The Localization of 1,25-Dihydroxycholecalciferol in Bone Cell Nuclei of Rachitic Chicks

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1. A simple technique has been developed to obtain subcellular fractions of chick bone. The method yielded 60–70% of total DNA in the nuclear debris fraction and 80–90% of total ^{14}C recovered in bone after a dose of radioactive vitamin D. 2. After a dose of $[4\text{-}^{14}\text{C}, 1,2\text{-}^3\text{H}]$ cholecalciferol ($0.5\text{ }\mu\text{g}$) was given to vitamin D-deficient chicks, the time-course of total ^{14}C radioactivity in the epiphysis, metaphysis and diaphysis of proximal tibiae was measured. The maximum concentrations were reached at 6 h, corresponding to a similar peak of radioactivity in blood, decreasing until 24 h and indicating the dependence on the circulating ^{14}C and on the blood supply of the three bone components. 3. The ^{14}C radioactivity of cholecalciferol and 25-hydroxycholecalciferol (expressed per mg of DNA) followed the pattern of incorporation of total ^{14}C radioactivity in all three bone components. The more polar metabolite fraction reached a peak of radioactivity at 6–9 h and maintained its concentration over the 24 h period studied in all anatomical bone components. 4. After a dose of $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]$ cholecalciferol ($0.5\text{ }\mu\text{g}$) was given to vitamin D-deficient chicks, the subcellular distribution was studied. At 24 h after dosing, the nuclear fraction contained 27% and the supernatant fraction had 67% of total ^{14}C recovered in the bone filtrate. When the ^{14}C in the residual bone fragments was included, the nuclear fraction contained up to 35% of the total radioactivity in the bone. 5. The subcellular distribution pattern of individual vitamin D metabolites indicated that the purified nuclear fraction concentrated the polar metabolite, which lost ^3H at C-1, so that 77% of the radioactivity could be accounted for by 1,25-dihydroxycholecalciferol. The supernatant fraction contained smaller amounts of 1,25-dihydroxycholecalciferol (9%), with 66% of 25-hydroxycholecalciferol forming the major metabolite, corresponding to its concentration found in blood at 24 h. 6. The preferential accumulation of 1,25-dihydroxycholecalciferol in the nuclear fraction and the overall pattern of other metabolites, found previously in intestinal tissue, suggests a similar mechanism of action in bone to that postulated for the intestinal cell in calcium translocation.

Since the preparation of radioactive vitamin D (Callow, Kodicek & Thompson, 1966; Neville & DeLuca, 1966), extensive study of its metabolism in vitamin D-deficient animals has been undertaken. Blunt, DeLuca & Schnoes (1968) made the important observation that cholecalciferol is hydroxylated at C-25 to 25-hydroxycholecalciferol and that liver is the major, if not the sole, site of synthesis. The latter compound was 1.4 times more active than cholecalciferol and it was originally considered to be the active form of vitamin D (for ref. see DeLuca, 1970), though it is now apparent

that it is a precursor of a further hormone-like metabolite which had an oxygen function introduced at C-1 (Lawson, Wilson & Kodicek, 1969*a,b*).

Lawson, Fraser, Kodicek, Morris & Williams (1971*a*) have identified the polar metabolite as $1\alpha,25$ -dihydroxycholecalciferol produced by the kidney only from 25-hydroxycholecalciferol (Fraser & Kodicek, 1970). It appears to be identical with the unidentified polar fractions described as 'peak 4B' (Haussler, Myrtle & Norman, 1968) and with peak V in intestinal extracts (Cousins, DeLuca, Suda, Chen & Tanaka, 1970*a*). It was found that 1,25-dihydroxycholecalciferol obtained *in vitro* from kidney preparations (Lawson *et al.* 1971*a*) and the

