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Regulation of extraembryonic calcium mobilization by the developing chick embryo

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SUMMARY

During development, the chick embryo mobilizes the calcium it needs from two extraembryonic sources, first the yolk and then the eggshell. Since previous studies have strongly suggested that vitamins D and K may regulate chick embryonic calcium metabolism, we have examined here how these vitamins might be involved in regulating the calcium mobilization processes. We used as our experimental system chick embryos which were maintained in long-term *in vitro* culture in the absence of the eggshell. Our results showed that exogenous vitamin D_3, in the form of the active 1,25-dihydroxylated metabolite, was hypercalcaemic in both control embryos and the calcium-deficient, shell-less embryos. Since the eggshell was absent in the latter, the vitamin D-induced hypercalcaemia must involve mobilization of calcium from the yolk and, or, the embryonic skeleton. The latter was unlikely since concomitant hyperphosphataemia was not observed. By radiolabelling the yolk with $^{45}Ca^{2+}$ and subsequently monitoring its distribution, we showed that vitamin D_3 stimulated yolk calcium mobilization. However, exogenous vitamin D_3 did not appear to influence the calcium uptake activity of the chorioallantoic membrane (CAM), the tissue responsible for translocating eggshell calcium. On the other hand, when embryos were rendered vitamin K deficient by the administration of its antagonist, Warfarin, CAM calcium activity was significantly depressed, an effect which was remedied by vitamin K supplementation. We conclude that, during normal chick embryonic development, vitamin D is primarily involved in regulating yolk calcium mobilization whereas vitamin K is required for eggshell calcium translocation by the CAM.

INTRODUCTION

Avian embryos, such as those of the domestic chicken, mobilize a large amount of calcium from extraembryonic sources during their development to meet the metabolic needs of skeletal growth, neuromuscular activities and other physiological functions. In the chick embryo, the yolk and the eggshell constitute the extraembryonic calcium sources (Simkiss, 1961). Of these, the eggshell (2–3 g of $CaCO_3$) is the major source since the hatchling has approximately 120–140 mg of calcium whereas the yolk contains only 20–25 mg of calcium (Romanoff, 1967). Early $^{45}Ca^{2+}$ tracer studies by Johnston & Comar (1955) clearly demonstrated that calcium is sequentially mobilized first from the yolk and then from the eggshell.

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Characteristically, yolk calcium is mobilized beginning around day 7–8 of incubation. By day 10, translocation of eggshell calcium is initiated which results in an increase in the calcium contents of both the embryo and the yolk. Calcium accumulation by the embryo continues until hatching on day 21 whereas the calcium content of the yolk is relatively stable at 30–35 mg after day 16 (see review by Packard & Packard, 1984).

To understand the mechanism and regulation of calcium accumulation by the developing chick embryo therefore requires the analysis of calcium translocation from both the yolk and eggshell. Much cellular and biochemical information has been gathered concerning the latter process. Eggshell calcium is transported actively and unidirectionally by the ectodermal cells of the chorioallantoic membrane (CAM) (Terepka, Coleman, Armbrecht & Gunther, 1976). A high-molecular-weight, vitamin K-dependent, calcium-binding protein (CaBP) (Tuan & Scott, 1977; Tuan, Scott & Cohn, 1978*a*,*b*; Tuan, 1980*a*), a Ca^{2+}-activated ATPase (Tuan & Knowles, 1984) and carbonic anhydrase (Gay, Schraer, Sharkey & Rieder, 1981; Tuan & Zrike, 1978; Tuan, 1984) have been implicated in the transport mechanism (Tuan *et al.* 1986*b*). The development-specific expression of calcium transport and the CaBP in the CAM also requires the continuous presence of the eggshell (Tuan, 1980*b*; Dunn, Graves & Fitzharris, 1981). Calcium transport by the yolk sac, on the other hand, has not received much experimental attention.

