Effect of Vitamin D Deficiency on Bone Formation in the Chick

By Ian R. DICKSON and Egon KODICEK

Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K.

(Received 12 December 1978)

1. The process of diaphyseal bone formation can be investigated by studying the rate of incorporation of radioactive precursors, administered *in vivo* into bone fractions of increasing density. 2. In the 4-week-old vitamin D-treated chick most of the osteoid becomes calcified within 12h and almost all within 2 days. The low-density calcified phase that is formed is converted into a higher density form and within 7 days the greater proportion of the calcified tissue is in the higher density form. 3. In the vitamin D-deficient chick of similar age the rate of calcification of osteoid is decreased, as is the rate of conversion into the higher density phase with the resultant accumulation of the lower density calcified form. 4. The higher density phase probably corresponds to hydroxyapatite and the lower density one to the ACP-phase described by Termine & Posner [(1967) *Calcif. Tissue Res.* 1, 8–23]. 5. The disorder in the process of calcification seems to be unrelated to the alteration in blood Ca^{2+} and phosphate concentrations, but related to the presence or absence of cholecalciferol.

Vitamin D deficiency or disorders of vitamin D metabolism result in a disorder of bone formation. Quantitative histological studies of the rachitic rat show decreased rate of initial mineralization and a decreased rate of maturation of osteoid (Baylink *et al.*, 1970). Although studies with the light and electron microscope can provide much valuable information about bone formation, there is a need to supplement this with information about the biochemistry and physiology of the various stages of the process. However, any biochemical investigation is complicated by the fact that a small area of tissue can contain bone at different stages of formation and newly forming bone may be adjacent to bone undergoing resorption. Attempts have been made to dissect individual osteons, but a more widely used technique has been density flotation of finely powdered bone. If bone tissue is decreased to a particle size smaller than that of osteons, the basic architectural units of bone, then individual particles are likely to be representative of tissue at one stage of calcification and their density will be related to the degree of calcification. Thus bone can be separated into fractions at different stages of maturation with the aid of a density gradient. This principle was first applied to the study of bone by Herman & Richelle (1961) and has subsequently been used in various forms to look at bone and dentine by other groups (Shapiro & Hartles, 1964; Coklica & Brudevold, 1966; Quinaux & Richelle, 1967; Fincham, 1969; Lapiere & Nussgens, 1970; Nussgens *et al.*, 1972; Russell & Avioli, 1972).

Most new bone formation in the skeletally immature animal occurs in the epiphyses during endo-

chondral ossification. However, it is not convenient to study osteoid calcification in this area by density flotation because of the presence of calcified and uncalcified cartilage. Within the diaphysis of long bones, bone formation also occurs as a consequence of remodelling and of widening of the shaft during growth. This does not involve a cartilage intermediary, as in endochondral ossification, so the only tissue present is osteoid at various stages of calcification.

In the present paper we describe the application of a density-flotation technique to the study of diaphyseal bone formation in chickens and show how this process is influenced by vitamin D deficiency.

Materials and Methods

Animals

One-day-old cockerels (Ranger cockerels; Ross Poultry, Woodhall Spa, Lincs., U.K.) were raised on a rachitogenic diet in a room lit by tungsten lights. The diet mix described by Barnes *et al.* (1973) was used; retinyl acetate, tocopheryl acetate and 2-methyl-1, 4-naphthoquinone were dissolved in arachis oil and incorporated into the diet mix, which was made up in small batches and kept refrigerated until used. A high- Ca^{2+} diet was prepared similarly with the addition of an extra 25g of CaCO_3 in each kg of diet mix.

Vitamin D-treated chicks were maintained on the same diet and were given cholecalciferol ($5\mu\text{g}$ in 0.5ml of arachis oil) perorally each week.

Materials

Cholecalciferol and other vitamins were obtained

from Sigma. L-[G- ^3H]Proline and $^{45}\text{CaCl}_2$ were obtained from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. Chemicals were of analytical grade or the best grade commercially available.