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polar material from intestinal tissue (Kodicek, Lawson & Wilson, 1970; Myrtle & Norman, 1971; Haussler, Boyce, Littledike & Rasmussen, 1971) were several times more active than cholecalciferol or 25-hydroxycholecalciferol in stimulating intestinal calcium transport. Only very small and limited amounts ($<0.3\text{ ng/g}$ of tissue) of 1,25-dihydroxycholecalciferol are produced from precursors *in vivo* and the identification was accomplished by using doubly labelled $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]$ cholecalciferol which lost its tritium on hydroxylation at C-1 (Lawson *et al.* 1969a,b; Kodicek, 1970a,b; Lawson, Pelc, Bell, Wilson & Kodicek, 1971b). It was found that this tritium-deficient substance 'P', now identified as 1,25-dihydroxycholecalciferol, was preferentially localized in the nuclei of intestinal cells; there were indications that a similar localization occurred in kidney cell nuclei and possibly in bone cells.

Since the biological response to vitamin D must result from an initial interaction of one or more of its metabolites with the target tissue, the time-course of their appearance in these tissues and a knowledge of their subcellular localization is fundamental to the interpretation of their individual functions. Such studies with physiological concentrations of vitamin D have been reported for rat and chicken intestine (Haussler & Norman, 1967; Stohs & DeLuca, 1967; Lawson *et al.* 1969b, 1971b).

Similar investigations on bone have been hampered by the technical difficulties of preparing tissue. In the present study methods of preparation of bone components were developed and applied to the study of the time-course and subcellular distribution of vitamin D and its metabolites in chicken bone.

EXPERIMENTAL

Animals. Day old 'Chunky' chicks (Sterling Poultry Services Ltd., Welwyn Garden City, Herts., U.K.) were fed on the vitamin D-deficient diet and supplements as described by Lawson *et al.* (1969a) and were used after 4 weeks, when they were vitamin D-deficient. The 4–6-week-old chicks were injected intracardially with $0.5\text{ }\mu\text{g}$ (20 i.u.) of $[4\text{-}^{14}\text{C}, 1,2\text{-}^3\text{H}]$ - or $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]$ -cholecalciferol in 0.2 ml of propylene glycol. The $^3\text{H}/^{14}\text{C}$ specific radioactivity ratios of the two forms of labelled vitamin D were 11.7 and 6.6 respectively. The birds were killed after periods of 3, 6, 9, 12, 18 and 24 h, blood was collected from each group and the tibiae were immediately freed from muscle. The perichondrium of the proximal epiphysis was trimmed and the articular cartilage peeled from the epiphyseal cartilage. The epiphyseal growth cartilage was separated from the metaphyseal bone by fracturing through the zone of provisional calcification (a natural cleavage plane). The metaphysis was separated from the diaphysis at the proximal limit of the marrow cavity. The epiphyseal growth cartilage, metaphysis and diaphysis were blotted free of blood and marrow and placed into separate 25 ml volumes of water at 0°C . Each of the three bone components was weighed,

chopped with scissors and scalpel, and finally homogenized in an MSE model 7700 homogenizer.

