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The role of vitamin D in chorioallantoic membrane calcium transport

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Abstract

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) is essential for the transport of eggshell calcium to the embryo across the chorioallantoic membrane (CAM). CAM contains the vitamin D receptor that increases following 1,25-$(OH)_2D_3$ injection into embryos at day 10 of incubation. Further, a single injection of 100 ng of 1,25-$(OH)_2D_3$ into vitamin D-deficient quail eggs at day 10 of incubation resulted in a significant increase in both body and yolk calcium. This is accompanied by an increase in carbonic anhydrase from low levels in deficiency to normal levels. Acetazolamide (AZ), a specific carbonic anhydrase inhibitor injected into the quail embryos, caused hypocalcemia and hyperphosphatemia. This is similar to the hypocalcemia and hyperphosphatemia found in vitamin D-deficient embryos. These results suggest that one mechanism of action of vitamin D in the mobilization of eggshell calcium is the activation of carbonic anhydrase that acidifies the calcium carbonate shell.

Key words: Vitamin D; Calcium; Embryonic development; Chorioallantoic membrane; Vitamin D receptor; Carbonic anhydrase; Acetazolamide

1. Introduction

Development of avian embryos requires that a substantial amount of calcium be transported from the eggshell to embryo to support rapid skeletal growth [1]. During embryonic skeletal growth and calcification, the chorioallantoic membrane (CAM) is responsible for mobilizing 80% of eggshell calcium [2]. The CAM, therefore, must dissolve eggshell calcium and transport it to the embryo [3]. The CAM is composed of the chorionic ectoderm and allantoic endoderm and connective tissue in between. The chorion contains the capillary plexus and comes into contact with the inner shell membrane during the last week of incubation to transport calcium from eggshell into embryonic circulation [3,4].

Vitamin D-deficient hens lay fertile eggs but the embryos fail to hatch [5]. The calcium content of these embryos is about one-half that of normal embryos. It was postulated that the embryos failed because of an inability to obtain sufficient calcium from the eggshell [5]. Irradiation of the vitamin D-deficient hens with ultraviolet light or administration of cod liver oil restored normal levels of hatching. Thus, vitamin D is implicated in the control of calcium transport from the eggshell to embryo.

The more recent observation that hens fed a diet containing 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) as the sole source of vitamin D lay fertile eggs that failed to hatch [6] because they were vitamin D deficient [7] indicated again a role for vitamin D in the regulation of eggshell calcium mobilization. It was shown that these embryos have severe hypocalcemia caused by vitamin D deficiency [8,9]. Injection of −D eggs (from 1,25-$(OH)_2D_3$-fed hens) with either vitamin D or one of its metabolites restores hatchability to normal values [10,11]. Furthermore, the administration of 1,25-$(OH)_2D_3$ to chick embryos produces hypercalcemia but not hyperphosphatemia [12], suggesting that the hormone stimulates calcium carbonate mobilization from the shell, rather than resorption of calcium phosphate from bone. It was first demonstrated by Narbaitz et al. [13] that the presence of target cells for 1,25-$(OH)_2D_3$ in the chorionic epithelium as well as other tissues was involved in calcium metabolism in the chick embryo. One component that was shown to increase in activity in the villus cavity cells of the CAM in response to 1,25-$(OH)_2D_3$ injection in ovo was carbonic anhydrase [14].

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The present investigation provides evidence for a direct role of vitamin D and carbonic anhydrase in eggshell resorption and transepithelial calcium transport by the CAM.

2. Experimental

Materials. All reagents used were of analytical grade and obtained from the standard suppliers. 25-OH-[26,27-^{3}H]D_3 (160 Ci/mmol) was obtained from DuPont/New England Nuclear (Boston, MA). Unlabeled vitamin D_3 metabolites were prepared by Tetrionics (Madison, WI). Acetazolamide was obtained from Sigma (St. Louis, MO).

