Vitamin D metabolism and its possible role in the developing chick embryo

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The relationship between bone formation and vitamin D metabolism was investigated in the developing chick embryo. Fertilized White Leghorn eggs were incubated at 38°C in an incubator for 21 days. The fresh weight and calcium content of embryonic tibiae began to increase at day 12 and attained maximal values at day 19. Bone alkaline phosphatase and citrate decarboxylation activities, both of which represent osteoblastic activity, also began to increase at days 10–12, reached maximal values at day 19 and sharply declined thereafter. Both bone enzyme activities were highly correlated with Ca^{2+} -binding activity in the chorioallantoic membrane measured by the Chelex 100 assay. When mesonephric and metanephric homogenates were incubated with 25-hydroxy[^3H]cholecalciferol, a marked and concomitant increase occurred in the metanephric 1α - and 24-hydroxylase activity after day 14. The production of $1\alpha,25$ -dihydroxycholecalciferol attained a maximal value at day 19 and decreased thereafter, whereas that of $24,25$ -dihydroxycholecalciferol continued to increase until hatching. The production rate of $1\alpha,25$ -dihydroxycholecalciferol by the metanephros coincided with the changes in Ca^{2+} -binding activity in the chorioallantoic membrane and osteoblastic activity. Since both intestinal calcium absorption and bone mineral mobilization do not occur in embryonic life, these results support the idea that $1\alpha,25$ -dihydroxycholecalciferol may be involved directly in bone formation or induction of a calcium-binding protein in the chorioallantoic membrane.

It is now well established that cholecalciferol is metabolized first in the liver, then in the kidney to $24,25(\text{OH})_2\text{D}_3$ or $1\alpha,25(\text{OH})_2\text{D}_3$ (Kodicek, 1974; DeLuca & Schnoes, 1976). The latter metabolite is known to be the active form of cholecalciferol in enhancing intestinal calcium transport (Holick *et al.*, 1971; Lawson *et al.*, 1971) and bone mineral mobilization (Tanaka & DeLuca, 1971; Raisz *et al.*, 1972). In addition, cholecalciferol has been thought to act directly on bone to facilitate mineralization. However, which metabolite(s) is the most responsible for bone mineralization is still controversial (Tanaka *et al.*, 1973; Wezeman, 1976; Ornoy *et al.*, 1978).

Avian embryos assimilate large amounts of calcium in their bones in a short time. The chicken embryo, for instance, accumulates over 100 mg of calcium from the egg shell across the chorio-

allantoic membrane from days 10–12 of embryonic life until hatching at day 21 (Simkiss, 1967; Tuan & Scott, 1977). It has been reported that chicken embryo kidneys are capable of producing $1\alpha,25(\text{OH})_2\text{D}_3$ as early as day 9 of embryonic life (Bishop & Norman, 1975). Neither intestinal calcium transport nor bone mineral mobilization, however, appear to occur in the chick embryo until hatching. Thus the avian embryo offers advantages in studying the relationship between vitamin D metabolism and bone formation.

The present paper describes the sequence of changes in the bone calcium content, osteoblastic activity of embryonic bone, Ca^{2+} -binding activity of the chorioallantoic membrane and renal 1α - and 24-hydroxylase activity of $25(\text{OH})\text{D}_3$ during the development of the chick embryo.

Materials and methods

Animals

Fertilized White Leghorn eggs were obtained from

Abbreviations used: $25(\text{OH})\text{D}_3$, 25-hydroxycholecalciferol; $1\alpha,25(\text{OH})_2\text{D}_3$, $1\alpha,25$ -dihydroxycholecalciferol; $24,25(\text{OH})_2\text{D}_3$, $24,25$ -dihydroxycholecalciferol.

a local distributor and incubated in a humidified egg incubator at 38°C. At selected days of incubation, the embryos were removed and weighed. The embryonic kidneys and tibiae were removed for following assays. The chorioallantoic membrane was also dissected from the egg shell and used to measure Ca^{2+} -binding activity.