Analytical methods

Ca^{2+} was estimated by using an atomic-absorption spectrometer, P, by a colorimetric method (Chen *et al.*, 1956) and alkaline phosphatase with a colorimetric reagent kit (Sigma). For hydroxyproline, samples were hydrolysed with 5.8M-HCl for 24h at 105°C, the acid was removed by evaporation and the hydrolysate was analysed (Woessner, 1961). Radioactivity counting was performed on a Packard Tri-Carb liquid-scintillation counter with a scintillation fluid containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene in solvent [70% (v/v) A.R. grade toluene and 30% (v/v) 2-ethoxyethanol]. Quench-correction curves were used to convert ^3H and ^{14}C counts into absolute values.

Preparation of diaphyseal bone

Diaphyseal bone from femurs and tibiae was scraped clean of adhering periosteum muscle and ligaments, split longitudinally and marrow removed by brushing and scraping in cold 0.15M-NaCl. The bone was washed in absolute ethanol and then air-dried. By using a stainless steel punch and die the bone was broken into small pieces that were pulverized in an impact mill (Spex Industries, Metuchen, NJ, U.S.A) cooled with liquid N_2 , to a particle size less than 45 μm . A 45 μm sieve was used to separate fine particles.

Fractionation of bone powder

1,2-Dibromoethane (sp.gr. 2.17 at 20°C) and chloroform (sp.gr. 1.48 at 20°C) were combined in various proportions to give a series of mixtures of sp. gr. approx. 1.47, 1.90, 2.00, 2.05, 2.10, 2.15 and 2.18. The exact values for each mixture were measured accurately with the aid of specific-gravity bottles.

Finely powdered bone (500–1000mg) was suspended in 5ml of chloroform in a borosilicate glass tube (10cm $\times$ 1cm) fitted with a Teflon-lined screw cap. The tube was centrifuged at 100g (MSE Mistral 4L; 2500rev./min) for 30min at 20°C. The temperature of the contents of the tube was checked at the end of the run. The liquid layer containing some suspended matter was withdrawn with a pipette, mixed with ethanol to decrease the density of the solution so that the suspended matter would sediment on centrifugation (fraction 1). The residual bone powder was treated in a similar manner with a further 5ml of chloroform and any slight trace of suspended matter remaining after centrifugation was pooled with fraction 1. The bone powder was suspended in 5ml of solvent mixture of sp.gr. 1.906 and the procedure was repeated, the suspended material in this case being

termed fraction 2. The procedure was repeated with mixtures of increasing specific gravity, the material sedimenting in pure 1,2-dibromoethane being denoted fraction 8. Fractions were washed with ethanol, air-dried and weighed.

Radioactivity studies

For studies of the incorporation of radioactive precursors, animals were injected intracardially with 100 μCi of [^3H]proline or $^{45}\text{CaCl}_2$ in 0.2ml of 0.15M-NaCl and then killed at designated times afterwards. The diaphyseal bone was cleaned, pulverized and fractionated as described above. Portions (approx. 5mg) of each fraction were weighed in duplicate, hydrolysed with 5.8M-HCl as previously described and the dry hydrolysate redissolved in 0.2ml of water, mixed with 10ml of scintillation fluid and the radioactivity was measured.

Results and Discussion

Fractionation procedure

The procedures we have used to fractionate bone powder are similar in principle to those used by other groups. Rather than the bromoform used by other workers we have used 1,2-dibromoethane, since this is more stable, economical and relatively little bone has a density higher than its specific gravity of 2.18; we have used this solvent in mixtures with chloroform rather than toluene used by other workers. We have also preferred to fractionate by using a series of solutions of increasing specific gravity rather than within a density gradient in a single tube, since the former method, though more laborious, permits a larger sample to be fractionated and with greater reliability.

Since bone is a metabolically active tissue with areas of formation and resorption in close proximity it is important to ensure that individual particles are likely to be homogeneous. The diameter of an osteon, the basic architectural unit of bone, has been estimated as 200 μm (Herman & Richelle, 1961). Thus material passing through a 45 μm sieve should only represent a small part of one osteon. In preliminary investigations with bovine cortical bone we measured bone particle sizes with the aid of a light microscope and found the average to be around 5 μm ; there was a greater proportion of small particles (less than 2 μm) in the highest density fraction than in the lowest density fraction, a reflection of the brittle nature of the more highly mineralized tissue. We observed also that the density-distribution profile of particles below 45 μm approximated to that of a 45–355 μm sieved fraction, supporting previous observations (Nusgens *et al.*, 1972) that the fractionation method is not critically dependent on the use of very small particles.