Animals. University of Wisconsin (Madison, WI) strain of Japanese quail (*Coturnix coturnix japonica*) were used in our experiments. All quail were maintained in galvanized wire floored cages in rooms with automatically controlled temperature, humidity and ventilation. Groups of 40 birds each were raised from hatching to sexual maturity on experimental diets. Feed and water were supplied ad libitum throughout the experiments. A diet was formulated that contained no vitamin D_3 [8]. The laying hen diet was initiated at 5 weeks of age. Vitamin D_3 (2200 IU/kg diet) or 1,25-$(OH)_2D_3$ (4 μg/kg diet) dissolved in corn oil was premixed with the other vitamins and added to 20-kg quantities of feed with thorough mixing.

Eggs were collected daily from both vitamin D-fed and 1,25-$(OH)_2D_3$-fed groups and stored at 12°C. At the end of each week, all eggs not cracked or deformed were incubated in a Roll-X incubator that is maintained at 37.2–37.8°C.

In ovo injections and blood and CAM tissue sampling. Injections were made as previously described [11]. Briefly, on day 10 or 11 of incubation, eggs were taken out of the incubator, candled and the exact location of the air cell was outlined with a marker. The egg surface over the air cell was wiped with 70% ethanol and the egg was injected with 10 μl dimethylsulfoxide (DMSO) with or without the vitamin D compound or carbonic anhydrase inhibitor acetazolamide. The injection site was sealed with Duco cement and the eggs returned back into the incubator.

Blood was collected from the extra embryonic blood vessels. Blood was manually pooled from 5 embryos and mixed in a heparin-coated tube, centrifuged and plasma collected for measurement of plasma calcium and phosphorus. The chorioallantoic membrane tissues were designated as −D when they were excised from eggs that were laid by hens maintained on diets containing only 1,25-$(OH)_2D_3$ and +D if the hens' diet contained vitamin D_3.

Mineral content of 1-day-old chick, embryo, incubated yolk sac, and whole eggs. 1-day-old chicks were sacrificed and placed in individual crucibles. Albumin and yolks of eggs were separated and also placed in crucibles. On the 10th day of incubation, changes in the calcium content of either the embryo or the yolk sac were determined. These embryos were killed and separated from their yolk sacs. Each yolk sac was placed in an individual crucible, while the embryos were washed with deionized water and placed in another crucible. Wet weights were taken for all samples before drying them for 24 h in an oven at 105°C. The dry weight was recorded followed by ashing of the contents in a muffle furnace for 24 h at 500°C to obtain the ash weight. After cooling, 3 ml of concentrated HCl were added to each crucible and heated until the ash was dissolved. This solution was used to measure calcium and phosphate content.

Calcium and phosphorus assays. Calcium was measured by atomic absorption spectrophotometry on samples to which was added lanthanum chloride to 0.1%. Inorganic phosphate was measured colorimetrically by the method of Itaya and Ui [15].

Carbonic anhydrase. The embryonic contents from +D and −D fertile eggs at days 10–14 of incubation were decanted from a hole made in the shell directly above the air cell. The interior of the egg was then rinsed with ice cold avian physiological saline and the CAM gently peeled from the shell. The CAM was weighed and minced into 2 mm^2 pieces with a scissor-like action of two scalpel blades. Samples were homogenized at 0°C in 0.02 M Tris-HCl buffer, pH 8.0 (250 mg tissue/ml buffer). Carbonic anhydrase was measured at 0°C by the electrometric method of Wilbur and Anderson [16] with CO_2 as the substrate. Determination of hemoglobin was carried out by the cyanomethaemoglobin method using a reagent kit supplied by Sigma. Hemoglobin concentrations were used to correct for the contamination of samples with carbonic anhydrase of erythrocytic origin. Protein was measured by the method of Lowry et al. [17] with bovine serum albumin (Sigma) as a standard. Other fertile eggs at day 11 of incubation were injected with 100 ng of 1,25-$(OH)_2D_3$ as described earlier. The CAMs were taken at 2, 4, 8, 12, and 24 h post-injection for the measurement of carbonic anhydrase. At each time point at least 5 samples were used.

