THE MECHANISM FOR CALCIUM CARBONATE DEPOSITION IN THE AVIAN EGGSHELL

THESIS

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INTRODUCTION

Eggshell composition and structure of the avian eggshell have been widely studied (Romanoff, 1949; Tyler, 1969; Mikhailov, 1987). These studies have concentrated on the physical properties and structural qualities of the eggshell. The mechanism for the deposition of calcium carbonate, the primary component of the eggshell, has been largely ignored. In order for mineralization to proceed at the rate of 4.45 mg/cm^2/hr (Lundholm, 1997) a ready supply of calcium and carbonate ions is necessary. During egg laying increased blood calcium levels supply the calcium ions. It has been suspected that carbonic anhydrase may play a role in maintaining bicarbonate levels and, therefore, providing carbonate ions for calcification of the eggshell (Robinson and King, 1963, and Lundholm, 1990). The extent of the role carbonic anhydrase plays in eggshell formation and where it can be located have not been fully explored.

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Egg Formation

The ova are produced in the ovary and then the egg passes through a series of reproductive organs known collectively as the oviduct. In the bird the ova are formed in the functional left ovary. At any time the ovary may hold ova in various stages of maturity. The maturation of the germ cell occurs in three phases: (1) differentiation of the primordial germ cells, (2) multiplication, and (3) growth and maturation (Romanoff

and Romanoff, 1949). This process is known as oogenesis and results in a mature ovum with yolk. At ovulation the mature follicle bursts and the mature ovum is released. The ovum is then engulfed by the infundibulum.

The infundibulum marks the beginning of the oviduct. The oviduct has extremely elastic walls and runs from the ovary to the cloaca. The first layer of albumin, the Chalaziferous Layer, is deposited in the posterior portion of the infundibulum (Scott and Huang, 1941). The egg is moved by peristalic motion to the albumin secreting region where the remainder of the albumin is added. The egg is propelled through the albumin secreting region to the isthmus. Here the inner and outer shell membranes are deposited. When the membrane bound egg reaches the uterus it takes on uterine secretions consisting of water and dissolved salts and becomes plump (Romanoff and Romanoff, 1949). Next, the formation of the shell proceeds. The matrix and the mineralized shell are deposited. Pigments are also added as the shell is formed. When the shell formation is complete a thin film is applied termed the cuticle. This material is added in the anterior portion of the oviduct. The fully formed egg is then expelled through the vagina into the cloaca.

Eggshell Formation

The formation of the inner and outer shell membranes marks the beginning of eggshell formation. These membranes are formed around the albumin enclosed ovum as it passes into the isthmus of the oviduct. The inner and outer shell membranes are composed of an elastin-like protein arranged in fibers running all directions (Leach, Rucker, and VanDyke, 1981). The membranes are a light pinkish color due to porphyrin (Klose and

Almquist, 1937). Mineralization initiates at the mammillary cores of the outer shell membrane (Tullett, 1987). The mammillary cores are protuberances of organic matter located on the outer surface of the membrane. They are firmly attached to the membrane (Tyler and Simkiss, 1959b; Terepka, 1963a,b). It is from these cores that the calcium carbonate crystals begin to form. Once crystal formation has begun it will continue until the calcium and carbonate supply is cut off. The mechanism initiating crystal formation is not known. Carbonic anhydrase has been isolated in mammillary core formations (Robinson and King, 1963), but its role in eggshell formation has not been studied.

In the uterus the true shell is deposited (Tyler, 1969). The shell has been described as calcium carbonate on an organic network of cross-linked fibrous sheets (Silyn-Roberts and Sharp, 1986). The matrix fibers have also been described as fine fibrills (Tullett, 1987). The matrix consists of anastomosing fibers with a proteinaceous core. Blood supply to the uterus is greatly increased during periods of eggshell formation. The uterine glands, which secrete a calcium rich solution into the uterine lumen, are supplied with calcium from the blood (Romanoff and Romanoff, 1949). The calcium lost from the blood to eggshell formation is replenished by absorption from the duodenum and jejunum (65-75%) and by resorption of the medullary bone (Simkiss and Taylor, 1971). During periods of non-laying calcium is redeposited in the bones.

It has been suspected that carbonic anhydrase may play a role in the formation of the avian eggshell. Lundholm (1990) proposed that due to this increase in carbonic activity bicarbonate is pumped out of the mucosa of the oviduct. The epithelium of the uterus is the layer most involved in the secretion of shell materials (Richardson, 1935). Robinson and King (1963) isolated carbonic anhydrase from mammillary core preparations.

Further, it has been found that administration of carbonic anhydrase inhibitors results in eggshell thinning (Lundholm, 1990).

Carbonic anhydrase is an interesting enzyme in that it catalyzes two reactions. Carbonic anhydrase catalyzes the formation of carbonic acid from water and carbon dioxide. This reaction is highly favored toward carbonic acid at physiological pHs. Carbonic anhydrase also catalyzes the formation of the bicarbonate ion from carbonic acid. The equilibrium between bicarbonate and carbonate is highly favored towards bicarbonate. High levels of bicarbonate are needed to enhance the formation of the carbonate ion. Carbonic anhydrase, by enhancing bicarbonate formation, maintains higher levels of the carbonate ion. Carbonic anhydrase may initiate or maintain mineralization by maintaining bicarbonate ions at the site of initiation. By maintaining the level of bicarbonate ions necessary for mineralization to progress carbonic anhydrase allows for rapid formation of calcium carbonate. Rapid crystalization is essential to eggshell formation.