Fig. 1 shows comparative density profiles for foetal calf jaw (membranous) bone and mature bovine diaphyseal cortical bone. Reproducibility of the fraction-

ation procedure was $\pm 5\%$ for most fractions; there was greater variation in fractions comprising only a small proportion by weight of the bone. The differences between foetal and adult bovine bone (three and seven fractionations respectively) were significant for every

Fig. 1. Distribution of bovine and chick bone powder between fractions of various densities
In each case bone from a number of animals was pooled, powdered and separated into fractions of increasing density as described in the text. Typical fractionation profiles are shown and results are expressed as a percentage of the total weight of bone fractionated. The density range of each fraction is given in Table 1. F=foetal calf bone; A=adult bovine bone; R=5-week-old rachitic chick bone; N=5-week-old vitamin D-treated chick bone.

fraction (Student's *t* test; $P<0.001$ for fractions 1–4, 6 and 7; $P<0.01$ for fraction 5). More than 95% by weight of the foetal bone has a density less than 2.05, most of it being present in the third fraction density range of 2.00–2.05. In comparison more than 95% of the mature bone is of density greater than 2.05. There is not such a marked distinction between profiles of pooled diaphyseal bone from vitamin D-deficient and vitamin D-replete chicks at 5 weeks of age. This is partly a reflection of the fact that the vitamin D-replete chicks are far from mature, in comparison with the profile from a 1-year-old vitamin D-replete chicken (result not shown), and secondly that the development of the lesions of vitamin D deficiency is gradual as the reserves of vitamin derived from the yolk become depleted. Analysis of profiles for individual chicks (five rachitic and ten vitamin D-treated) aged 5 weeks showed significant differences between all fractions except fraction 3 (Student's *t* test; for fractions 1, 2 and 4, $P<0.001$; for fraction 5, $P<0.01$). These studies show that in vitamin D deficiency there is an accumulation of a low-density form of bone and that the changes have a superficial similarity to those between immature and mature bone tissue.

The composition of fractions of increasing density (Table 1) shows a progressive increase in the proportion by weight of Ca^{2+} , and of phosphorus (results not shown), and a corresponding decrease in the proportion of the organic matrix. There was no marked difference between the vitamin D-treated and vitamin D-deficient fractions.

Incorporation of [^3H]proline and $^{45}\text{Ca}^{2+}$

Information about the alterations in bone formation as a result of vitamin D deficiency can be obtained by studying the incorporation of $^{45}\text{Ca}^{2+}$ and [^3H]proline into diaphyseal bone. Since this is a reflection of the present state of the animal in the vitamin D-replete chick the Ca^{2+} activity 2 days after administration parenterally is in fractions 3 and 4 (see Fig. 2) and at the end of 7 days there has been a progression of activity towards higher density frac-

Table 1. Composition of chick bone fractions of various densities
The organic matrix consists of EDTA-insoluble and EDTA-soluble, but non-dialysable, material.

		Content (% by wt. of undecalcified bone powder)			
Fraction	Density	Cholecalciferol-treated		Cholecalciferol-deficient	
		Ca^{2+}	Organic matrix	Ca^{2+}	Organic matrix
1	<1.474	4.2	73.9	4.3	72.3
2	<1.906	16.9	40.1	14.7	48.9
3	<2.003	21.2	24.9	20.0	27.9
4	<2.055	21.9	21.9	21.1	24.0
5	<2.102	21.0	21.4	23.6	23.7
6	<2.152	23.2	22.0	29.0	18.6

Fig. 2. ⁴⁵Ca²⁺ Incorporation into chick diaphyseal bone. Chicks (4 weeks old) were given a single intracardial injection of ⁴⁵CaCl₂ and killed 2 or 7 days later. Diaphyseal bone was cleaned, powdered and fractionated as described in the text. Radioactivity in each fraction was determined and expressed as a percentage of the total amount of radioactivity in the bone sample. Each histogram shows the mean value for two chicks for each experiment, except for the vitamin D-treated 7 day experiment; the range of individual values is shown by the vertical bars. The density range of each fraction is given in Table 1.