Vitamin D receptor determination. Fertile +D eggs were injected with 1,25-$(OH)_2D_3$ (100 ng/egg) at day 11 of incubation, and the CAMs collected after 2, 4, 8, 12 and 24 h post-injection (7–10 eggs/time point). To serve as a control, a group of non-injected +D eggs were used as the 0 time point. The CAMs while still adhering to the inside of the eggshell were placed in 50 mM Tris-HCl/1.5 mM EDTA/5 mM dithiothreitol/150 mM NaCl (pH 7.4 at room temperature) ($TEDNa_{150}$) on ice. The tissue was washed twice with $TEDNa_{150}$ and once with 50 mM Tris-HCl/1.5 mM

EDTA/5 mM dithiothreitol (pH 7.4 at room temperature) (TED) after being removed from the shell. The tissue was homogenized with five strokes of a glass and Teflon homogenizer in 2 volumes (v/v) of 50 mM Tris-HCl/1.5 mM EDTA/5 mM dithiothreitol/300 mM KCl/5 mM diisopropylfluorophosphate (DFP) (pH 7.4 at room temperature) on ice. The homogenates were centrifuged at 4°C for 1 h at 120000 × *g* in a Beckman ultracentrifuge (Beckman Instruments, Fullerton, CA). The samples were divided into aliquots, frozen in liquid nitrogen and stored at −80°C until analysis. CAM homogenates were thawed and receptor levels measured using an immunoradiometric assay previously described [18]. The protein concentration of the CAM homogenates was measured by the method of Bradford [19] using the BioRad microassay kit (Bio-Rad, Melville, NY). Bovine serum albumin was used as a standard. Developmental changes in the receptor levels were determined similarly.

Statistical analyses. Results are presented as means ± S.E. Statistical analyses were conducted using the ANOVA General Linear models, procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Significant differences between groups were determined by Fisher's least significant differences and Student's *t*-test [20].

3. Results

Calcium was measured in the hatched control chick from vitamin D3-fed hens and in two different components of fresh + D eggs (albumin and yolk). These determinations were undertaken to determine how much each part of the egg contributes to the mineralization of the chick. Fig. 1 shows the results of measuring calcium in the ash of egg components and in day-old chicks. 1-day-old chicks contain 25.9 mg of calcium, while both yolk and albumin contain only 6.4 mg. Therefore, during embryogenesis, it is estimated that this difference (19.5 mg of Ca) was mobilized from the eggshell and accumulated in the embryo.

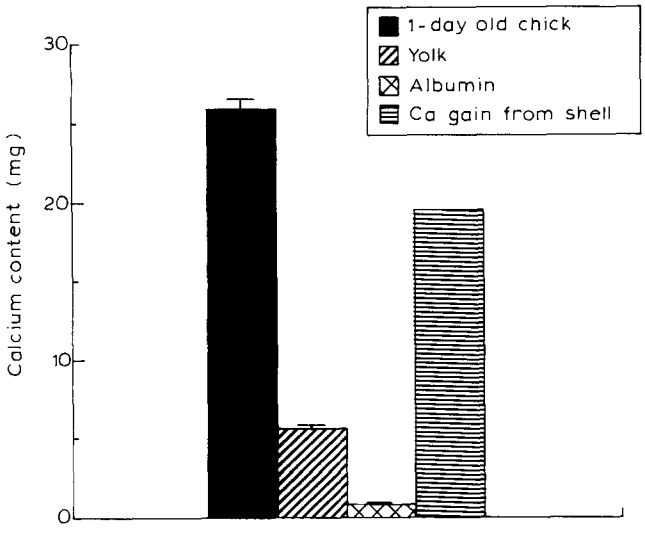

Fig. 1. Calcium content of 1-day-old chicks and the components of unincubated eggs from vitamin D_3-fed hens. Data are means ± S.E. of 10 chicks and egg contents. Calcium gain represents the transport of calcium from eggshell to embryo during incubation, and it is the calculated difference between the calcium content of the 1-day-old chick and the combined yolk and albumin calcium of the unincubated egg.