To understand how the transport substrate itself, the eggshell, may regulate calcium transport by the CAM, we and other investigators (Tuan, 1980*b*, 1983; Dunn & Fitzharris, 1979; Dunn *et al.* 1981) have studied chick embryos by maintaining them in long-term shell-less culture. It is found that the CAM of these shell-less embryos fails to show the development-specific onset of calcium transport activity and expresses an inactive form of the CaBP. Lacking their primary calcium source, these shell-less embryos are highly hypocalcaemic (Burke, Narbaitz & Tolnai, 1979; Ono & Tuan, 1986) and show clear signs of malformations and abnormal cytodifferentiation in their skeletal components (Slavkin, Slavkin & Bringas, 1980; Narbaitz & Jande, 1983; Tuan & Lynch, 1983; Jacenko & Tuan, 1986). The shell-less embryo therefore represents a useful experimental system to study the relationship between embryonic calcium homeostasis and skeletogenesis.

In this report, we have made use of the shell-less chick embryo to understand how the mobilization of extraembryonic calcium is regulated during embryonic development. Specifically, we have examined vitamins D and K as possible regulatory agents in the mobilization of calcium from the yolk and the eggshell. This investigation is prompted by two sets of findings: (1) the classic study of Hart *et al.* (1925) and the studies from the laboratories of DeLuca (Ameenuddin *et al.* 1982; Hart & DeLuca, 1984, 1985; Sunde, Turk & DeLuca, 1978) and Norman (Henry & Norman, 1978) have strongly suggested that proper vitamin D metabolism is required for normal embryonic development and skeletal formation; and

(2) our recent demonstration of the functional involvement of the vitamin K-dependent CaBP in CAM calcium transport (Tuan *et al.* 1986*b*).

The shell-less chick embryo serves as an ideal experimental animal for this study since it possesses only one mobilizable source of extraembryonic calcium, the yolk, making it possible to identify unequivocally the target tissue under regulation. In this study, we have monitored calcium mobilization by means of $^{45}Ca^{2+}$ tracer analysis and have characterized CAM calcium transport using recently developed protocols to measure calcium uptake (Tuan *et al.* 1986*a*). We report here results that strongly suggest that vitamin D is involved in regulating yolk calcium mobilization whereas vitamin K is required in CAM calcium transport.

MATERIALS AND METHODS

Chick embryos and shell-less culture

Fertile White Leghorn eggs obtained from Truslow Farms (Chestertown, MD) were used throughout the study. They were incubated at 37·5 °C in a humidified commercial egg incubator. The procedure of shell-less culture has been described previously (Dunn & Boone, 1976; Tuan, 1980*b*; Watanabe & Imura, 1983; Ono & Tuan, 1986). Briefly, embryonated eggs were cracked open aseptically after 3 days of incubation *in ovo*, and transferred to a hemispherical pouch made of transparent plastic kitchen wrap suspended within a ring stand. The culture was loosely covered with a 100 mm Petri dish lid and then placed in a humidified tissue culture incubator at 37 °C with constant air flow.

Embryo injections

Embryos were injected, *in ovo* and in shell-less cultures, with 1,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$) and Warfarin (and vitamin K_1) using the following protocols. For $1,25(OH)_2D_3$, each embryo received 125 μg (300 pmoles) of the vitamin (in 50 μl of 95 % ethanol) injected into the egg yolk on day 16 of incubation (Narbaitz & Fragisko, 1984). Control embryos received 95 % ethanol alone. For Warfarin, injections (in 50 μl of physiological saline per embryo) were made into the allantoic sac of embryos *in ovo* using a previously published regimen (Tuan, 1979): 100 $\mu g\,day^{-1}$ on incubation days 11, 12, 13 and 14; and 200 $\mu g\,day^{-1}$ on days 15, 16 and 17. In some experiments, vitamin K_1 was coinjected with Warfarin using the same regimen and dosage.