Subcellular distribution. Vitamin D-deficient chicks (20) were injected as before with $0.5\text{ }\mu\text{g}$ (20 i.u.) of $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]$ cholecalciferol having a $^3\text{H}/^{14}\text{C}$ specific radioactivity ratio of 6.6. Metaphyses and diaphyses were removed and weighed in cold 0.25 M -sucrose in TKM buffer [60 mM -tris-HCl (pH 7.5)– 25 mM -KCl– 5 mM - MgCl_2]. These combined bone components were repeatedly ground at 4°C with a mincer fitted with openings down to $3/32\text{ in}$. This finely minced bone was suspended in 0.25 M -sucrose and filtered through nylon bolting cloth (St Martins 10N). Nuclear, mitochondrial, microsomal and soluble subcellular fractions were prepared by a modified method of Schneider & Hogeboom (1950), as described by Wilson, Lawson & Kodicek (1967) as follows. The filtrate was centrifuged for 10 min at $800g$ and the nuclear-debris pellet resuspended and washed three times with 0.32 M -sucrose in TKM buffer. For further purification, this pellet was resuspended in 10 ml of 0.32 M -sucrose, mixed slowly with 80 ml of 2.4 M -sucrose and centrifuged for 1 h at $53\,500g$ in a Spinco SW 25.1 rotor to obtain purified nuclei (Lawson, Wilson, Barker & Kodicek, 1969c). The supernatant from the original $800g$ centrifugation was then centrifuged at $20\,000g$ for 10 min in an MSE 18 centrifuge to yield a mitochondrial fraction. The resulting supernatant was then centrifuged at $105\,000g$ for 1 h in an MSE 65 centrifuge to yield a microsomal fraction. The residual bone material left on the nylon bolting cloth was also extracted as described below. All procedures were carried out at 4°C .

Extraction of tissue. Homogenate preparations were extracted by shaking with 10 vol. of chloroform-methanol (1:1, v/v) for 16 h at 4°C . Chloroform was separated by adding 2 vol. of water and the aqueous methanol was re-extracted with a further 8 vol. of chloroform. Total radioactivity was measured in 10% of the chloroform extract.

Chromatography. Lipid extracts were chromatographed on silica gel G thin layers, 0.5 mm thick, with ethylacetate-*n*-heptane (1:1, v/v) as developing solvent. Unlabelled marker cholecalciferol and 25-hydroxycholecalciferol were co-chromatographed and detected with iodine vapour. Radioactivity along the plate was estimated after elution with diethyl ether-ethanol (3:1, v/v).

Materials. $[1,2\text{-}^3\text{H}_2]$ cholecalciferol (508 mCi/mmol) and $[1\text{-}^3\text{H}]$ cholecalciferol (211 mCi/mmol) were prepared by Dr P. A. Bell and Dr B. Pelc (Lawson *et al.* 1971b). $[4\text{-}^{14}\text{C}]$ cholecalciferol was obtained from Phillips-Duphar, Amsterdam, The Netherlands, (19.2 mCi/mmol) and The Radiochemical Centre, Amersham, Bucks., U.K. (32.3 mCi/mmol).

Chemical determinations and measurement of radioactivity. DNA was determined by the method of Burton (1956). Radioactivity was measured in a Packard Tri-Carb Model 3375 automatic liquid-scintillation spectrometer. The absolute number of disintegrations per minute was derived by using automatic external standardization and correlation curves for ^3H in the presence of ^{14}C . From the $^3\text{H}/^{14}\text{C}$ specific radioactivity ratios the relative amount of the ^3H -deficient vitamin D metabolite, $1\alpha,25$ -dihydroxycholecalciferol, was calculated thus: $[(R_1 - R_2)/R_1] \times 100 \times f$; where R_1 , original $^3\text{H}/^{14}\text{C}$ specific radioactivity ratio administered; R_2 , extract $^3\text{H}/^{14}\text{C}$

specific radioactivity ratio; *f*, correction factor of 100/85, allowing for the distribution of ^3H in the α position (85%) from which the loss of ^3H occurred (Bell & Kodicek, 1970).

RESULTS

Total radioactivity of bone components with time after injection of $[4\text{-}^{14}\text{C}, 1,2\text{-}^3\text{H}_2]\text{cholecalciferol}$. Fig. 1 shows the total d.p.m. of ^{14}C /mg of DNA extracted from each bone component as a function of time after dosing. The average amount of DNA/g for each bone component is shown in Table 1.