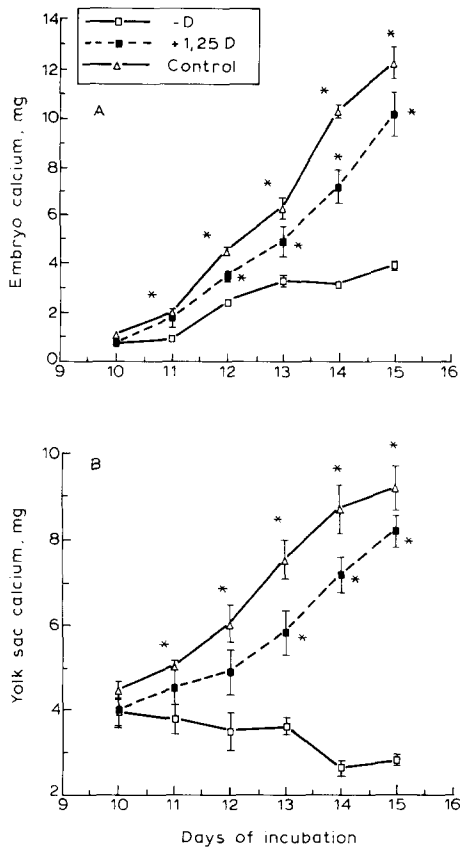

Fig. 2. Changes in the calcium content of the developing Japanese quail embryo (A) and incubated yolk sac (B) of −D; 1,25-$(OH)_2D_3$-injected, deficient eggs; and control + D eggs. Data are means ± S.E. of 5–10 embryos at each time point. Where S.E. is not visible, the variance is less than the area occupied by the symbol. * $P < 0.05$ when compared to the vitamin D-deficient embryo (A) or yolk sac (B).

Total calcium content of + D and −D embryos was not different at 10 days of incubation, whereas the −D embryos became progressively deficient in calcium thereafter when compared with + D embryos (Fig. 2A). Total calcium content of 15-day-old + D embryos reached 12.3 mg, while that of the −D embryos was approximately 4 mg, indicating the inability of −D embryos to accumulate calcium in their skeleton at the same rate as the + D embryos. When 1,25-$(OH)_2D_3$ was injected into −D eggs at day 10 of incubation, the embryos started to accumulate calcium, and by 48 h after the injection, they had significantly higher body

calcium. The injected embryos had a 10 mg body calcium at day 15 of incubation, as compared to the 4 mg in the −D, suggesting that shell calcium was mobilized as a result of 1,25-$(OH)_2D_3$ injection. At day 10 of incubation, there was no significant differences in calcium content of the yolk sac. All embryos had about 4 mg of calcium (Fig. 2B). After day 10, the + D yolk sacs increased their content of calcium until they reached more than 9 mg at day 15. During the same time, −D yolk sacs were depleted of calcium. Injecting 1,25-$(OH)_2D_3$ resulted in a marked increase in yolk calcium.

Vitamin D_3 acts through the interaction of its active form, 1,25-$(OH)_2D_3$, with a nuclear receptor protein [21]. Vitamin D receptor is present in the CAM [22] and develops rapidly at 14–19 days of chick embryo development. We analyzed the receptor concentration in + D and −D CAMs and in response to 1,25-$(OH)_2D_3$ injection (Fig. 3). Vitamin D receptor was higher (but not significant) in the −D CAM at 8 days of incubation; however, the + D CAM had significantly higher receptor levels thereafter (Fig. 3A). The vitamin D receptor level increased significantly 4 h after treatment with 1,25-$(OH)_2D_3$, and continued to increase until it peaked at 8 h to a level that is twice as much as that at the 0 time point. It started to decline thereafter, but after 24 h, it was still higher than the initial value.