tions. In the vitamin D-deficient chick most of the radioactivity at the end of 7 days remains in fractions 2 and 3. A small amount of the ⁴⁵Ca²⁺ activity present in fractions may result from exchange with Ca²⁺ in previously formed bone and this could explain the existence of activity in the high-density fractions of vitamin D-deficient bone, since hyperparathyroidism, secondary to the hypocalcaemia of vitamin D deficiency, will result in increased Ca²⁺ reabsorption and a relatively long half-life for ⁴⁵Ca²⁺ in plasma.

Studies of proline incorporation eliminate problems of interpretation owing to exchange and results are essentially similar to those obtained with ⁴⁵Ca²⁺. In the vitamin D-replete chick most of the activity 12h after administration is already in fraction 3 (Table 2). There is a progression of activity towards higher density fractions with time, but the most marked change occurs between 4 and 7 days. In the vitamin D-deficient chicks most of the radioactivity remains in fraction 2, but between 3 and 7 days, there is a marked transition into fraction 3. Alterations in the specific activity of [³H]proline in the fractions during these

Table 2. Incorporation of [³H]proline into the diaphyseal bone of chickens. Chicks aged 5 weeks were given a single intracardial dose of [³H]proline and killed at various time intervals afterwards. Powdered diaphyseal bone was fractionated and the amount of ³H radioactivity in each fraction was determined. Results are the amount of radioactivity in each fraction expressed as a percentage of total radioactivity in the bone sample. Because of the small amount of material in the lowest density fractions of the vitamin D-treated chick bone values for fractions 1 and 2 were combined. Values for individual chicks are shown with the mean values in parentheses. The density range of the fractions is given in Table 1.

Fraction	³ H radioactivity in fraction (% of total)									
	Vitamin D-treated					Vitamin D-deficient				
	Time of killing (days)	1	2	4	7	1	3	7		
1	32.6, 21.5 (27.1)	24.2, 19.8 (22.0)	11.7, 5.1 (8.4)	3.2, 1.6 (2.4)	4.2, 1.3 (2.8)	14.2, 15.8 (15.0)	11.2, 21.7 (16.5)	1.3		
2	—	—	—	—	—	60.5, 70.0 (65.3)	57.4, 62.2 (59.8)	23.5		
3	55.3, 65.4 (60.4)	64.5, 68.8 (66.7)	71.4, 67.0 (69.2)	82.8, 60.0 (71.4)	44.2, 31.4 (37.8)	21.9, 11.8 (16.9)	28.7, 10.1 (19.4)	62.9		
4	9.1, 10.9 (10.0)	9.5, 9.9 (9.7)	14.5, 23.2 (18.9)	34.4, 14.7 (24.6)	48.3, 59.4 (53.9)	2.7, 2.0 (2.4)	2.6, 4.1 (3.4)	11.4		
5-7	3.0, 2.2 (2.6)	1.9, 1.6 (1.8)	2.5, 4.7 (3.6)	2.4, 0.2 (1.3)	3.4, 8.0 (5.7)	0.8, 0.4 (0.6)	0.2, 1.9 (1.1)	0.9		

changes provides additional confirmation of the transitions.

These results suggest that there are two pools of calcified tissue: a low-density form is rapidly produced initially and in the normal chick this appears to be converted after a number of days into a higher density form. Electron-microscopic studies of rachitic and normal chick bone density fractions (S. Jande, unpublished work) show that the calcified tissue exists in two physically different states: the higher density fractions contain large amounts of hydroxyapatite-like crystals that are only present in relatively small amounts in the lower density calcified fractions. There is abundant evidence that bone exists in more than one mineralized phase (for a recent discussion and references see Smith & Smith, 1976). Whilst there is general agreement that the most abundant phase in the normal skeletally mature animal is of a hydroxyapatite type, there is dispute about whether the remaining material is amorphous or of a different crystalline form. The reported increase in the proportion of the 'amorphous calcium phosphate' or ACP-phase in the bone of rachitic chickens (Termine & Posner, 1967) is consistent with our findings of an increased proportion of the lower density form in chick bone under similar experimental conditions.