Determination of the calcium content of the bone

The tibiae removed from the embryos of selected ages were cut with scissors into several pieces. The bone fragments were homogenized in 1 ml of 0.1 M-acetic acid, with a glass homogenizer to pulverize the bone completely. After standing for several days at 4°C, the homogenates were centrifuged at 3000 rev./min for 10 min. The calcium content of the extracts was measured by the colorimetric method using *o*-cresolphthalein complex formation (Connerty & Briggs, 1966).

Measurement of alkaline and acid phosphatase activity of the bone

The minced tibiae (approx. 200 mg) were homogenized in 2 ml of 0.25 M-sucrose solution containing 0.3 M-KCl and 0.1% (v/v) Triton. The homogenates were centrifuged at 3000 rev./min at 4°C for 10 min. The supernatant was used for the assay of alkaline and acid phosphatase activity by the method of Kind & King (1954) with phenyl phosphate as substrate at pH 10.15 and 4.90 respectively. The enzyme activities were expressed as μg of phenol released/min per 100 μg of DNA. The DNA content was measured by the method of Burton (1956).

Measurement of citrate decarboxylation activity of the bone

Bone citrate decarboxylation activity was measured by a modified method of Wolinsky & Cohn (1969). The tibiae were cut into several pieces and transferred to a glass tube containing 0.4 ml of a Ca^{2+} - and Mg^{2+} -free phosphate-buffered solution with 4 mM-EDTA and 0.1 μCi of $[6\text{-}^{14}\text{C}]\text{citric acid/ml}$ (sp. radioactivity 4.5 Ci/mol; New England Nuclear) as substrate. The glass tube was connected by a 4 cm-long rubber tube to a counting vial to which had been affixed on the inside surface a Whatman 3MM filter paper (3 cm $\times$ 8 cm) soaked in phenethylamine. The contents of the tube were incubated for 45 min at 37°C. The incubation medium was then acidified by injecting 0.5 ml of 20% trichloroacetic acid through the connecting rubber tube. Incubation continued at 37°C for 1 h to allow the filter paper to absorb evolved $^{14}\text{CO}_2$. The counting vial was then removed and filled with 10 ml of a Triton scintillation solution containing 12 g of 2,5-diphenyloxazole and 0.6 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of Triton X-100 and 2

litres of toluene. The radioactivity was counted in a Packard liquid-scintillation counter (model 3255). The citrate decarboxylation activity was expressed as c.p.m. of $^{14}\text{CO}_2$ released/45 min per 100 μg of DNA of the bone.

Measurement of renal 25(OH) D_3 1 α - and 24-hydroxylase activity in vitro

The mesonephros and metanephros were separately removed under a dissecting microscope and collected from eight to 20 chicken embryos at selected ages. A 20% homogenate was prepared in 0.2 M-sucrose solution containing 15 mM-Tris/HCl (pH 7.4), 2 mM-MgCl₂ and 5 mM-sodium succinate. Of the homogenate 1.5 ml portions were incubated with 1.8 nmol of (0.1 μCi) of 25(OH)[26,27- ^3H] D_3 (The Radiochemical Centre, Amersham, Bucks., U.K.) for 40 min at 37°C under 100% O_2 in a 20 ml Erlenmeyer flask. The reaction was terminated by the addition of 9 ml of methanol/chloroform (2:1, v/v). Extraction was performed by the method of Bligh & Dyer (1959). The extracts were chromatographed on Sephadex LH-20 columns (1 cm $\times$ 30 cm) equilibrated and eluted with 35% hexane in chloroform (v/v) by the method of Holick & DeLuca (1971). Fractions (3 ml) were collected. Each radioactive peak on the Sephadex column was then applied with authentic 1 α ,25(OH) $_2\text{D}_3$ or 24,25(OH) $_2\text{D}_3$ to a high-pressure liquid chromatograph (Waters model 204) equipped with a Zorbax-Sil column (4.6 mm $\times$ 15 cm; Du Pont). Dr. M. R. Uskoković (Hoffman-LaRoche Inc., Nutley, NJ, U.S.A.) kindly donated the reference compounds. The column was eluted with 9% propan-2-ol in hexane (Jones & DeLuca, 1975) or 2% methanol in dichloromethane (Ikekawa & Koizumi, 1976). The radioactivity was counted in a toluene scintillation solution containing 2 g of 2,5-diphenyloxazole in 1 litre of toluene. The production rate of the metabolites of 25(OH)[^3H] D_3 was expressed as percentages of 1 α ,25(OH) $_2$ [^3H] D_3 or 24,25(OH) $_2$ [^3H] D_3 of the total radioactivity recovered per embryo.