Objectives

The objectives of this research were: 1) to isolate and demonstrate carbonic anhydrase from the extracellular eggshell, 2) determine whether or not the enzyme was active, 3) determine the effects of the carbonic anhydrase inhibitor, acetazolamide and 4) estimate the molecular weight of isolated carbonic anhydrase isozymes. With answers to these questions the role of the proteinaceous matrix of the eggshell in eggshell mineralization may be ascertained. The matrix may play an important role in eggshell formation in

providing a meshwork infused with carbonic anhydrase, capable of maintaining high levels of bicarbonate, and consequently carbonate, at levels necessary for mineralization. As the shell is formed and the enzyme entombed more matrix is laid down, and with it more enzyme.

MATERIALS AND METHODS

Extraction of Carbonic Anhydrase

The procedure for extraction of the enzyme is similar to that used by Miyamo to, et al. (1996), while the purification procedure is similar to that of Osbourne and Tashian (1975), Chegwidden (1991), Bering, et al. (1998), and Lane and Morel (2000). Eggshells of the fowl *Gallus gallus* and of the emu *Dromaius* were used. The eggs were obtained from local grocery stores or from individuals.

Egg contents were removed, as are the inner and outer shell membranes. Due to porphrin in the membranes they have a light pinkish hue. The shells were soaked in water, which loosened the membrane from the shell, and then the membranes were peeled off. The shells were soaked 6 hrs in 1.5% sodium hypochlorite solution, rinsed with DI water, and again soaked 6 hrs in 1.5% sodium dodeylsulfate (SDS), rinsed with DI water and then dried at room temperature and ground using a Thomas Wiley Laboratory Mill model 4. The ground shells were then weighed. The powder was then placed in 1000 ml (0.025 M Tris buffer, pH 8.0, 0.01% sodium azide, 1 mM 2-mercaptoethanol). Equal equivalents plus 10% Na_2H_2EDTA (372 g/ 100 g $CaCO_3$) was then added while stirring at room temperature to dissolve the calcium carbonate. When decalcification had occurred (bubbling stops) and the solution had cleared, the pH was adjusted to pH 9 using NaOH.

The shell extract was then dialyzed three times against 0.025 M Tris buffer (pH 8.0, 0.01% sodium azide, 1 mM 2-mercaptoethanol) in a cold room (4°C) to remove CaEDTA. Each dialysis was for 6 hours against 10x fresh buffer.

Total proteins were then precipitated at 4°C with cold 80% saturated ammonium sulfate (0.025 M Tris, pH 7.0, 0.01% sodium azide, 1 mM 2-mercaptoethanol), and then centrifuged at 13,000 rpms for 20 minutes in a Sorvall RC5B refrigerated centrifuge. Ammonium sulfate precipitate was collected, dissolved in 50 ml buffer (0.025 M Tris, pH 7.0, 0.01% sodium azide, 1 mM 2-mercaptoethanol), and dialyzed three times. The first and second dialyses were against 2000 ml 0.025 M Tris buffer (pH 7.0, 0.01% sodium azide) at 4°C for 6 hours each. In the third dialysis 1 mM $ZnCl_2$ is added to replace zinc lost to EDTA. The dialysate was centrifuged at 13,000 rpms to separate water soluble proteins (WSPs) from water insoluble proteins (WIPs). The WSP supernatant was retained, the precipitate was washed with buffer (0.025 M Tris, pH 7.0, 0.01% sodium azide, 1 mM 2-mercaptoethanol) twice, centrifuged and combined with the initial supernatant.

The combined supernatants (WSP) were placed in a 50 ml beaker with one gram wetted (0.025 M Tris, 0.25 M K_2SO_4, pH 7.0) affinity beads specific for carbonic anhydrase (Bio-Gel A: carboxymethyl agarose containing a ligand of 4-aminomethylbenzenesulfonamide; BIO-RAD) and were stirred for 2-3 hours before being placed on a gravity pre-packed column (25 x 1.5 cm) with 8 cm wetted affinity resin beads in place (Bering, et al., 1998). Following sample introduction the column was washed with 6 volumes of 0.2 M K_2SO_4, 0.025 M Tris buffer (pH 7.0) and collected. All WSP excepting resin bound carbonic anhydrase were eluted. Carbonic anhydrase

was eluted with 4 volumes (approximately 60 ml) of 0.4 M KSCN, 0.025 M Tris buffer (pH 7.0) by competitive inhibition and collected. The KSCN collected was concentrated to 200 µl using Millipore ultrafree-4 centrifugal filters, washed three times with 4 ml of buffer (0.025 M Tris buffer, pH 7.0) to remove KSCN.

Three replicates of carbonic anhydrase extraction were performed using chicken eggs (C1, C2, C3). All three samples were used in spectrophotometric and SDS/PAGE gel tests. Additionally, an Enzyme Linked Immunosorbent Assay (ELISA) was run on C1 and E1, and a Dot Blot and an enzyme activity assay were run on C3. For comparison one sample was prepared using emu eggs. This sample was used for spectrophotometric and ELISA tests.