Effect of cholecalciferol on 'maturation' of bone

When vitamin D-deficient chicks are given a single intracardial dose of [^3H]proline, most of the ^3H radioactivity in bone after 5 days is present in the lower

density calcified fractions. If at this stage the chicks are given a single ($5\mu\text{g}$) dose of cholecalciferol and killed 48 h later much of the radioactivity can be found in the higher density fractions, whereas in the control chicks injected with vehicle alone the radioactivity is predominantly present in the lower density fractions (see Table 3). The effect of cholecalciferol administration appears to be the promoted conversion of calcified bone collagen from the low-density into the high-density form. This change is unlikely to be due to an alteration in the $[\text{Ca}^{2+}] \times [\text{phosphate}]$ product. Studies of the effect of cholecalciferol administered, under conditions similar to those in the present experiment, to a group of rachitic chicks showed that the $[\text{Ca}^{2+}] \times [\text{phosphate}]$ product had risen to about half of the normal value 24 h after giving the vitamin and by 48 h the value of the product had fallen, a temporary consequence of the return of $[\text{Ca}^{2+}]$ to near normality on secretion of Ca^{2+} - and phosphate-regulating hormones: after 48 h there was a progressive rise in the value of the product until normal values were reached several days later. It is therefore apparent that the increase in density of the bone after cholecalciferol administration does not coincide with a return to normality of the $[\text{Ca}^{2+}] \times [\text{phosphate}]$ product in blood.

Effect of correcting hypocalcaemia in vitamin D-deficient chicks

The abnormality in the process of calcification could be related to the disturbance in Ca^{2+} homeostasis secondary to vitamin D deficiency rather than to a direct effect of the vitamin or its metabolites. By altering the Ca^{2+} content of the rachitogenic diet it was possible to raise the blood plasma $[\text{Ca}^{2+}]$ in vitamin D-deficient chicks to normal values (see Table 4). Under these conditions the incorporation

Table 3. *Effect of a single dose of cholecalciferol on 'maturation' of bone in rachitic chicks*

A single dose of [^3H]proline ($100\mu\text{Ci}$) was given to all 5-week-old vitamin D-deficient chickens 8 days before killing. At 2 days before killing one group (three animals) was given intracardially $5\mu\text{g}$ of cholecalciferol in 0.2 ml of propylene glycol; the controls (three animals) were injected with propylene glycol alone. Although a full fractionation of the bone was performed, values for fractions 1 and 2 and 4-7 have been combined for ease of comparison. Results are expressed as means $\pm$ s.d. The differences between cholecalciferol-treated and control animals were all significant (for fractions 1-3, $P < 0.05$; for fractions 4-7, $P < 0.1$ by Student's t test). The density range of the fractions is given in Table 1.

Fraction	^3H Radioactivity in fractions of diaphyseal bone (% of total)	
	Control	Cholecalciferol-treated
1 and 2	61.8 $\pm$ 14.7	26.5 $\pm$ 13.8
3	34.7 $\pm$ 15.0	63.3 $\pm$ 9.3
4-7	3.7 $\pm$ 1.5	10.1 $\pm$ 4.6

Table 4. *Effect of modifying the diet on blood serum biochemistry of chicks*

The age of the chickens was 4 weeks and the number in each group was six. Chicks in the group marked +D were given $5\mu\text{g}$ of cholecalciferol in arachis oil orally each week. High Ca^{2+} refers to the normal Ca^{2+} diet supplemented with 1% Ca^{2+} as CaCO_3 . Results are expressed as means $\pm$ s.d.