Measurement of Ca^{2+} -binding activity of the chorioallantoic membrane

The dissected chorioallantoic membranes were rinsed and homogenized in 4 vol. of the TAD buffer containing 13.7 mM-Tris/HCl (pH 7.4), 0.12 M-NaCl, 4.74 mM-KCl, 98.5 μM -glucose, 0.71 mM-dithiothreitol and 0.02% NaN_3 with a loose-fitting Teflon homogenizer. The homogenates were centrifuged at 31 000 g for 20 min and the supernatant was stored at -20°C until they were assayed. Ca^{2+} -binding activity was measured by the Chelex 100 ion-exchange method as described by Wasserman *et al.* (1968). The activity was calculated as radioactivity in the supernatant/radioactivity retained by Chelex

100 resin (100–200 mesh, Na^+ form; Bio-Rad Laboratories, Richmond, CA, U.S.A.), and expressed as percentages per mg of protein. The protein content was measured by the method of Lowry *et al.* (1951).

Results

Fig. 1 shows the changes in embryo weights and the weights and calcium content of tibiae during incubation. The embryos developed and increased their weights linearly from day 12 until hatching at day 21, whereas both the weights and calcium content of the bone began to increase at day 12 and almost reached maximal values at day 19 (2 days before hatching).

The changes in the citrate decarboxylation activity and in alkaline and acid phosphatase activities of tibiae during embryonic development are shown in Fig. 2. The bone citrate decarboxylation and alkaline phosphatase activities are thought to represent osteoblastic activity (Luben *et al.*, 1976).

Both bone enzyme activities began to increase at days 10–12 and attained maximal values on or about day 19. The maximal value of the bone citrate decarboxylation activity showed a greater than 30-fold increase over the basal value at days 6–8. The maximal value of the bone alkaline phosphatase activity was also more than 200 times higher than the basal value at day 6. Both bone enzyme activities, however, sharply declined thereafter. The bone acid phosphatase activity, on the other hand, was very low and did not change appreciably during embryonic development.

Most of the avian embryonic bone calcium is known to be derived from the egg shell across the chorioallantoic membrane, which contains a calcium-binding protein (Tuan & Scott, 1977). Fig. 3 shows a relationship between the calcium content of tibiae and the Ca^{2+} -binding activity of the chorioallantoic membrane. The Ca^{2+} -binding activity of the chorioallantoic membrane began to increase at day 12 synchronously with the onset of calcium deposition into the bone. It attained a maximal value at day 19 when the bone calcium content almost reached a maximum. The maximal value of Ca^{2+} -binding activity of the chorioallantoic membrane was about 10 times higher than the basal value at

Fig. 1. Changes in embryo weights, and the weights and calcium content of tibiae during incubation

Incubation was carried out at 38°C in a humidified incubator. Embryo weights (○), and the weights (●) and calcium content (■) of tibiae were measured from day 6 until day 21 of incubation. The values are means $\pm$ S.E.M.