^{45}Ca radiotracer studies

Approximately 20 μCi of carrier-free $[^{45}Ca]CaCl_2$ in 50 μl of physiological saline was injected into the yolk *in ovo* or in shell-less cultures on day 16 of incubation. After 24 h, the following components, yolk, long bones (femurs and tibia) and sera, were collected for calcium and phosphate analyses and radioactivity determination. Serum samples were obtained by bleeding at the extraembryonic arteries or veins. The solid components (yolk and bones), after drying and weighing, were either dry-ashed (650 °C, 4 h; Tuan, 1983) and then solubilized in 6 N-HCl for calcium determination, or wet-ashed in perchloric–sulphuric acid (Kirkpatrick & Bishop, 1971) for phosphate (P_i) determination. Radioactivity of $^{45}Ca^{2+}$ in the sera and the solubilized tissue samples was determined by liquid scintillation counting. For all assays, triplicates were performed and all calculations of specific activities were based on the values of each individual embryo and then pooled for statistical analysis.

Calcium and phosphate analyses

Total calcium was measured fluorometrically with an automated calcium analyser (CALCETTE, Precision Instruments, Sudbury, MA) using the fluorescent dye, calcein (Borle

& Briggs, 1968; Ono & Tuan, 1986). Total phosphate (P_i) was estimated using the molybdate–malachite green method as described previously (Tuan & Knowles, 1984; Ono & Tuan, 1986).

CAM calcium uptake assays

An *in vivo* method (by constructing an uptake chamber on top of the CAM; Crooks & Simkiss, 1975; Tuan & Zrike, 1978) and two *in vitro* methods (using tissue disks and cell-free microsomes; Tuan *et al.* 1986*a*) were used to assay calcium uptake by the CAM. All assays used triplicate-to-quadruplicate samples for each time point.

Statistical analysis

Data were analysed using Student's *t*-test. Differences were regarded as statistically significant at $P < 0{\cdot}05$ (Dowdy & Wearden, 1983).

Materials

The active metabolite of vitamin D_3, $1,25(OH)_2D_3$, was kindly provided by Dr Milan Uskokovic of Hoffman-La-Roche Inc. (Nutley, NJ, USA). Vitamin K_1 and Warfarin (sodium) were obtained from Sigma Chemicals (St Louis, MO, USA); carrier-free $[^{45}Ca]CaCl_2$ was from Amersham (Chicago, IL, USA); trace-mineral-free concentrated HCl was from Baker Chemical Co. (Philipsburg, NJ, USA); molybdic acid and malachite green are from Aldrich Chemical Co. (Milwaukee, WI, USA).

RESULTS

Effect of vitamin D on embryonic calcium homeostasis

Vitamin D was administered in the form of its active metabolite, $1,25(OH)_2D_3$, to control and shell-less chick embryos on day 16 of incubation. After 24 h, serum calcium and phosphate contents were determined. As shown in Fig. 1A, vitamin D was clearly active in increasing serum calcium in both normal and shell-less embryos. That the shell-less embryos also responded to the hypercalcaemic action of vitamin D was particularly interesting since these embryos lacked the eggshell and thus the influx of calcium could not possibly be derived from the CAM–eggshell pathway. Therefore, of the three calcium compartments (yolk, embryo proper and eggshell), vitamin D did not need to act on the eggshell but perhaps instead on the yolk and, or, the embryo itself to bring about the increase in serum calcium. The data in Fig. 1B showed that the vitamin D-induced rise in serum calcium was not accompanied by a significant concomitant increase in serum phosphate. This finding suggested that bone demineralization was unlikely to be a major contribution to the vitamin D effect. In fact, as shown in Fig. 2, no apparent calcium loss could be detected in embryonic bones as a result of vitamin D administration. Taken together, these observations therefore suggested that vitamin D might act by stimulating the mobilization of yolk calcium.