At all time-periods the amount of ^{14}C /mg of DNA was greatest in the metaphysis and diaphysis and least in the epiphysis. The peak of radioactivity in the metaphysis and diaphysis was reached at 6h, followed by a rapid decrease, particularly in the metaphysis. The peak of radioactivity in the epiphysis was slightly delayed and less pronounced. It is most likely that the time-course of ^{14}C incorporation depends on the relative blood supply, which is abundant in the trabecular bone of the metaphysis, but is somewhat limited in the epiphysis. After 9h, when the effect of difference in blood supplies had disappeared, the time-course of ^{14}C radioactivity was, excepting the epiphysis, essentially the same. Between 18 and 24h there was little, if any, decrease in radioactivity in the metaphysis and diaphysis, which had a virtually identical ^{14}C count.

Time-course of vitamin D metabolites in bone components. Fig. 2 shows the amount of cholecalciferol, 25-hydroxycholecalciferol and the more polar metabolites/mg of DNA at various times after dosing with 20 i.u. of $[4\text{-}^{14}\text{C}, 1,2\text{-}^3\text{H}_2]\text{cholecalciferol}$. In the metaphysis (Fig. 2a) and diaphysis (Fig. 2b) the cholecalciferol concentration is seen to decrease from 3h onwards. This reflects the decreasing concentrations of cholecalciferol in the blood shown in Fig. 2(d) indicating that bone shows little ability to actively retain cholecalciferol. 25-Hydroxycholecalciferol was the major contributor to the 6h peak observed in the curve of total ^{14}C radioactivity (Fig. 1) and its concentration also follows the amounts found in blood (Fig. 2d). The more polar metabolites of vitamin D reach a maximum concentration at 6–9h in the metaphysis and 9–12h in the diaphysis. Since chicken blood contains only very small amounts of the polar metabolites (Lawson *et al.* 1971a), this appearance of relatively large amounts in bone suggests a high binding affinity.

The epiphysis (Fig. 2c) showed a similar distribution pattern of metabolites, but at markedly lower concentrations. The peak of the curves shifted to later times, with vitamin D reaching its maximum concentration at 6h. This might have been caused by the limited blood circulation within the epiphysis.

In this experiment, it was found that the changes of $^3\text{H}/^{14}\text{C}$ ratios after $[4\text{-}^{14}\text{C}, 1,2\text{-}^3\text{H}_2]\text{cholecalciferol}$ treatment were associated with greater errors and had little significance in view of the relatively small amounts of radioactivity recovered in the tissues examined, and also because of the relatively small change in the ratio to be expected from the tritium distribution (Bell & Kodicek, 1970). Therefore, no calculation of the tritium-deficient polar substance was made and in the subsequent experiments, dosage with $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]\text{cholecalciferol}$ was preferred.

Vitamin D metabolites in bone components. Table 2 shows the distribution of vitamin D and its metabolites 24h after dosing with $0.5\text{ }\mu\text{g}$ of $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]\text{cholecalciferol}$. It can be seen that 25-hydroxycholecalciferol accounted for about 60% of the recovered ^{14}C radioactivity in all three bone components. The polar peak P, containing 1,25-dihydroxycholecalciferol, ranged from 26 to 35% and the rest of ^{14}C radioactivity was found in the tibia in the cholecalciferol and ester region. The doubly labelled cholecalciferol, with ^3H in position C-1 only, should have allowed for an improved measure of the tritium-deficient material in peak P. However, considerable difficulties were encountered in obtaining reliable $^3\text{H}/^{14}\text{C}$ ratios due to excessive trailing, particularly in the more polar regions.

Fig. 1. Time-course of appearance of total radioactivity in bone components of vitamin D-deficient chicks after a $0.5\text{ }\mu\text{g}$ dose of $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]\text{cholecalciferol}$. $\circ$, Metaphysis; $\square$, diaphysis; $\triangle$, epiphysis.

Table 1. Average distribution of DNA in bone components of vitamin D-deficient chicks

Each result is the mean of six samples $\pm$ s.e.m.