Fig. 2. Changes in the enzyme activities in bone during incubation

Alkaline phosphatase (○) and acid phosphatase (■) activities in bone were measured by the method of Kind & King (1954) and expressed as μg of phenol released/min per $100\mu\text{g}$ of DNA of the bone. The bone citrate decarboxylation activity (●) was measured as described in the Materials and methods section and expressed as c.p.m. of $^{14}\text{CO}_2$ released/45 min per $100\mu\text{g}$ of DNA of the bone. The values are means $\pm$ S.E.M., except those obtained from bones at earlier incubations. The bones before day 10 were pooled at each day and analysed together.

Fig. 3. Changes in Ca^{2+} -binding activity in the chorioallantoic membrane and the calcium content of tibiae during incubation

Ca^{2+} -binding activity (●) in the chorioallantoic membrane was measured by the Chelex 100 ion-exchange method. Ca^{2+} -binding activity was calculated as radioactivity in the supernatant/radioactivity retained by Chelex 100 resin, and expressed as a percentage per mg of protein. The calcium content of tibiae (■) shown in Fig. 1 has been redrawn. The values are means $\pm$ S.E.M.

days 6–8. The Ca^{2+} -binding activity of the chorioallantoic membrane sharply decreased thereafter. These results are consistent with those of Tuan & Scott (1977).

It has been reported that the yolk contains about $2\mu\text{g}$ of cholecalciferol (Fraser & Emtage, 1976) and that chick embryos are capable of metabolizing cholecalciferol to $25(\text{OH})\text{D}_3$, $1\alpha,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ (Moriuchi & DeLuca, 1974; Bishop & Norman, 1975). Three kinds of kidney tissue are formed in the chicken embryo: pronephros (non-functional), mesonephros (intermediate kidney) and metanephros (definitive kidney). The mesonephric tissue begins excretory function at day 6, reaches its maximal function at day 10 and gradually disappears thereafter. The metanephric tissue appears at day 10 and functions as the definitive kidney thereafter (Romanoff, 1960). The metabolism of $25(\text{OH})[^3\text{H}]\text{D}_3$ *in vitro* by the mesonephros and metanephros was investigated from day 6 of incubation until hatching. Fig. 4 demonstrates Sephadex LH-20 column-chromatographic profiles of extracts of the mesonephros from embryos at day 8 and the metanephros at day 19. Both the mesonephros and metanephros metabolized $25(\text{OH})[^3\text{H}]\text{D}_3$ to at least four radioactive peaks. In both the mesonephros and metanephros the major

Fig. 4. Sephadex LH-20 chromatographic profiles of extracts of the mesonephros from embryos at day 8 (a) and the metanephros from those at day 19 (b)

The homogenates of the mesonephros and metanephros were incubated for 40 min with $0.1\mu\text{Ci}$ of $25(\text{OH})[^3\text{H}]\text{D}_3$. The columns were eluted with chloroform/n-hexane (65:35, v/v), and 3 ml fractions were collected.

radioactive metabolites appeared in fractions 18–25 (peak A) and 32–42 (peak B).

When the radioactive peaks on Sephadex columns (fractions 18–25 and 32–42) from either the mesonephros or metanephros were individually applied to high-pressure-liquid-chromatography columns, most of the radioactivity of peak A co-migrated to exactly the same position as authentic $24,25(\text{OH})_2\text{D}_3$ and that of peak B to the same position as authentic $1\alpha,25(\text{OH})_2\text{D}_3$ (Fig. 5).

