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In vitro synthesis of 1α,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol by isolated calvarial cells

(1,25-dihydroxyvitamin D_3/24,25-dihydroxyvitamin D_3/vitamin D metabolism)

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ABSTRACT **The question of whether the skeleton metabolizes 25-hydroxycholecalciferol [25(OH)D_3] to more-polar products was studied. Calvarial cells were dispersed from 16-day old chicken embryos by using collagenase and then grown in culture in serum-free medium. Confluent cell cultures were incubated with 7 nM 25(OH)[^{3}H]D_3 for 2 hr, and the vitamin D metabolites were then extracted. At least four polar metabolites were produced. Based on separation by Sephadex LH-20 chromatography followed by high-pressure liquid chromatography, two of these metabolites were identified as 1,25-dihydroxycholecalciferol [1,25$(OH)_2D_3$] and 24,25-dihydroxycholecalciferol [24,25$(OH)_2D_3$]. These metabolites were also produced by cultured kidney cells but not by liver, heart muscle, or skin cells isolated from the same embryos. The specific activities of the calvarial 1- and 24-hydroxylases were similar in magnitude to those in isolated kidney cells. The specific activity of the calvarial 25(OH)D_3:1-hydroxylase was inhibited by an 8-hr preincubation with 1,25$(OH)_2D_3$, whereas the 24-hydroxylase was enhanced. It is concluded that (*i*) vitamin D metabolism by isolated cells is organ-specific, (*ii*) calvarial cells produce active metabolites of vitamin D in significant amounts, (*iii*) vitamin D metabolism by calvarial cells is regulated by 1,25$(OH)_2D_3$, and (*iv*) locally produced, active metabolites could act locally, thereby adding a new dimension to the regulation of mineral metabolism by vitamin D metabolites.**

1α,25-Dihydroxycholecalciferol [1,25$(OH)_2D_3$] is a potent mineral homeostatic hormone. It acts on the skeleton to mobilize bone mineral and on the intestine to enhance absorption of calcium and phosphorus (1–3). In addition, it appears to act on the kidney, in concert with parathyroid hormone (PTH), to promote calcium reabsorption (4). It was formerly believed that 1,25$(OH)_2D_3$ is synthesized exclusively in the kidney but recent evidence suggests that it may be synthesized in placenta as well (5–7). In this paper, we report that cells isolated from calvaria of chicken embryos and grown in culture metabolize 25-hydroxycholecalciferol [25(OH)D_3] to several more-polar metabolites, including 1,25$(OH)_2D_3$. These findings suggest that the tissue distribution of 1,25$(OH)_2D_3$ production may be wider than previously thought and that locally produced as well as systemically derived hormone may be important in mediating vitamin D action in target organs such as the skeleton.

MATERIALS AND METHODS

Isolation of Cells. Cells were isolated from calvaria of 15- to 16-day chicken embryos (*Gallus gallus*, Fors Farms Hatchery, Puyallup, WA) by a modification of the method developed for fetal rat calvaria (8). Briefly, the frontal and parietal bones and intervening suture line area were aseptically dissected, rinsed, and incubated for up to 120 min in Tris·HCl/NaCl, pH 7.4 (four calvaria per ml) containing collagenase (2 mg/ml; Worthington, type II). The cells were recovered as described (8), washed twice in enzyme-free Hanks' buffer (pH 7.4), and diluted in BGJ_b medium (Fitton-Jackson modification, GIBCO) (9) containing 100 units of penicillin and 100 μg streptomycin per ml. Cell number was determined by counting with a hemacytometer.

Kidney, skin, liver, and heart tissue were aseptically dissected from 15- to 16-day chicken embryos and minced into pieces no larger than 1 mm^3, and the cells were isolated as described (10).

Culture Conditions. Calvarial cells were suspended in serum-free BGJ_b medium, plated at a density of 1200–1500 cells per mm^2 of culture dish, and incubated at 37°C in 5% CO_2/95% air. The medium (2.5 ml/60 mm dish) was changed after 48 hr and again before assaying vitamin D metabolism in confluent cultures (approximately 72–96 hr).

Kidney, liver, skin, and heart cells were grown in the same manner as the calvarial cells. Kidney, liver, and skin cells became confluent within 4 days. Heart cells did not reach confluency; however, they remained viable and retained differentiated function (beating) for 4 days in culture.