Vitamin D stimulation of yolk calcium mobilization

To test this hypothesis, direct measurements were made of the yolk calcium contents. The data in Fig. 3 revealed that (1) normal embryos in general contained more calcium in their yolk than the shell-less embryos; and (2) upon vitamin D

administration, calcium was indeed mobilized from the yolk of the shell-less embryos, although no apparent change in yolk calcium content was observed in normal embryos (see below).

To identify unequivocally the calcium source involved in the vitamin D effect, we radiolabelled the yolk with $^{45}Ca^{2+}$ to trace the traffic of yolk calcium in the embryo. The results in Fig. 4 showed that vitamin D indeed elicited the mobilization of yolk calcium into both the serum and the bone tissue compartments of the embryo. Again, in each case, the extent of yolk contribution, with or without vitamin D administration, was greater in the shell-less embryos. This would be consistent with the shell-less embryos being totally dependent on the yolk reserve as the only calcium source. On the other hand, the normal embryos are able to accumulate eggshell calcium, part of which is used to replenish the yolk calcium reserve and accounts for the unchanged yolk calcium content with vitamin D administration (see above). Interestingly, the relative increase in specific activities upon vitamin D administration were similar in both the serum and the long bones. This finding suggested that the vitamin-induced contribution of yolk calcium was probably totally responsible for the increase in serum calcium and its subsequent deposition into the growing skeleton. Thus, even for the normal embryos, little contribution of the unlabelled calcium from the eggshell was needed for the vitamin D effect. In other words, vitamin D was unlikely to influence the calcium transport ability of the CAM.

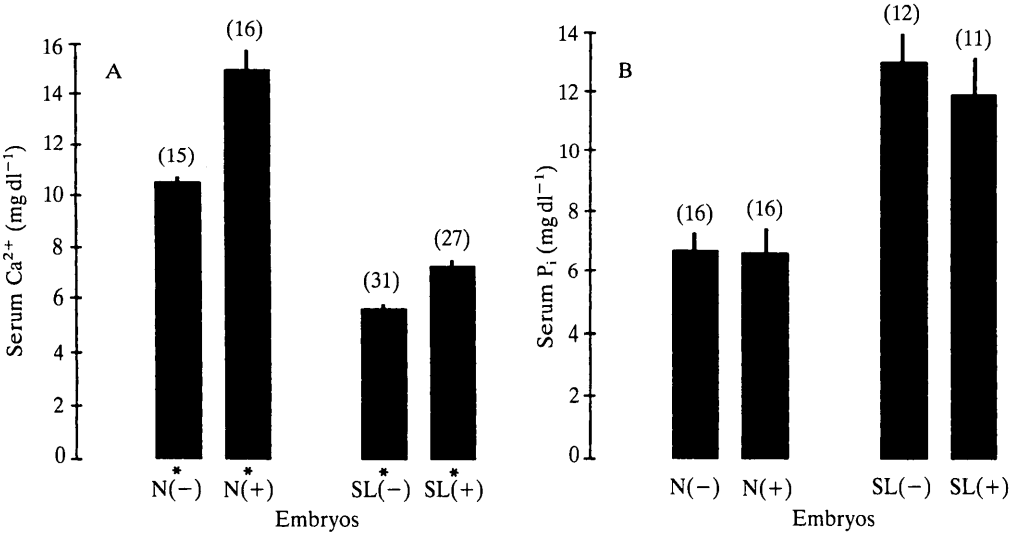

Fig. 1. Serum (A) calcium (Ca^{2+}) and (B) phosphorus (P_i) of normal (N) and shell-less (SL) day 17 chick embryos with (+) or without (−) $1,25(OH)_2D_3$ treatment. The number of embryos analysed in each group is shown in parentheses and the values represent the mean ± S.E.M. For Ca^{2+}, the + and − values are statistically significantly different ($P < 0{\cdot}05$) within each group of embryos (N or SL). For P_i, there is no significant difference between + and − values within each group of embryos. Asterisks indicate embryo groups with statistically different + and − values ($P < 0{\cdot}05$).