Fig. 6 shows the variations in the production of $1\alpha,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ by the mesonephros and metanephros. The mesonephric tissue produced $1\alpha,25(\text{OH})_2\text{D}_3$ as early as day 6 of incubation, reaching a maximum at day 8. In contrast, the production of $24,25(\text{OH})_2\text{D}_3$ by the mesonephros was not significant throughout the functional period of the mesonephros (Fig. 6a). The

Fig. 5. The high-pressure-liquid-chromatography profiles of peaks A and B collected from the Sephadex LH-20 columns shown in Fig. 4

The profiles of peak A are shown in (a) (mesonephros) and (c) (metanephros) and those of peak B are presented in (b) (mesonephros) and (d) (metanephros). Before applying to high-pressure-liquid-chromatography columns, 500 pmol of authentic $24R,25(OH)_2D_3$ (a and c) or $1\alpha,25(OH)_2D_3$ (b and d) were added to each sample. The columns were eluted with 9% propan-2-ol in n-hexane (a and c) or 2% methanol in dichloromethane (b and d). The solid line indicates A_{254} , and the bars represent radioactivity in the 1 min-fractions.

Fig. 6. Changes in the production of $1\alpha,25(OH)_2D_3$ and $24,25(OH)_2D_3$ by the mesonephros and metanephros. The production of $1\alpha,25(OH)_2D_3$ (○) and $24,25(OH)_2D_3$ (●) was expressed as percentages of the total radioactivity recovered per embryo. The values are means $\pm$ S.E.M.

metanephros, on the other hand, metabolized $25(OH)[^3H]D_3$ to both $1\alpha,25(OH)_2[^3H]D_3$ and $24,25(OH)_2[^3H]D_3$. The production of $1\alpha,25(OH)_2D_3$ by the metanephros began to increase at day 16, reached a maximum at day 19 and declined thereafter. Synthesis of $24,25(OH)_2D_3$ by the metanephros showed a marked increase during the last 6 days before hatching. At day 21 the production rate of $24,25(OH)_2D_3$ by the metanephros was about six times higher than that of $1\alpha,25(OH)_2D_3$ (Fig. 6b).

Discussion

The avian embryo utilizes calcium contained in the egg shell and yolk for bone mineralization. The yolk has about 20 mg of calcium, which is not enough to mineralize the embryonic skeletal system, which contains 120–130 mg of calcium at hatching (Simkiss, 1967). Therefore, the greater part of calcium has to be supplied from the egg shell.

Calcium is transported from the egg shell to embryo by the chorioallantoic membrane through its circulatory system. The chorioallantoic membrane lines the interior of the egg shell and beings to transport calcium at days 10–12 of incubation. The

onset of calcium transport by the chorioallantoic membrane is coincident with the appearance of a calcium-binding protein (Tuan & Scott, 1977). The Ca^{2+} -binding activity assayed by Chelex 100 also began to increase at day 12, attained a maximal value at day 19 and decreased thereafter (Fig. 3). This coincides with the calcium accumulation in the bone. In addition to calcium, the embryo takes up O_2 by simple diffusion across the chorioallantoic membrane through microscopic pores in the egg shell. On day 19, the chicken embryo penetrates the air sac with its beak, a process called internal pipping. The embryo then begins to breathe air from the sac. About 6 h later the chick breaks through the egg shell with the egg tooth at the tip of its upper beak and starts to breathe atmospheric air (Rahn *et al.*, 1979). Concomitantly chorioallantoic function begins to wane. The reason that embryonic bone formation has to be completed by day 19, therefore, appears to be closely related to chorioallantoic function. Also the sharp decrease after day 19 of the bone citrate decarboxylation and alkaline phosphatase activities, which reflect osteoblastic activity, indicates the completion of the embryonic bone formation by day 19.