Tests for the Presence of Carbonic anhydrase

Total WSP in the combined 0.2 M K_2SO_4 / 0.025 M Tris buffer (pH 7.0) and the carbonic anhydrase in the 0.4 M KSCN / 0.025 M Tris (pH 7.0) eluates were estimated spectrophotometrically and the percentage carbonic anhydrase in the total WSP calculated (µg-protein / ml = 183 x A230 nm - 75.8 x A260 nm; Kalb and Bernlohr, 1977).

Aliquots of the concentrated carbonic anhydrase were used for SDS/PAGE, Dot Blot, and ELISAs (Chemicon #AB1243). The reaction was followed with a Rabbit / Mouse Rapid Staining Kit (Sigma Chemical #89H4879). Polyacrilimide gel electrophoresis (SDS/PAGE, 12.5% acrylamide) was used to ascertain purity and estimate relative molecular mass of the carbonic anhydrase (Laemmli, 1970). As set of two molecular weight standards (10 to 25 kD; BIO-RAD 161-0362) was run in two lanes along with the

concentrated sample. To locate proteins the gels were stained with Coomassie blue, and then destained.

Activity of Carbonic anhydrase

The enzyme assay for carbonic anhydrase is similar to that of Rickli, et al. (1964). Aliquots of 10 µl of sample were added to 100 µl Tris-phenol red solution (pH 8.3), and then 400 µl substrate (CO_2 saturated solution, pH < 4) was added, all maintained at 0°C in an ice bath. Upon adding the substrate, timing began with a stopwatch. The reaction is followed by a color change from red to yellow-orange (pH 7.3). Blanks with heat denatured protein and samples plus acetylzolamide were used for comparison with the catalyzed reaction. Activity units = 2 x (1/tc – 1/tu) x 1000/µg-protein; where tu = time of the uncatalyzed reaction and tc = time of the reaction with the enzyme (Wilbur and Anderson, 1948).

RESULTS

Spectrophotometry readings for the samples (C1, C2, C3, and E1) were consistent across all samples. Total water soluble proteins (WSP), total carbonic anhydrase, and percentage of eggshell weight that is carbonic anhydrase were calculated from the spectrophotometry data. The spectrophotometric method of Kalb and Bernlohr (1977) for estimating protein was used to estimate the concentrations of carbonic anhydrase and water soluble protein (WSP). The samples of the eggshell were found to consist of 92 mg/100 g, 52 mg/100 g, 119 mg/100 g and 32 mg/100 g eggshell and the WSP was found to consist of 0.090%, 0.087%, 0.084% and 0.130% carbonic anhydrase respectively (Table 1).

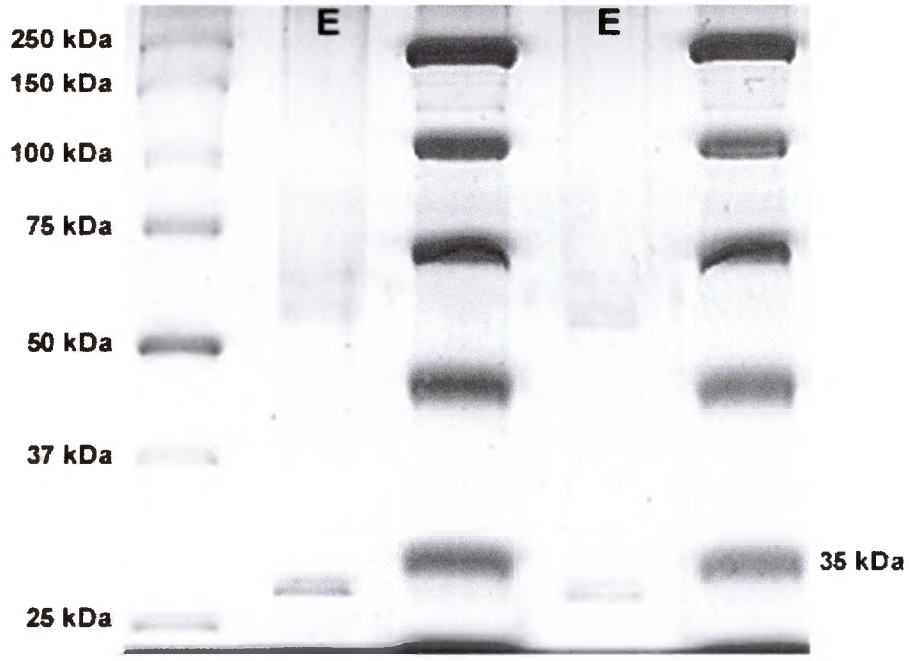
All four samples were run on SDS/PAGE (12.5%) gels and molecular mass was estimated by comparing Rf values of carbonic anhydrase with those of standard proteins. Three isozymes for carbonic anhydrase were detected that had estimated molecular weights of 31, 54 and 66 kDa. (Figure 1). The two larger proteins/isozymes may be dimers of the smaller isozyme of 31 kDa. The inconsistent quantities of the smaller isozyme in relation to the two larger proteins in the different samples suggested that dimerization may have taken place. Due to the fact that the proteins had been isolated using an affinity column specific to carbonic anhydrase it is likely that all proteins isolated are isozymes of carbonic anhydrase. The estimated molecular weights were