	Concn. of DNA (mg/g of wet tissue)
Epiphysis	0.223 $\pm$ 0.016
Metaphysis	0.589 $\pm$ 0.046
Diaphysis	0.367 $\pm$ 0.037

Fig. 2. Time-course of appearance of radioactive vitamin D metabolites in lipid extracts of (a) metaphysis, (b) diaphysis, (c) epiphysis and (d) plasma after a $0.5\mu\text{g}$ dose of $[4\text{-}^{14}\text{C}, 1, 2\text{-}^3\text{H}_2]\text{cholecalciferol}$. $\circ$, 25-Hydroxycholecalciferol; $\square$, cholecalciferol; $\triangle$, peak P.

Table 2. *Distribution of radioactivity of lipid extracts of chick bone components after a dose of $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]\text{cholecalciferol}$*

Vitamin D-deficient chicks were given $0.5\mu\text{g}$ of $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]\text{cholecalciferol}$ intracardially and killed after 24 h. Tibial epiphyses were obtained from 60, metaphyses and diaphyses from 20 chicks. Lipid extracts were chromatographed on t.l.c. plates, with ethyl acetate-*n*-heptane (1:1, v/v) as developing solvent. Results are expressed as % of ^{14}C radioactivity recovered from the chromatogram.

	Epiphysis (% ^{14}C)	Metaphysis (% ^{14}C)	Diaphysis (% ^{14}C)
Total ^{14}C chromatographed (d.p.m.)	343.4	282.9	347.8
Peak P	25.6	34.6	35.2
25-Hydroxycholecalciferol	65.9	58.9	52.0
Cholecalciferol	6.9	6.1	12.0
Cholecalciferol esters	1.5	0.6	0.8

This was caused by the presence of other bone lipids contaminating the extracts that had to be obtained from a large number of samples. For instance, the chromatogram for the epiphysis required the pooling of 120 tibial epiphyses to yield sufficient quantities of polar metabolites to allow reliable calculations. Therefore calculations of $^3\text{H}/^{14}\text{C}$ ratios were abandoned since a clearer and more exact distribution pattern was obtained with the subcellular fraction (see below).

Subcellular distribution of cholecalciferol and its metabolites in bone at 24 h. Table 3 shows the sub-

cellular distribution of ^{14}C in bone of vitamin D-deficient chicks that had been given $0.5\mu\text{g}$ of $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]\text{cholecalciferol}$ intracardially. Metaphyses and diaphyses were combined before fractionation since no significant differences in amount or proportion of the metabolites were found (Figs. 1 and 2) or in preliminary subcellular distribution experiments (results not shown). T.l.c. of the purified nuclear bone fraction (Fig. 3a) shows that 81% of the ^{14}C was in the form of a polar metabolite of which 95% was calculated to be 1,25-dihydroxycholecalciferol (with a change in

Table 3. Subcellular distribution of cholecalciferol and its metabolites in bone after a dose of [4-¹⁴C,1-³H]-cholecalciferol

Vitamin D-deficient chicks (20) were given 0.5 μg of [4-¹⁴C,1-³H]cholecalciferol intracardially and killed after 24 h. Results are expressed as % of ¹⁴C radioactivity recovered in the bone filtrate and from the t.l.c., respectively. The percentage of 1,25-dihydroxycholecalciferol, detected in peak P, was calculated from the change in ³H/¹⁴C ratio (for calculation see text). The total ¹⁴C recovered in the bone filtrate was 1310 d.p.m.