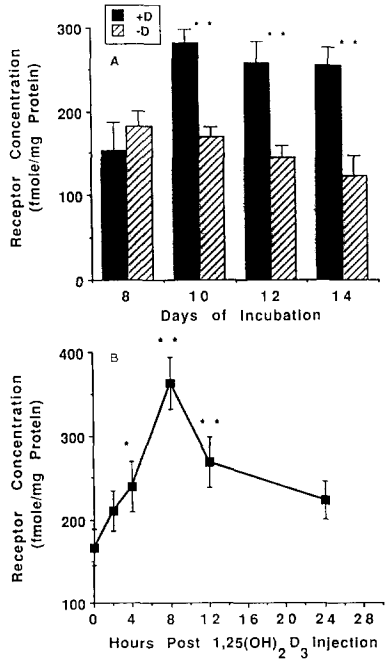

Fig. 3. Changes in the level of the vitamin D receptor in the CAMs of the developing −D and + D eggs (A) and in response to injection of 100 ng of 1,25-$(OH)_2D_3$ into + D eggs at day 11 of incubation (B). At least five individual CAM tissues were used per time point. The data are presented as the means ± S.E. * $P < 0.05$ when compared to −D (A) or the zero time point (B). ** $P < 0.01$ when compared similarly.

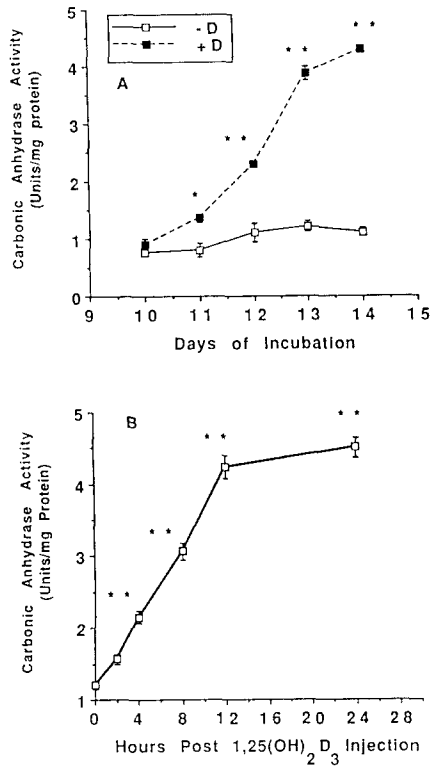

Fig. 4. Carbonic anhydrase activity of −D and + D CAM at the later stages of development when eggshell calcium mobilization takes place (A) and the effect of injecting 1,25-$(OH)_2D_3$ into + D eggs at day 11 of incubation on carbonic anhydrase activity (units/mg protein) (B). Values are means of 5–10 determinations ± S.E. * $P < 0.05$ and ** $P < 0.01$ compared to −D (A) or to zero time point (B).

Carbonic anhydrase activity was demonstrated in the chick embryonic CAM and was correlated with the Ca transport activity of the membrane [23]. Fig. 4A shows the age profile of carbonic anhydrase activity of CAM extracts from −D and + D embryos. In + D CAMs, the carbonic anhydrase activity was significantly higher at day 11 and continued to increase thereafter. The −D CAMs did not show a similar increase in the enzymatic activity and this defect might be one of the major reasons why −D embryos fail to resorb eggshell calcium. Injecting 1,25-$(OH)_2D_3$ into embryos markedly increased carbonic anhydrase activity (Fig. 4B). It increased significantly already at 4 h post-injection and reached a maximum by 24 h. This strongly suggests that the action of the hormone in the CAM involves stimulation of the synthesis and/or stabilization of this enzyme.