Diet type	... Normal -D	Normal +D	High Ca^{2+} -D
Weight (g)	162 $\pm$ 27	314 $\pm$ 16	250 $\pm$ 48
Serum Ca^{2+} (mg/dl)	6.8 $\pm$ 1.2	10.6 $\pm$ 1.1	12.2 $\pm$ 0.6
Serum phosphorus (mg/dl)	6.2 $\pm$ 1.4	8.9 $\pm$ 0.9	2.9 $\pm$ 0.9
Alkaline phosphatase (units/ml)	350 $\pm$ 127	43 $\pm$ 14	222 $\pm$ 45

of $^{45}\text{Ca}^{2+}$ or of $[^3\text{H}]$ proline into bone fractions was found to be identical with those in the chicks on a normal Ca^{2+} diet, with accompanying hypocalcaemia, and different from those in vitamin D-replete chicks (see Fig. 3). This makes it unlikely that the changes relate directly either to changes in plasma $[\text{Ca}^{2+}]$ or to those of parathyrin, since we would expect secretion of the latter to be minimal under the normocalcaemic conditions. The results also suggest that the changes do not relate directly to alterations in plasma [phosphate] either. Plasma [phosphate] is high even in the vitamin D-deficient chicks fed on the usual rachitogenic diet, but on the high- Ca^{2+} diet there is a very marked decrease in plasma [phosphate], with no apparent effects on the degree of the maturational defect.

A bone maturational defect with some similar characteristics to that of the vitamin D-deficient chick can be induced in rats with experimental chronic renal insufficiency (Russell & Avioli, 1972); in these experiments blood $[\text{Ca}^{2+}]$ and pH of the animals were similar to those of controls and [phosphate] was actually slightly higher than in controls. Although the experimental conditions are not directly comparable with those used by ourselves the defect in maturation in chronic renal insufficiency appears to be more severe than that observed in the vitamin D-deficient chicks.

There are other similarities between the bone lesions in chronic renal insufficiency and in vitamin D deficiency. In X-ray-diffraction studies of bone from rachitic chicks (Termine & Posner, 1967) there was an increase in an amorphous low-density-bone phase relative to the crystalline high-density phase, and a similar effect has been observed in chronically uremic rats (Russell *et al.*, 1973).

Vitamin D deficiency in chickens also results in an increase in the ratio of the reduced cross-links dihydroxylysine norleucine and hydroxylysine norleucine (Mechanic *et al.*, 1975) in bone collagen and a similar change has been found in bone from chronically uremic rats (Russell *et al.*, 1975). Other observations (I. R. Dickson & D. R. Eyre, unpublished work) show that the major cause of the increased proportion of the dihydroxylated cross-link in vitamin D-deficient chicks is the accompanying hypocalcaemia rather than altered extracellular activities of lysyl oxidase (Gonnerman *et al.*, 1976), so this apparent similarity between the two experimental models may partially result from different causes.

It was originally concluded (Russell & Avioli, 1972) that vitamin D was not implicated in these changes in experimental chronic renal insufficiency, since replacement therapy with cholecalciferol or 25-hydroxycholecalciferol failed to reverse the lesion, but more recent information about the role of the kidney in the hydroxylation of 25-hydroxycholecalciferol (Kodicek, 1974) makes it reasonable to assume that metabolism

Fig. 3. $[^3\text{H}]$ Proline incorporation into vitamin D-deficient chick diaphyseal bone

All chicks in this experiment were maintained on a vitamin D-deficient diet. One group (normal- Ca^{2+}) was fed on the usual diet containing 1.2% Ca^{2+} . The other group (high- Ca^{2+}) was fed on diet supplemented with 1% Ca^{2+} . At 5 weeks chicks were given a single intracardial injection of $^{45}\text{CaCl}_2$ or $[^3\text{H}]$ proline, killed 1 week later and the diaphyseal bone powder was fractionated. The amount of radioactivity in each fraction is expressed as a percentage of the total amount of radioactivity in the bone. Each histogram shows the mean value for two chicks for each experiment. The range of individual values is shown by the bars. The differences between chicks on the high- Ca^{2+} diet and those on the normal- Ca^{2+} diet are not significant. The density range of each fraction is given in Table 1. (a) shows $[^3\text{H}]$ proline incorporation and (b) shows $^{45}\text{Ca}^{2+}$ incorporation. The shaded column show results with a high- Ca^{2+} diet and the open columns show results with a normal- Ca^{2+} diet.

of cholecalciferol would have been impaired under the experimental conditions used. Further evidence for a role of vitamin D metabolites has been obtained by treatment of parathyroidectomized rats with 25-

hydroxycholecalciferol (Russell & Avioli, 1975); these animals showed evidence of accelerated bone mineral and matrix maturation compared with parathyroid-ectomized control animals.