Metabolism of 25-Hydroxy-[26(27)-*methyl*-^{3}H]cholecalciferol [25(OH)[^{3}H]D_3]. 25(OH)[^{3}H]D_3 (7 nM; 9 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; Amersham) was incubated with the cultured cells for 2 hr (with ethanol at a final concentration of 0.1% as the carrier) (10). In one experiment, isolated calvarial cells were preincubated with 10 nM of 1,25$(OH)_2D_3$ for 8 hr before addition of 25(OH)[^{3}H]D_3. Dichloromethane extracts of the cultured cells and medium were evaporated to dryness under a gentle stream of N_2, resuspended in a small volume of hexane/chloroform/methanol (9:1:1), and chromatographed on Sephadex LH-20 columns (Pharmarcia) (1 × 56 cm; 15 g of LH-20) previously equilibrated with the same solvent system. Three-milliliter fractions were collected for determination of the radioactivity (10); 80–95% of the radioactivity added to calvarial cells was recovered after separation of vitamin D metabolites by Sephadex LH-20 column chromatography.

Polar metabolites of 25(OH)[^{3}H]D_3 were identified by comparing their elution volumes with those of authentic standards on Sephadex LH-20 columns, by periodate (aqueous) cleavage of the peak fractions (11), and by high-pressure liquid chromatography (HPLC) of the peak fractions.

HPLC of Polar Metabolites. Fractions that migrated in the 1,25$(OH)_2D_3$ and 24,25-dihydroxycholecalciferol

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Abbreviations: 25(OH)D_3, 25-hydroxycholecalciferol; 1,25$(OH)_2D_3$, 1α,25-dihydroxycholecalciferol; 24,25$(OH)_2D_3$, 24,25-dihydroxycholecalciferol; PTH, parathyroid hormone; HPLC, high-pressure liquid chromatography.

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[24,25(OH)$_2$D$_3$] regions after Sephadex LH-20 column chromatography were pooled, dried under N_2, and redissolved in the appropriate solvent. These pooled fractions were cochromatographed with synthetic 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$, respectively (donated by M. Uskokovic, Hoffmann-La Roche, Nutley, NJ), on an HPLC system (Laboratory Data Control, Riviera Beach, FL) using a Zorbax-Sil (4.6 mm × 25 cm) column and the following solvent systems: (*i*) hexane/isopropanol (90:10) at a flow rate of 1 ml/min and (*ii*) hexane/isopropanol (87:13) at a flow rate of 1 ml/min. 1,25(OH)$_2$[^{3}H]D$_3$ was recycled on the latter system by rechromatographing the 1,25(OH)$_2$D$_3$ region four times. An aliquot of the coeluting fraction was then subjected to HPLC on a reverse-phase column (0.8 × 10 cm cartridge, Radial-Pak A, Waters Associates) with a mobile phase of methanol/water (gradient of 75:25 to 95:5 over 30 min) at a flow rate of 1 ml/min.

RESULTS

Characterization and Isolation of Calvarial Cells. A longitudinal section through a calvarium from a 16-day embryo showed that osteoblasts (plump cells having negative Golgi lying immediately adjacent to bone) lined most of the endosteal and periosteal surfaces of the calvarium (Fig. 1). Osteocytes were numerous in the intact calvarium. In contrast, osteoclasts [identified by staining for acid phosphatase (12)] were relatively rare (1% of the osteoblast number). In addition, the calvaria contained bone precursor cells as well as connective tissue fibroblasts and capillary endothelial cells. There was no bone marrow. The effect of collagenase treatment on calvaria was to release all of the cells except the deepest osteocytes (Fig. 1*B*). However, it is not clear whether all of the cell types that were released attached to tissue culture dishes or remained viable in culture.

Metabolites of 25(OH)[^{3}H]D$_3$ Produced by Isolated Calvarial Cells. Vitamin D metabolism was assayed in calvarial cells after the cultures reached confluence. Calvarial cells metabolized 25(OH)[^{3}H]D$_3$ to more-polar compounds; these metabolites were produced without failure in more than 70 separate preparations of calvarial cells but were not produced in medium that did not contain cells. Two of the metabolites were identified on the basis of elution volume on Sephadex LH-20 as 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ (Fig. 2). The 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ regions appeared to be homogeneous with respect to reaction with periodate: on periodate treatment, 93 ± 1% (mean ± SD; n = 3) of the radioactivity in the 24,25(OH)$_2$D$_3$ region and 0 ± 1% of the radioactivity in the 1,25(OH)$_2$D$_3$ region was lost. Furthermore, the material in the 24,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$ regions comigrated exactly with crystalline standards when cochromatographed on several HPLC systems (Figs. 3 and 4, respectively). The structures of two polar metabolites, IV and V, have not yet been determined.