The plasma calcium and phosphorus responses to a single injection of either the vehicle (DMSO), 100 ng 1,25-$(OH)_2D_3$ or 300 μg of acetazolamide are summarized in Fig. 5. After acetazolamide treatment, embryos exhibited a significant decline in plasma calcium and a significant elevation in plasma phosphate at 2 and 4 h post-injection compared to control embryos. However, this effect of acetazolamide in inducing these changes

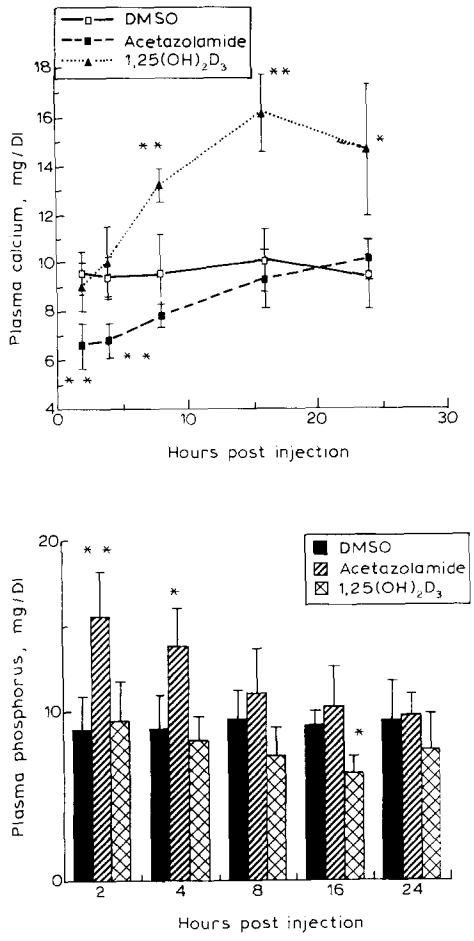

Fig. 5. Plasma levels of calcium and phosphorus of +D embryos treated with DMSO (vehicle), 1,25-$(OH)_2D_3$ (100 ng/egg) or acetazolamide (300 μg/egg). Embryos were bled 2, 4, 8, 16 and 24 h later. Data are means ± S.E. of five pooled (three or more) plasma samples. * $P < 0.05$ and ** $P < 0.01$ compared to DMSO-treated.

in plasma calcium and phosphate started to decline thereafter, and by 24 h post-injection, plasma levels were almost normal. Treatments with 1,25-$(OH)_2D_3$ produced the opposite effect of acetazolamide, namely hypercalcemia and hypophosphatemia. Plasma calcium levels peaked at 16 h post hormone injection, while phosphate was at its lowest at that time point.

4. Discussion

The results of Fig. 1 clearly demonstrate the importance of shell calcium in the mineralization of embryonic skeleton in Japanese quail. More than 75% of the total calcium in 1-day-old quail chicks (26 mg) comes from the shell, the rest is obtained from the yolk. Rapid calcification of the skeleton takes place during the last week of incubation [24] and these authors as well as others have shown also that the yolk accumulates calcium at a high rate during this period [2]. This was shown to occur in + D embryos, as their yolk accumulated calcium, while the −D yolk sacs failed to do so. Injection of 1,25-$(OH)_2D_3$ enabled the −D embryos to accumulate calcium in their yolk sacs. Calcification failure in −D embryos was not manifested until the embryo became dependent on shell calcium at day 11 of incubation. Before that, the embryo relies on the yolk to satisfy its calcium needs. Thus, a lack of calcium from the eggshell in the −D embryo is the main cause of the impaired development.