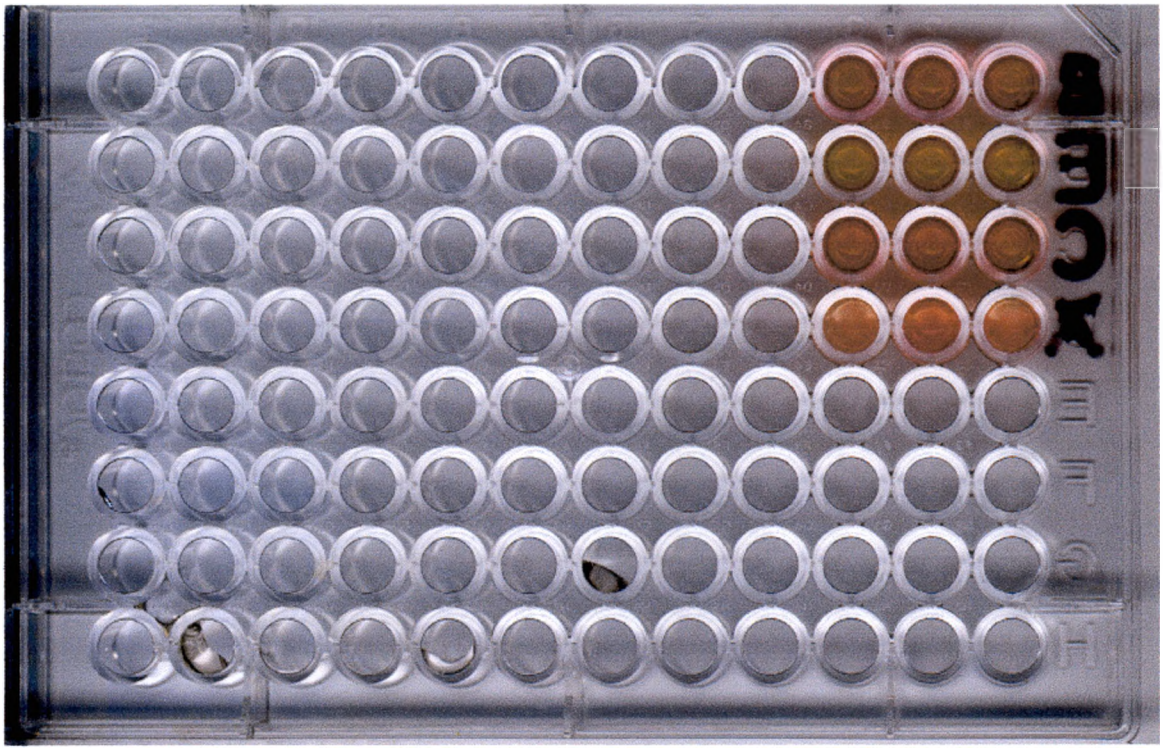
	SOLUBLE SHELL PROTEIN (EDTA EXTRACTION)	
	TOTAL WSP* (mg/100 g-shell)	CARBONIC ANHYDRASE µg/100 g shell (%WSP)
Chicken Eggshell:		
C1 (shell = 108 g)	92 mg/100 g shell	86 µg/100 g shell (0.090%)
C2 (shell = 94.8 g)	52 mg/100 g shell	45 µg/100 g shell (0.087%)
C3 (shell = 191.7 g)	119 mg/100 g shell	99 µg/100 g shell (0.084%)
Emu Eggshell:		
E1 (shell = 130.61 g)	32 mg/100 g shell	47 µg/100 g shell (0.130%)

***Total shell soluble protein ; **Carbonic anhydrase as a total % of shell soluble protein. Protein isolated by affinity chromatography and estimated by UV absorbance.**

Figure 1. Polyacrilamide (12.5%) Gel Electrophoresis. Three isozymes of carbonic anhydrase were isolated having molecular weights of 31, 54 and 66 kDa. The two heavier bands may be the result of dimerization.

Figure 2. ELISA performed on samples C1 and E1. This antibody test provides further evidence that the enzymes isolated by affinity chromatography are isozymes of carbonic anhydrase. Both the bovine carbonic anhydrase positive control (top) and the C1 sample (3rd from top) produced a strong positive result. The E1 sample (2nd from top) produced a moderate positive response, though significantly different from the negative control (bottom).


E
E
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
35 kDa



consistent and similar across all gels in that the three proteins were found in all gels, but not necessarily in the same ratios.

Antibody tests performed on samples C1, C2 and C3 provide further evidence that the isolated proteins are carbonic anhydrase. ELISAs performed on samples C1 and E1 produced a positive reaction to antibodies of bovine carbonic anhydrase II (Figure 2). The Dot Blot performed using an antibody to bovine carbonic anhydrase II on sample C3 also demonstrated positive results (Figure 3). Two negative controls that were run were solutions of 1) only PBS (phophate buffer and saline) and 2) albumen in PBS. Neither of the negative controls reacted with the antibody to bovine carbonic anhydrase II.