Fraction	Percentage distribution of ¹⁴ C in bone filtrate	Percentage distribution of ¹⁴ C recovered from t.l.c. plates			
		Cholecalciferol	25-Hydroxy-cholecalciferol	1,25-Dihydroxy-cholecalciferol	Rest of peak P
Nuclear	27.4	9	6	77	4
Mitochondrial	3.0	—	—	—	—
Microsomal	2.7	—	—	—	—
Supernatant	66.9	4	66	9	3

Fig. 3. Thin-layer chromatographic distribution of vitamin D metabolites in the lipid extracts of (a) bone cell nuclei, (b) supernatant fraction, (c) residual bone and (d) blood from vitamin D-deficient chicks 24 h after a 0.5 μg dose of [4-¹⁴C,1-³H]cholecalciferol. The silica gel G thin layer was developed with *n*-heptane-ethyl acetate (1:1, v/v). The ³H/¹⁴C ratios are given in parentheses when the quantity of radioactivity allows reliable ratio calculations. The shading in the polar peak represents the amount of 1,25-dihydroxycholecalciferol calculated from the change in the ³H/¹⁴C ratio (for calculations see text). D, Cholecalciferol; HCC, 25-hydroxycholecalciferol; P, peak P.

the ³H/¹⁴C ratio from 6.6 to 1.3). The remaining 19% of the ¹⁴C was distributed among the cholecalciferol and 25-hydroxycholecalciferol bands. The nuclear fractions from bone contain quantities of bone mineral which pass through the filter and sediment with nuclei. All attempts to remove this

filtered mineral have resulted in proportional loss of nuclear DNA, but the mineral contamination did not interfere with subsequent procedures.

T.l.c. of the bone supernatant fraction (Fig. 3b) indicates that 67% of the ¹⁴C was present as 25-hydroxycholecalciferol and a further 12% in the

polar band, of which 70% was defined as 1,25-dihydroxycholecalciferol (calculated from the change in the $^3\text{H}/^{14}\text{C}$ ratio of the polar band from 6.6 to 2.7). The chromatograms of bone supernatant, and of plasma at the same time period (Fig. 3d), are almost identical except for the polar peak containing 1,25-dihydroxycholecalciferol. This further suggests that 25-hydroxycholecalciferol readily diffuses into and out of cells according to the extracellular concentration, whereas the polar metabolite has a stronger intracellular binding, particularly in the nucleus. Fig. 3(c) shows the thin layer chromatogram of the extract from the non-filtrable residual bone of the mince. The distribution pattern resembled that of the nuclear fraction (Fig. 3a) in that the major component was 1,25-dihydroxycholecalciferol (73%), but differed from it in containing somewhat greater amounts of cholecalciferol (9%) and 25-hydroxycholecalciferol (17%). This non-filtrable fraction of the residual bone extract, in which a further 295 d.p.m. of ^{14}C was recovered, consisted mainly of nuclear material, since it contained a further 50% of DNA found in the bone filtrate.

Table 3 shows the subcellular distribution of total ^{14}C in the bone filtrate, indicating that 27.4% of ^{14}C was recovered in the nuclear fraction. If the residual ^{14}C in the non-filtrable bone material is taken into account, the recovery in the nuclear fraction could be as high as 35%. The distribution pattern of vitamin D metabolites in the nuclear fraction shows that they consist preferentially of 1,25-dihydroxycholecalciferol, accounting for almost all the radioactivity in the polar peak P. The supernatant had 66.9% of ^{14}C of the filtrate, consisting mainly of 25-hydroxycholecalciferol, while here the polar peak P was only a minor fraction; the latter, within the experimental error of the method of calculation, consisted almost entirely of 1,25-dihydroxycholecalciferol. If any other dihydroxy analogues were present, they were in negligible amounts.

DISCUSSION

The technique described above for subcellular fractionation of bone yielded consistently about 60–70% of the bone DNA in the nuclear fraction and 80–90% of the total ^{14}C bone radioactivity in the subcellular fractions. It indicated that for these particular investigations there was no need to isolate bone cells first, as in the more elaborate preparation of Nichols & Rogers (1971). This simple technique left about 10–20% of total bone ^{14}C behind in the non-filtrable residual bone material. This radioactivity appeared to be mainly, but not entirely, nuclear material. The reason for this retention is not clear, but the incomplete

yield will depend on the structural properties of bone with the occlusion of bone cells in the mineral matrix.