The CAM is an extra embryonic membrane that lines the internal surface of the inner shell membrane and eggshell and functions to translocate shell calcium into the chick embryo [1]. The present study has clearly reaffirmed the essentiality of vitamin D for shell calcium mobilization. The presence of 1,25-$(OH)_2D_3$ receptors in the CAM was previously demonstrated [22]. The developmental increase of the vitamin D receptor also strongly suggests a role for 1,25-$(OH)_2D_3$ in the regulation of shell calcium mobilization [25]. This is supported by previous work which showed that injected vitamin D compounds restore hatchability of vitamin D-deficient embryos [10,11]. Also, the finding that 1,25-$(OH)_2D_3$ administration to chick embryos induced a marked hypercalcemia that is associated with hypophosphatemia (Fig. 5), suggests that the injected metabolite stimulated the absorption from the $CaCO_3$ of shell rather than resorption of both from bone. In fact, 1,25-$(OH)_2D_3$ injection into −D eggs corrected the distortion of calcium to phosphorus ratio in −D embryos [10,11]. The receptor results presented here show that vitamin D and 1,25-$(OH)_2D_3$ up-regulate the CAM 1,25-$(OH)_2D_3$ receptor in vivo in quail embryos (Fig. 3). Evidence suggests that 1,25-$(OH)_2D_3$ stabilizes the receptor; thus, increasing its in vivo lifetime [26]. This was further confirmed in Fig. 3B when 1,25-$(OH)_2D_3$ was injected in vitamin D-sufficient eggs, as previously stated, 1,25-$(OH)_2D_3$ increased CAM receptor to its highest level at 8 h post-treatment. The increase in the receptor at day 10 occurred immediately before induction of shell resorption as a necessary preliminary event, suggesting regulation of shell calcium mobilization.

The mechanism of shell resorption and calcium transport by the CAM is unclear. Shell resorption apparently occurs by a two-step process, involving degradation of calcium-binding shell matrix glycoproteins and proteoglycans [27], followed by calcium and carbonate removal. Elaroussi and DeLuca [28] have cloned a cDNA for a novel metalloendopeptidase from CAM that is thought to participate in the matrix degradation process. This cDNA is regulated by vitamin D and increases at the time of shell calcium mobilization. Carbonic anhydrase has been shown to be functionally involved in the calcium transport function of the CAM [23]. At day 10, the membrane had low levels of carbonic anhydrase as shown in Fig. 4A.

The rise in carbonic anhydrase in CAM precedes uptake of calcium from the shell. Terepka et al. [1] have shown, by means of an Ussing Chamber-type apparatus, that the rate of Ca transport by the CAM measured in vitro exhibits an age-dependent increase. Vitamin D-deficient embryos were found to have a reduced carbonic anhydrase activity which explains in part the failure of these embryos to obtain calcium from the shell. This conclusion is consistent with the results in Fig. 4B and shows an increased carbonic anhydrase activity in response to 1,25-$(OH)_2D_3$ and agrees with others [14]. Carbonic anhydrase has been shown also to play a role in the process of bone resorption [29], and carbonic anhydrase deficiency was identified as the defect responsible for one form of osteopetrosis [30]. Similarly, 1,25-$(OH)_2D_3$ increases carbonic anhydrase in the osteoclasts of bone [31].

To further investigate the role of carbonic anhydrase in shell calcium mobilization, we used the specific carbonic anhydrase inhibitor, acetazolamide. Carbonic anhydrase is the only enzyme that could be inhibited by acetazolamide [32]. Acetazolamide decreased plasma calcium and increased plasma phosphorus (Fig. 5). This plasma profile is quite similar to that produced by vitamin D deficiency in avian embryos [8,9]. Thus, it is postulated that both acetazolamide treatment and vitamin D deficiency inhibit carbonic anhydrase activity that is required for shell calcium transport. The results shown here suggest that one factor in 1,25-$(OH)_2D_3$ stimulated shell calcium mobilization is the induction of CAM carbonic anhydrase that acidifies fluid bathing the shell and solubilizes the $CaCO_3$ of the shell. Therefore, the process of dissolution and/or active transport of shell calcium may have an absolute dependence on vitamin D in the avian embryo.

Acknowledgments

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