Enzyme activity assays performed on sample C2 demonstrated that carbonic anhydrase isolated by affinity chromatography was active (AU = 163.0 U/mg-protein). A series of dilutions of the well-known carbonic acid inhibitor was used to evaluate its effect on the enzyme activity of carbonic anhydrase. Carbonic anhydrase activity increased as the concentration of acetazolamide decreased from 0.01 M to 0.000001 M (Figure 4). At a concentration of 0.01 M acetazolamide enzyme activity was reduced to 0% of the initial uninhibited activity.

Figure 3. Dot Blot performed on sample C3. All three drops of the C3 sample reacted positively with the bovine carbonic anhydrase antibody. Neither of the negative controls (PBS in lower left and albumen in lower right) reacted with the antibody.

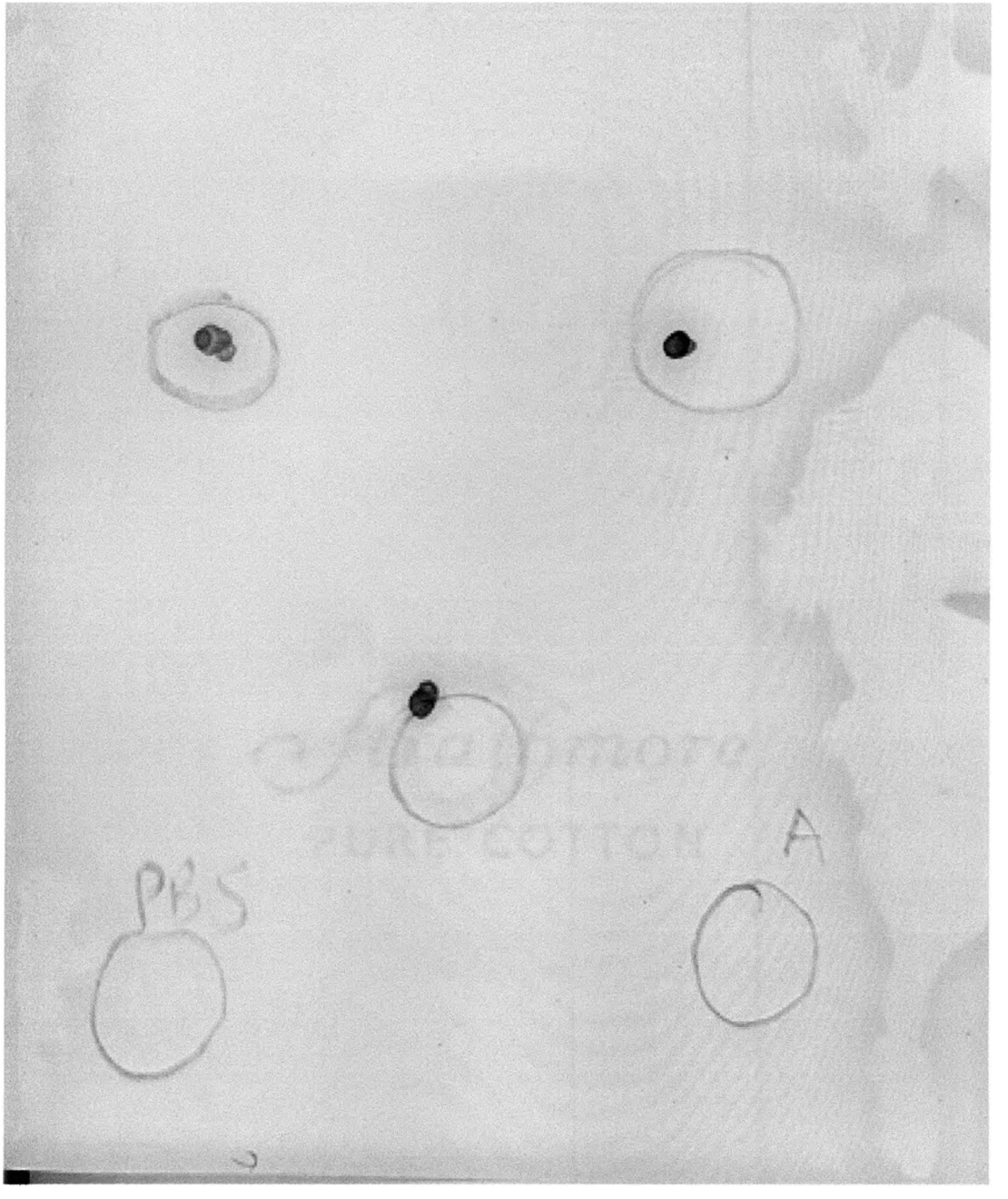
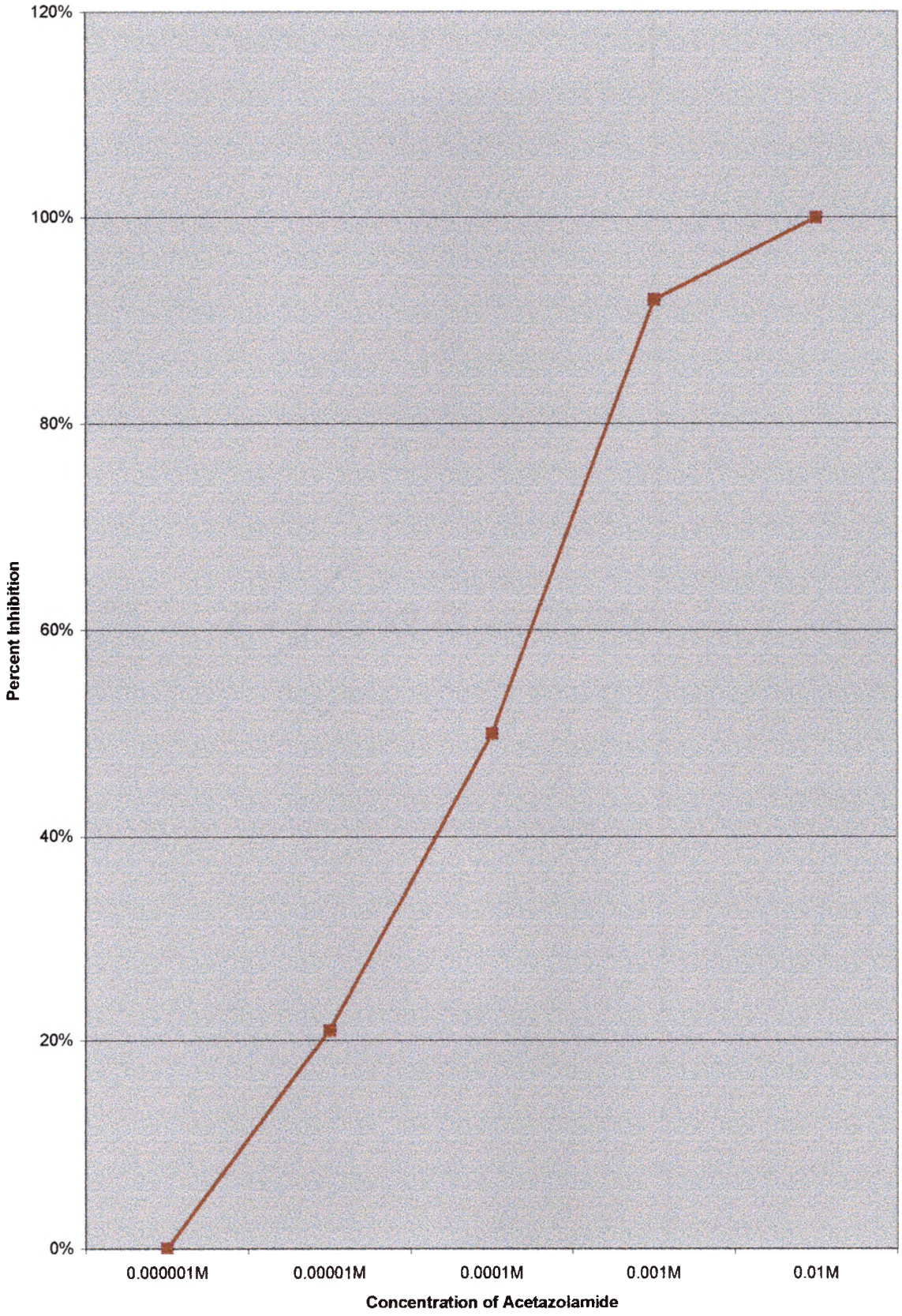