The time-course and subcellular distribution of cholecalciferol and its metabolites after administering $[4\text{-}^{14}\text{C}, 1,2\text{-}^3\text{H}_2]\text{cholecalciferol}$ or $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]\text{cholecalciferol}$ to vitamin D-deficient chicks, indicates that bone cells have a distribution pattern similar to that of intestinal cells in comparable situations. The percentage distribution of total vitamin D metabolites in bone cells and in their subcellular fractions is very similar to that in the intestine (Lawson *et al.* 1969b, 1971b; Cousins *et al.* 1970a).

In rachitic chick and rat bone (Lawson *et al.* 1969b), after a dose of $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]\text{cholecalciferol}$ or 25-hydroxy $[4\text{-}^{14}\text{C}, 1\text{-}^3\text{H}]\text{cholecalciferol}$, about 20% of the bone ^{14}C radioactivity was found in peak P, with a significantly lowered $^3\text{H}/^{14}\text{C}$ specific radioactivity ratio, and is now identified as 1,25-dihydroxycholecalciferol (Lawson *et al.* 1971b). As much as 35–40% of the ^3H in rat bone, after a dose of 25-hydroxy $[26,27\text{-}^3\text{H}_2]\text{cholecalciferol}$, was found by Cousins, DeLuca & Gray (1970b) in the fraction they termed peak V, which is identical with peak P under certain conditions. Our results agree with these findings and, further, show that peak P or peak V in bone consists almost entirely of 1,25-dihydroxycholecalciferol. Our present results give further evidence of the localization of vitamin D metabolites in bone anatomical components (i.e. epiphysis, metaphysis and diaphysis) and add new information regarding the specific distribution pattern of vitamin D metabolites in subcellular fractions of bone. In particular, they demonstrate, by the use of doubly labelled cholecalciferol, the specific accumulation of 1,25-dihydroxycholecalciferol in bone cell nuclei.

Because small amounts of 1,25-dihydroxycholecalciferol can be accumulated in the cell nuclei of intestinal tissue, and also because of its effects on nuclear metabolism (Lawson *et al.* 1969a,b; Hallick & DeLuca, 1969; Norman, 1968; Stohs, Zull & DeLuca, 1967), an active role in calcium transport mechanisms has been postulated for this metabolite (see Kodicek *et al.* 1970). The similar distribution of the metabolite in bone cells leads us to suggest that 1,25-dihydroxycholecalciferol has a similar role in this tissue.

21,25-Dihydroxycholecalciferol has been identified in the plasma of pigs given large amounts of cholecalciferol (Suda *et al.* 1970a). This metabolite, although less active than cholecalciferol in intestinal transport and the cure of rickets, was as active as 25-hydroxycholecalciferol in the mobilization of bone mineral. Similarly a further polar metabolite was identified as 25,26-dihydroxycholecalciferol (Suda, DeLuca, Schnoes, Tanaka & Holick, 1970b),

which was virtually inactive in mobilization of bone calcium and cure of rickets, but had some activity in intestinal calcium transport. The specific localization of these two compounds in cells of target organs has so far not been reported. As to their involvement in bone metabolism these derivatives of cholecalciferol can account for only a small proportion of the polar metabolites in bone cells under our conditions, if indeed they are present at all.

25-Hydroxycholecalciferol was the main constituent of our supernatant fraction. It was virtually absent in the bone-cell nuclei, where the major metabolite was 1,25-dihydroxycholecalciferol. In view of the scarcity of information concerning the exact mechanism of bone formation and resorption, the question whether 25-hydroxycholecalciferol has, under physiological conditions, a direct role to play in bone metabolism *in vivo*, has to await further studies. This is particularly important, since Trummel, Raisz, Blunt & DeLuca (1969) found a direct effect on calcium release from bone *in vitro*, despite the fact that no 1,25-dihydroxycholecalciferol could have been present.

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