Conclusions

The density-fractionation technique is the only procedure available permitting certain aspects of bone formation to be studied. It provides an extremely useful adjunct to histological studies for investigating bone formation under normal and pathological conditions. Although applied in the present paper to an examination of the role of vitamin D in bone formation the technique can be applied more widely to study the effects of other factors on this process as well as to examine the composition and structure of bone at different stages of formation.

The experimental evidence suggests that the differences in bone formation found in the vitamin D-deficient state are not related to the abnormalities in plasma $[Ca^{2+}]$ or of the $[Ca^{2+}] \times [\text{phosphate}]$ product. Although there is a circumstantial relationship between the administration of cholecalciferol to the rachitic chick and the maturation of the low-density calcified fraction to a higher density form, it is unlikely that the vitamin or its metabolites directly influence this transition. It is more likely that any action of a cholecalciferol metabolite will be on the bone-forming cell, to stimulate synthesis of a factor that will mediate this transition. Phospholipids can influence the transformation from amorphous calcium phosphate to hydroxyapatite *in vitro* (Wuthier & Eanes, 1975; Boskey & Posner, 1977), but there is as yet no evidence that similar effects occur *in vivo* or that cholecalciferol metabolites can influence phospholipid metabolism in bone cells.

We thank Miss Pamela Newport for her skilled assistance. This research was supported by a grant from the Medical Research Council.

References

- Barnes, M. J., Constable, B. J., Morton, L. F. & Kodicek, E. (1973) *Biochim. Biophys. Acta* **328**, 373–382
- Baylink, D., Stauffer, M., Wergedal, J. & Rich, C. (1970) *J. Clin. Invest.* **49**, 1122–1134
- Boskey, A. L. & Posner, A. S. (1977) *Calcif. Tissue Res.* **23**, 251–258
- Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758
- Coklica, V. & Brudevold, F. (1966) *Arch. Oral Biol.* **11**, 1261–1268
- Fincham, A. G. (1969) *Calcif. Tissue Res.* **3**, 327–339
- Gonnermann, W. A., Toverud, S. U., Ramp, W. K. & Mechanic, G. L. (1976) *Proc. Soc. Exp. Biol. Med.* **151**, 453–456
- Herman, H. & Richelle, L. (1961) *Bull. Soc. Chim. Biol.* **43**, 273–282
- Kodicek, E. (1964) *Lancet* **i**, 325–329
- Lapiere, Ch. M. & Nusgens, B. V. (1970) in *Chemistry and Molecular Biology of the Intracellular Matrix: Collagen Basal Laminæ and Elastin*, pp. 55–79, Academic Press, London and New York
- Mechanic, G. L., Toverud, S. U., Ramp, W. K. & Gonnermann, W. A. (1975) *Biochim. Biophys. Acta* **393**, 419–425
- Nusgens, B., Chantaine, A. & Lapiere, Ch. M. (1972) *Clin. Orthop.* **88**, 252–274
- Quinaux, N. & Richelle, L. J. (1967) *Isr. J. Med. Sci.* **3**, 667–689
- Russell, J. E. & Avioli, L. V. (1972) *J. Clin. Invest.* **51**, 3072–3079
- Russell, J. E. & Avioli, L. V. (1975) *J. Clin. Invest.* **56**, 792–798
- Russell, J. E., Termine, J. D. & Avioli, L. V. (1973) *J. Clin. Invest.* **52**, 2848–2852
- Russell, J. E., Avioli, L. V. & Mechanic, G. (1975) *Biochem. J.* **145**, 119–121
- Shapiro, I. M. & Hartles, R. L. (1964) *Arch. Oral Biol.* **10**, 155–159
- Smith, C. B. & Smith, D. A. (1976) *Calcif. Tissue Res.* **22**, 219–226
- Termine, J. D. & Posner, A. S. (1967) *Calcif. Tissue Res.* **1**, 8–23
- Woessner, J. F. (1961) *Arch. Biochem. Biophys.* **93**, 440–447
- Wuthier, R. E. & Eanes, E. D. (1975) *Calcif. Tissue Res.* **19**, 197–210