PBS
A

Figure 4. Acetazolamide inhibition curve from the results of activity tests on sample C3. Carbonic anhydrase is known to be inhibited by acetazolamide. As the concentration of acetazolamide is increased carbonic anhydrase activity is decreased. Activity tests performed on this sample gave results of AU = 163.0 U/mg-protein. This level of activity was reduced to 0% with an acetazolamide concentration of 0.001M.


120%
100%
80%
60%
40%
20%
0%
Percent Inhibition
0.000001M
0.00001M
0.0001M
0.001M
0.01M
Concentration of Acetazolamide

DISCUSSION

In chickens the rate of shell deposition at the height of mineralization is 4.45 $mg/cm^2/hr$ (Lundholm 1997). To maintain this rapid rate of formation a steady supply of calcium and carbonate ions is necessary. Both ions must be supersaturated at the site of mineralization. Calcium ions are provided by increased blood flow and blood calcium levels to the tissues of the uterus. Eggshell formation in the domestic fowl, *Gallus gallus*, requires 2 grams of calcium (Lundholm, 1997), which is acquired from the blood. Transport of calcium from the blood to the shell gland lumen is increased by the presence of sodium and bicarbonate in the shell gland lumen (Eastin and Spaziani, 1978a and Pearson and Goldner, 1974). Blood calcium is replenished and maintained from their diet by absorption from the duodenum and jejunum (65-75%) and from medullary bone (Simkiss and Taylor, 1971).

The source and location of carbonate ions is not well understood, but it has been assumed that the carbonate ions come from bicarbonate via carbonic anhydrase in the oviduct (Lundholm, 1990). The occurrence of carbonic anhydrase in the extracellular matrix of the eggshell as shown in the current study would indicate a more defined role for carbonic anhydrase in eggshell mineralization. That is, bicarbonate would be maintained at the site of mineralization in the external matrix. Eggshell carbonic anhydrase was isolated and identified from eggshells of both the chicken and the emu.

Identification of an avian eggshell carbonic anhydrase is supported by positive results from the 1) affinity chromatography, 2) ELISA, 3) Dot blot test, 4) enzyme activity and 5) carbonic inhibition by acetazolamide. All tests preformed on the eggshell samples in this study were positive for the enzyme, carbonic anhydrase.

