T. H. SHEPARD, *J. Embryol. Exptl. Morphol.*, 10 (1962) 191.
21 K. C. LEIBMAN AND F. E. GREENE, *Proc. Soc. Exptl. Biol. Med.*, 125 (1967) 106.
22 A. DOUNCE *et al.*, *J. Biophys. Biochem. Cytol.*, 1 (1955) 139.
23 W. HOHMAN AND H. SCHRAER, *J. Cell Biol.*, 30 (1966) 317.
24 H. A. KREBS AND F. J. W. ROUGHTON, *Biochem. J.*, 43 (1948) 550.
25 V. I. OYAMA AND H. EAGLE, *Proc. Soc. Exptl. Biol. Med.*, 91 (1956) 305.
26 O. H. LOWRY *et al.*, *J. Biol. Chem.*, 193 (1951) 265.
27 G. MILLONIG, *J. Appl. Phys.*, 32 (1961) 1637.
28 J. H. LUFT, *J. Biophys. Biochem. Cytol.*, 9 (1961) 409.
29 M. L. WATSON, *J. Biophys. Biochem. Cytol.*, 4 (1958) 475.
30 J. H. VENABLE AND J. H. COGGESHALL, *J. Cell Biol.*, 25 (1965) 407
31 J. WINKELSTEIN, M. G. MENEFEE AND A. BELL, *Stain Technol.*, 38 (1963) 202.
32 K. PAIGEN, *Biochim. Biophys. Acta*, 19 (1956) 297.
33 W. C. SCHNEIDER AND G. H. HOGEBOOM, *J. Biol. Chem.*, 198 (1952) 155.
34 U. BLEYL, *Histochemie*, 4 (1964) 286.
35 W. J. MUELLER, *Poultry Sci.*, 41 (1962) 1792.
36 T. H. MAREN, *Physiol. Rev.*, 47 (1967) 752.
37 G. EHRENSPECK, H. SCHRAER AND R. SCHRAER, *Proc. Soc. Exptl. Biol. Med.*, 126 (1967) 392.
38 D. D. SABATINI, K. BENSCH AND R. J. BARRNETT, *J. Cell Biol.*, 17 (1963) 19.
39 A. R. SEAMAN AND T. M. HIMELFARB, *Am. J. Ophthalmol.*, 56 (1963) 278.
40 T. H. MAREN, E. MAYER AND B. C. WADSWORTH, *Bull. Johns Hopkins Hosp.*, 95 (1954) 199.
41 A. CESTARI, R. LASCHI AND G. MURARI, *Sperimentale*, 114 (1964) 153.
42 G. E. PALADE, *J. Biophys. Biochem. Cytol.*, Suppl. 2 (1956) 85.
43 C. V. GAY AND H. SCHRAER, *Federation Proc.*, 26 (1967) 660.
44 M. H. ELJACK AND P. E. LAKE, *J. Reprod. Fertility*, 13 (1967) 127.
45 B. W. BEADLE, R. M. CONRAD AND H. M. SCOTT, *Poultry Sci.*, 17 (1938) 498.