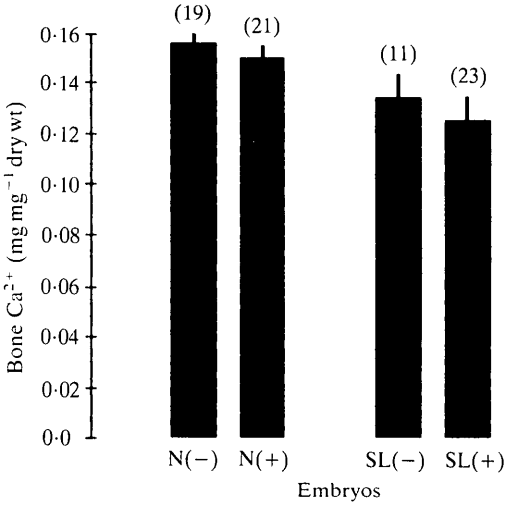

Fig. 2. Bone Ca^{2+} content in normal (N) and shell-less (SL) day 17 chick embryos with (+) or without (−) $1,25(OH)_2D_3$ treatment. The number of embryos analysed in each group is shown in parentheses and the values represent the mean ± S.E.M. There is no statistically significant difference between + and − embryos: N embryos, $P > 0{\cdot}1$; SL embryos, $0{\cdot}1 > P > 0{\cdot}05$.

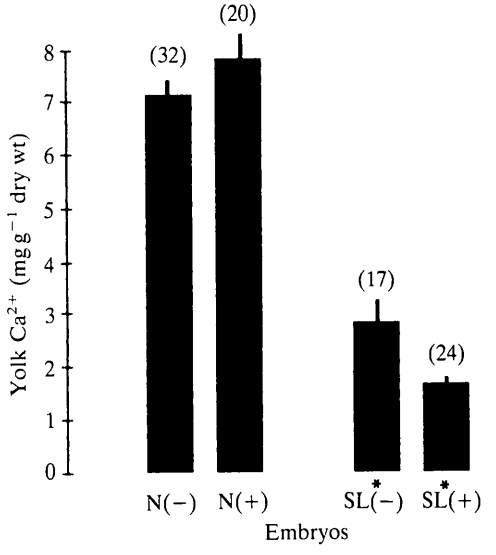

Fig. 3. Yolk Ca^{2+} content in normal (N) and shell-less (SL) day 17 chick embryos with (+) or without (−) $1,25(OH)_2D_3$ treatment. The number of embryos analysed in each group is shown in parentheses and values represent the mean ± S.E.M. The values of + and − embryos were statistically different only in the SL embryos (indicated by asterisk): N embryos, $0{\cdot}1 > P > 0{\cdot}05$; SL embryos, $0{\cdot}001 < P < 0{\cdot}01$.

Effect of vitamin D on CAM calcium uptake activity

This was tested by directly assaying the calcium uptake activity of the CAM of normal and shell-less embryos after vitamin D administration. Using both *in vivo* and *in vitro* assays, vitamin D treatment did not affect the intrinsic calcium uptake

activity of the CAM in either normal (see Fig. 5) or shell-less embryos (data not shown). Also, as reported earlier (Tuan, 1980*b*; Dunn *et al.* 1981), the calcium uptake activity of the CAM from normal embryos was significantly higher than that of the shell-less embryos (data not shown).

Effect of vitamin K on CAM calcium uptake activity

Our previous finding of the vitamin K dependence of the CAM CaBP (Tuan *et al.* 1978*b*; Tuan, 1980*a*), a functional component of the calcium transport pathway (Tuan *et al.* 1986*b*), strongly suggested that vitamin K might play a regulatory role in CAM calcium transport. This was tested here by the administration of the vitamin K antagonist, Warfarin, to the chick embryo followed by measurement of the calcium uptake activity of the CAM. The results in Fig. 6 showed that Warfarin treatment of the embryo significantly decreased CAM calcium uptake activity, measured by both the tissue disk and cell-free membrane vesicle assays. Coinjecting the embryo with vitamin K, however, restored CAM calcium uptake, indicating the specificity of the Warfarin effect. These results therefore strongly indicated that vitamin K was required for normal CAM calcium transport.