Assessment of the purity and the Mr of the enzyme was accomplished by SDS-PAGE gel electrophoresis. A major protein band of molecular weight 31 kDa. was detected along with two larger bands with molecular weights of 54 and 66 kDa. (Figure 1). Enzyme activity for the affinity chromatographically purified carbonic anhydrase fraction that contained all three isozymes was shown to be 163.0 U/mg-protein. The carbonic anhydrase activity was also found to be inhibited by acetazolamide, a well-known carbonic anhydrase inhibitor. Complete inhibition was obtained with an acetazolamide concentration of 0.01 M. It is not known which of the three SDS/PAGE Gel isozymes was enzymatically active or if all were active. A Dot Blot of the eggshell carbonic anhydrase sample showed that at least one of the three isolated proteins is a form of carbonic anhydrase since the combined sample reacted with bovine carbonic anhydrase II (Figure 3). It is not known for certain if the two larger proteins are actually isozymes of carbonic anhydrase, or are proteins formed by dimerization. Although a Western Blot by Horne (personal communication) has shown that all three eggshell proteins reacted with the antibody to bovine carbonic anhydrase II and that the 31 kDa protein reacted most intensely, it is still not certain that the two larger proteins are isozymes. They could be just dimers. In any case, these data lead to the conclusion that the eggshell avian carbonic anhydrase plays an important role in eggshell formation, and that it assists in mineralization in the acellular matrix of the eggshell.

Among the various taxa some species have several carbonic anhydrase isozymes and many others have carbonic anhydrases that are sensitive to oxidation. Three isozymes of carbonic anhydrase have been isolated in birds. These isozymes differ in the tissues in which they are located, their sensitivity to sulphonamides and their level of activity (Holmes, 1977). Isozymes "A", "B" (also denoted CA I) and "C" (also denoted CA II) were isolated from the domestic fowl by Holmes (1977). Isozyme "A" was isolated from red skeletal muscle and was found to have a molecular weight of 30 kDa. Isozyme "B" was isolated from the digestive tract and was found to have a molecular weight of 30 kDa. Isozyme "C" was found to be widely distributed and to also have a molecular weight of 30 kDa. Isozyme "B" is similar to the low activity CA I isolated in mammals and "C" is similar to the high activity CA II isolated in mammals. Type "A" is believed to be a modified "B" resulting from amide group hydrolysis (Funakoshi and Deutsch, 1968). An isozyme "A" with a molecular weight of 34 kDa. has been isolated from the red skeletal muscle of sheep. The molecular weight of 34 kDa. is believed to be the result of protein binding small amounts of blood carbonic anhydrase "C" to the red skeletal muscle carbonic anhydrase "A" with molecular weight 30 kDa. (Holmes, 1977). Results of this study which detected isozymes of molecular weights of 54 and 66 kDa. could perhaps be explained in this same manner. An isozyme of molecular weight 35 kDa., termed CA IV, has been isolated from human renal tissue (Wistrand and Knuuttila, 1989).

Extensive research has been conducted on the affects of sulfhydryl reducing agents on the behavior of carbonic anhydrase purification and activity. Bernstein and Schraer (1972) were the first to purify an avian carbonic anhydrase to homogeneity. They found

it was necessary to use a reducing agent to maintain full activity of the enzyme. Hall and Schraer (1978) purified low and high activity isozymes of carbonic anhydrase from the erythrocytes of the diamondback terrapin. Mercaptoethanol was required for full activity. A high activity isozyme purified from bullfrog erythrocytes required a thiol agent to maintain full activity (Hall and Schraer (1978). Purification of carbonic anhydrase (similar to CA II in mammals) in the oyster required the use of a reducing agent to maintain full activity (Nielsen and Frieden, 1972). In contrast carbonic anhydrase isolated from the teleost and hagfish were found not to require use of a reducing agent (Hill, Power and Gilbert, 1977). And Stratakis and Linzen (1984) found carbonic anhydrase isolated from the tarantula to be inhibited by mercaptoethanol. The high activity isozymes isolated from the turtle, chicken and frog have multiple cysteine residues and therefore require use of a reducing agent for full activity (Sanyal, 1984). The use of a reducing agent has also been found to be necessary to eliminate dimerization of the enzyme. Bernstein and Schraer (1972) observed a single band in polyacrylamide gel electrophoresis with a molecular weight of 30 kDa. when mercaptoethanol was used. In the absence of the reducing agent two active components were observed with molecular weights of 30 and 60 kDa. The carbon dioxide hydrase activity was lower for the 60 kDa. form of the enzyme. Also, the 30 kDa. enzyme isolated using mercatoethanol had higher activity than the 30 kDa. enzyme obtained without mercaptoethanol. The dimerization is the result of disulfide bonding (Sanyal, 1984). Eisenberg (1983) found that dimerization in cat muscle carbonic anhydrase was associated with a significant activity loss. Dimerization of mammalian muscle carbonic anhydrase (III) has also been observed in the absence of a reducing agent (Register,

Koester and Noltmann, 1978). Even when dimerization does not occur a loss of 97% of activity has been obtained with an extended absence of mercaptoethanol (Bernstein and Schraer, 1972). This loss of activity was partially reversed by the addition of an excess of mercatoethanol (gain of 47-67% of activity). The high content of half-cystine in avian carbonic anhydrase makes it necessary to use a reducing agent to maintain purity and full activity (Bernstein and Schraer, 1972). In this study the presence of possible dimers (54 and 66kDa. proteins) and the occasional low level of activity can be explained by the dynamics of carbonic anhydrase half-cystines maintained in a medium without ample reducing agent or kept for prolonged periods without mercaptoethanol.