It is well known that $1\alpha,25(\text{OH})_2\text{D}_3$ stimulates intestinal calcium transport and bone mineral mobilization activity. However, intestinal Ca^{2+} -binding activity does not appear until 1 day after hatching and it is believed that intestinal absorption of calcium does not occur in embryonic life (Moriuchi & DeLuca, 1974). Bone resorption also does not seem to occur, since bone acid phosphatase activity is very low throughout embryonic life. Irrespective of the absence of both vitamin D-dependent activities in embryonic life, renal $25(\text{OH})\text{D}_3$ 1α -hydroxylase activity was significantly enhanced at day 8 in the mesonephros and at day 19 in the metanephros. The reason why renal 1α -hydroxylase activity is enhanced in embryonic life is still obscure. Two possibilities may be considered. First, the metanephric 1α -hydroxylase activity was changed in parallel with bone citrate decarboxylation and alkaline phosphatase activity. Thus, $1\alpha,25(\text{OH})_2\text{D}_3$ may be important directly for bone formation. Secondly, $1\alpha,25(\text{OH})_2\text{D}_3$ may be involved in the induction of calcium-binding protein in the chorioallantoic membrane. Tuan *et al.* (1978) and Tuan (1979) reported that the developmental expression of calcium-binding protein in the chorioallantoic membrane is vitamin K-dependent and that calcium-binding protein remains immunologically detectable in the chorioallantoic membrane of warfarin-treated embryos. These results suggest that vitamin K is involved in the synthesis of calcium-binding protein in a post-translational state. $1\alpha,25(\text{OH})_2\text{D}_3$, if it functions in a fashion similar to the induction of intestinal calcium-binding protein,

may be involved in the synthesis of chorioallantoic calcium-binding protein in a pre-translational state.

Moriuchi & DeLuca (1974) reported that chick embryos are capable of metabolizing cholecalciferol to $25(\text{OH})\text{D}_3$, $1\alpha,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ *in vivo*. It has also been reported that the renal $25(\text{OH})\text{D}_3$ 1α - and 24-hydroxylase activities measured *in vitro* are in accord with the production rates of $1\alpha,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ *in vivo* estimated from the appearance of radioactive metabolites in plasma after administration of $25(\text{OH})[^3\text{H}]\text{D}_3$ (Pike *et al.*, 1978; Abe *et al.*, 1979). To further confirm this point, we attempted to measure metabolites of cholecalciferol in plasma collected from fifty 19-day-old chicken embryos by competitive protein-binding assays (Eisman *et al.*, 1976; Kano *et al.*, 1979). Preliminary results showed that the plasma concentrations of cholecalciferol metabolites in the 19-day-old chicken embryo were 2.3 ng/ml in 25-hydroxy vitamin D, 0.6 ng/ml in 24,25-dihydroxy vitamin D and 300 pg/ml in $1\alpha,25$ -dihydroxy vitamin D (M. Kubota, E. Abe, T. Shinki & T. Suda, unpublished work). These results clearly demonstrate that the 1α - and 24-hydroxylase enzyme activities are functionally active in the developing chick embryo.

Our results on the renal metabolism of $25(\text{OH})\text{D}_3$ in embryonic life are inconsistent in part with those of Bishop & Norman (1975). They did not find any detectable stimulation of $24,25(\text{OH})_2\text{D}_3$ production. In our hands, $25(\text{OH})\text{D}_3$ 24-hydroxylase activity in the metanephros continued to increase until hatching. At day 21, the production rate of $24,25(\text{OH})_2\text{D}_3$ by the metanephros was about six times higher than that of $1\alpha,25(\text{OH})_2\text{D}_3$. Sunde *et al.* (1978) suggested that metabolite(s) of cholecalciferol other than $1\alpha,25(\text{OH})_2\text{D}_3$ are also necessary for normal chick embryo development. Henry & Norman (1978) reported the interesting result that $24,25(\text{OH})_2\text{D}_3$ is essential, along with $1\alpha,25(\text{OH})_2\text{D}_3$, for chicken egg hatchability. Thus, it is interesting to consider that the marked increase of the $24,25(\text{OH})_2\text{D}_3$ production for the last 6 days before hatching may be related to avian hatchability. Whether the metabolites of cholecalciferol are directly involved in bone formation or calcium-binding protein synthesis in the chorioallantoic membrane requires elucidation by further experiments *in ovo* and *in vitro*.

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