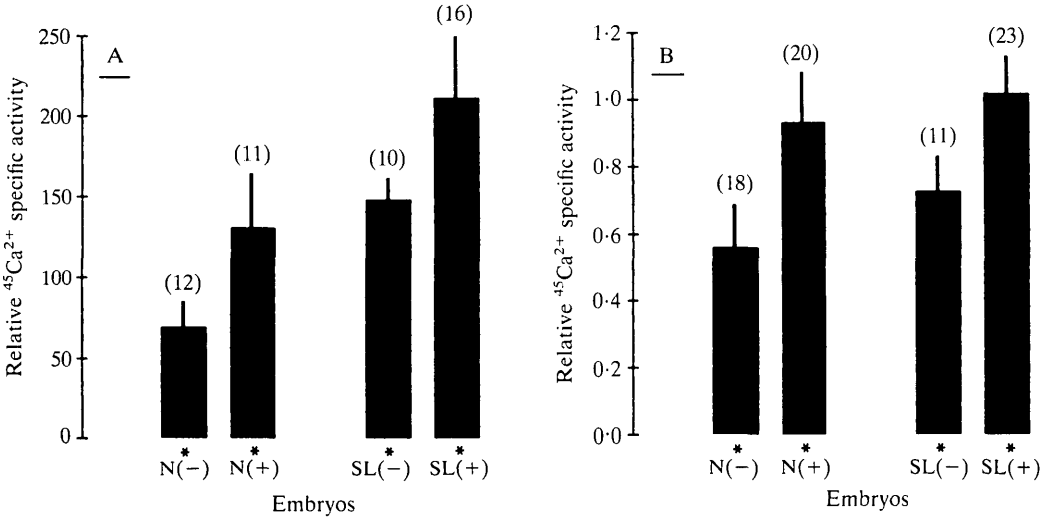

Fig. 4. Effect of $1,25(OH)_2D_3$ on the mobilization of yolk calcium into (A) the embryonic circulation (serum) and (B) the embryonic bones (tibia and femur). The content of Ca^{2+} and level of $^{45}Ca^{2+}$ radioactivity in the serum, the yolk and the bones were determined as described in Materials and Methods. The values represent the specific activity, $^{45}Ca^{2+}(cts\,min^{-1})/Ca^{2+}(mg)$, in the serum (A) or bone (B) relative to that in the yolk of normal (N) and shell-less (SL) day 17 embryos with (+) or without (−) $1,25(OH)_2D_3$ treatment. The number of embryos analysed in each group is shown in parentheses and values represent the mean ± S.E.M. In both A and B, the values for + and − embryos are statistically significant in each embryo group (indicated by asterisks): N embryos, $P < 0{\cdot}05$; SL embryos, $P < 0{\cdot}05$.

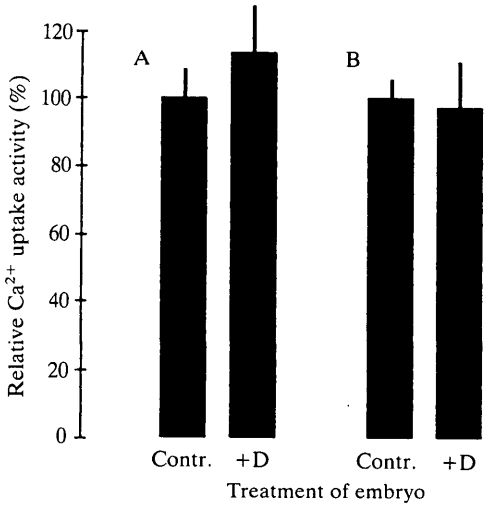

Fig. 5. Effect of $1,25(OH)_2D_3$ on CAM calcium uptake activity. The calcium uptake assays were carried out both (A) *in vivo* and (B) *in vitro* (tissue disks) using day 17 N embryos as described in Materials and Methods. The activities (mol calcium min^{-1} cm^{-2}) represent the mean ± S.E.M. of two to three separate experiments and are expressed as a percentage of control. No statistical significance was apparent between the control and $1,25(OH)_2D_3$-treated embryos.