Avian carbonic anhydrase is not found only in the tissues of the uterus. The enzyme also seems to be laid down throughout the fibrous matrix of the eggshell as it is formed. In the matrix the enzyme catalyzes the formation of carbonic acid from carbon dioxide and water and then the formation of bicarbonate from carbonic acid. An equilibrium is reached between bicarbonate and carbonate in which bicarbonate is highly favored. But by maintaining high levels of bicarbonate, carbonate ion formation is increased by the uncatalysed reaction. Calcium ions that are provided via increased blood flow react with the carbonate ions to form calcium carbonate. This reaction takes place at specific sites in the matrix where carbonic anhydrase has been deposited. Crystallization proceeds as long as calcium and carbonate concentrations are adequate. As the enzyme is entombed by crystal formation, mineralization slows and then ceases. Horne, et al. (In Review) have proposed a similar mechanism for the initiation and cessation of formation of the exoskeleton of the spiny lobster (*Panulirirs argus)*, blue crab (*Callinectes sapidus*), dungeness crab (*Cancer borealis*), and the jonah crab (*Cancer magister* Proteins were

isolated from the matrix of the exoskeletons of all four species that were found to be carbonic anhydrase. ELISA tests also showed the presence of carbonic anhydrase.

This mechanism for mineralization of the eggshell probably can be applied to oviparous reptiles as well as birds. Reptilian egg structure and formation is similar to that seen in avian species in many respects. The reptilian egg consists of an organic membrane enveloped by an inorganic outer layer. In most species the organic layer consists of one to many layers of fibers interconnected to create a single shell membrane (Packard and DeMarco, 1991). The number of layers composing the membrane differs among species. Some species' eggs, however, contain an air cell as seen in avian eggs leading to the concept of an inner and outer shell membrane (Packard and Packard, 1979). The outer inorganic layer, the true shell, is calcite in crocodilians and oviparous squamates, but aragonite in testudinians (Ferguson, 1982 and Deeming, 1988). Reptilian eggshells can be rigid as in avian species or flexible. In rigid shells the shell units are closely packed in the calcareous layer, in flexible shells the shell units are discrete (Packard and DeMarco, 1991). In contrast to avian species' sequential ovulation reptiles show simultaneous ovulation in which all eggs are ovulated at the same time. As a result the process of shell mineralization takes longer than the 24 hours common in birds. The albumin, shell membranes, and the true shell are formed in the oviduct as in birds. Formation of the shell membranes and true shell occurs in the uterus (Palmer and Guillette, 1988). Probable site of initiation of eggshell mineralization differs between species. Organic cones, similar to the mammillary cores observed in avian shells, have been observed on the shell membranes eggs typical of rigid-shell laying turtles (Packard, et al. 1984*a*). Membranous structures called central plaques have been seen in the

flexible shells of painted turtles (*Chrysemys picta*) and the snapping turtle (*Chrysemys serpentina*) (Agassiz, 1857), and saucer shaped pits in the shell membrane have been described from the shells of sea turtles (*Chelonia mydas*). The organic cones are very similar in structure to the mammillary cores of the avian shell membrane. These structures serve as sites of initiation for mineralization. The shell units initiate at the site and grow a short distance into the shell membrane. As a result the organic cone and the membrane fibers are enclosed ceasing crystal formation (Silyn-Roberts and Sharp, 1986 and Packard and Packard, 1979). If these units grow to closely abut one another the shell is rigid, if they are spaced apart the shell is flexible. The second scenario results in a thin crust of calcium carbonate atop the shell membrane (Packard and Hirsch, 1986).

The eggshell matrix has many functions in shell formation. One of its important roles in eggshell formation may be that it plays a role in providing a meshwork infused with carbonic anhydrase, capable of maintaining high levels of bicarbonate, and consequently carbonate ions at levels necessary for mineralization. As the shell is formed and the enzyme entombed more matrix is laid down, and with it more enzyme. This mechanism of eggshell formation may also be important to reptiles.

The presence of carbonic anhydrase in the extracellular eggshell may reveal the mechanism for mineralization in both the avian and reptilian eggshell. By increasing the formation of carbonate ions carbonic anhydrase allows eggshell formation to proceed at a very fast pace.

CONCLUSION

Carbonic anhydrase may be the guiding factor for the initiation, maintenance and the cessation of eggshell mineralization. By maintaining high levels of bicarbonate carbonate ion levels are maintained. As the necessary levels of carbonate ions (in equilibrium with bicarbonate) and calcium ions (increased blood calcium levels and blood flow) are reached crystallization begins at the initiation sites. As mineralization proceeds carbonic anhydrase is entombed, bicarbonate levels drop off and carbonate ion formation stops. With the supply of carbonate ions depleted crystallization ceases. This mechanism explains how eggshell mineralization is initiated, maintained and stopped and how all this is accomplished in such a short period of time.

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