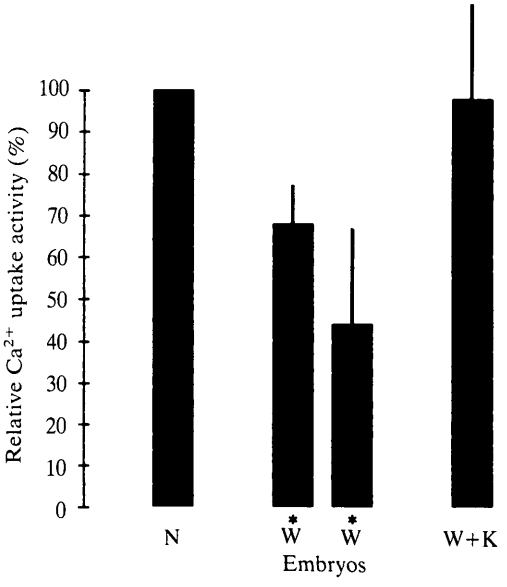

Fig. 6. Vitamin K dependence of CAM calcium uptake activity. Day 17 N chick embryos were injected with Warfarin (W) and, or, vitamin K_1 (K) and their CAM was assayed for calcium uptake activity *in vitro* as described in Materials and Methods. The activities (mol calcium min^{-1} cm^{-2} for tissue disks, and mol calcium mg^{-1} protein for membrane vesicles) are expressed as a percentage of control and represent the mean ± S.E.M. of two to three separate experiments. Asterisks indicate statistically significant differences from control.

DISCUSSION

The results presented here have provided evidence strongly indicating that vitamin D, in the form of $1,25(OH)_2D_3$, is biologically active in the developing chick embryo. Based on the $^{45}Ca^{2+}$ tracer studies on the shell-less chick embryo, we conclude that the primary target for the vitamin D action is the yolk calcium reserve. The CAM, which is responsible for mobilizing eggshell calcium, does not appear to be functionally influenced by the level of vitamin D. On the other hand, the calcium uptake activity of the CAM is dependent on vitamin K.

During chick embryonic development, the maternally derived vitamin D stored in the yolk is gradually mobilized and is hydroxylated to various active metabolites, including $1,25(OH)_2D_3$ (Moriuchi & DeLuca, 1974; Bishop & Norman, 1975; Kubota, Abe, Shink & Suda, 1981; Abbas, Fox & Care, 1985). Since the intestinal mucosa, the primary target tissue for vitamin D action (DeLuca & Schnoes, 1983), is presumably quiescent with respect to nutrient uptake during embryonic life owing to the absence of food ingestion, the exact functional involvement and necessity of vitamin D in embryonic calcium homeostasis have been subjects of speculation (Kubota *et al.* 1981; Hart & DeLuca, 1984, 1985; Abbas *et al.* 1985). Narbaitz & Tolnai (1978) were the first to show that chick embryos are indeed responsive to the hypercalcaemic action of vitamin D. Since these investigators administered the vitamin on incubation day 15–16, when the chick embryo was utilizing the eggshell as the principal calcium supply, this observation would suggest that perhaps eggshell calcium mobilization is enhanced by vitamin D. However, Narbaitz (1979) later observed that shell-less embryos also became hypercalcaemic when injected with $1,25(OH)_2D_3$, a finding which is confirmed in this paper. The similarity in the vitamin D effects in both normal and shell-less embryo (which has no eggshell calcium to mobilize) is strongly suggestive that vitamin D exerts its action on other embryonic calcium transporting tissues/cells, instead of the CAM as suggested by several workers (Kubota *et al.* 1981; Hart & DeLuca, 1984, 1985). The $^{45}Ca^{2+}$ tracer studies presented here have conclusively demonstrated that this is indeed the case. Thus, hypervitaminosis D would result in increased serum calcium (derived from the yolk) which may then be incorporated into the mineralized matrix of the growing bone.

We have also directly studied the effect of vitamin D, in the form of $1,25(OH)_2D_3$, on the calcium transport function of the CAM. No apparent vitamin D stimulation of CAM calcium uptake activity is observed. Crooks, Kyriakides & Simkiss (1976) also previously reported that administration of 600 pmoles of 1-hydroxy-vitamin D_3 to day 15 chick embryos did not enhance CAM calcium uptake activity as assayed *in vivo*. On the other hand, our data show that when embryos are rendered severely deficient in vitamin K, CAM calcium uptake activity is significantly decreased, an effect which may be blocked by the administration of exogenous vitamin K. It thus appears that the CAM calcium transport function is indeed dependent on vitamin K but unlikely to be under

vitamin D regulation. These findings are thus consistent with our previous observations which showed that the CaBP, a functional component of the CAM calcium transport mechanism (Tuan *et al.* 1986*b*), is a vitamin K-dependent protein (Tuan *et al.* 1978*b*; Tuan, 1979, 1980*a*,*b*) whereas vitamin D has no effect on its expression (Tuan *et al.* 1978*b*).

The functional involvement of vitamin D in embryonic calcium homeostasis and its action on the mobilization of yolk calcium reserve agree well with the overall developmental profiles of the contents of the calcium compartments in the egg (see Introduction). Taking into consideration all the available information, we propose the following temporal scheme to model the regulation and the processes of calcium accumulation into the embryonic circulation during chick development.

(A) Phase 1 (approximately incubation days 7 to 10). Calcium is mobilized exclusively from the yolk, the calcium content of which shows a gradual decrease during this time period (Romanoff, 1967; Crooks & Simkiss, 1974). The tissue responsible for calcium uptake is presumably the yolk sac epithelium. There is at present no experimental information on how the calcium translocation process is regulated, although vitamin D is a likely regulatory factor.

(B) Phase 2 (approximately incubation days 10 to 14). The eggshell calcium reserve begins to be mobilized by the CAM. The CAM calcium transport function is dependent on vitamin K, presumably derived from the yolk (Bolton, 1961). The transport pathway involves at least the CaBP, the Ca^{2+}-ATPase and carbonic anhydrase (Tuan *et al.* 1986*b*). The influx of calcium appears to exceed that actually accumulated in the embryo and some of the mineral is deposited in the yolk, whose calcium content begins to rise during this period.

(C) Phase 3 (incubation days 14 to 20). Active CAM calcium transport continues. A significant portion of the calcium mobilized from the shell to the circulation does not appear to be accumulated directly into the embryo but is instead stored at least temporarily in the yolk compartment and then remobilized by the yolk sac. During this period, the yolk sac calcium transport mechanism is under vitamin D regulation.

(D) Phase 4 (day 20 to hatching). The CAM becomes necrotic and its calcium transport function ceases. The embryo begins to resorb the yolk and, by the time of hatching, the entire yolk is retracted, thus providing nutrients for the hatchling during its first few days of postembryonic life. At this point, the vitamin D-dependent calcium transport system of the intestine is also initiated (Moriuchi & DeLuca, 1974).

There are obvious, outstanding questions in this proposed scheme that remain to be resolved, particularly concerning the cellular mechanism and temporal regulation of the calcium-mobilizing functions of the yolk sac (both into and out of the yolk). We believe our findings have provided a direction for further experimental characterization of these functions.

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