We do not know what cells from calvaria metabolized 25(OH)D$_3$ or whether the 25(OH)D$_3$:1-hydroxylase and 25(OH)D$_3$:24-hydroxylase are located in the same cells. However, it is likely that these enzymes were located in cells unique

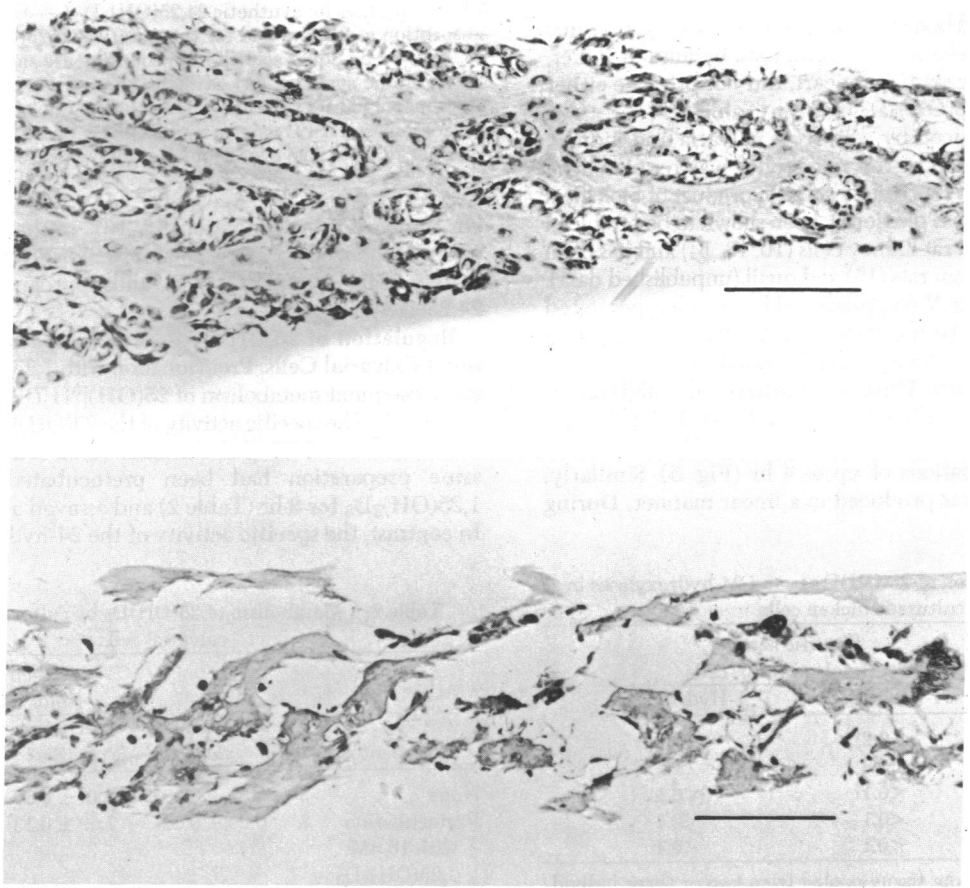

FIG. 1. Transverse sections through calvaria from 16-day embryos. (*A*) Sections (5 μm) were stained with methyl green and thionine. Osteoblasts cover all surfaces of the bone. (*B*) Digested with collagenase for 120 min. With the exception of the deepest osteocytes, all of the cells in the calvarium have been released. The length of the bar equals 100 μm.

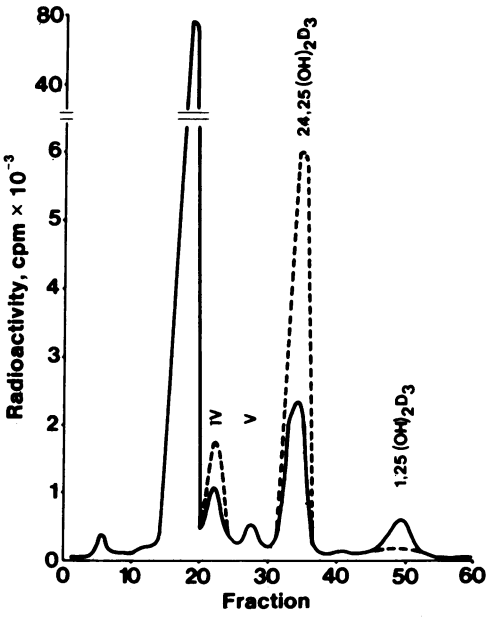

FIG. 2. Polar metabolites of 25(OH)[^{3}H]D_3 produced by primary cultures of chicken embryo calvarial cells. Cells (8–10 × 10^6) were incubated for 2 hr with 25(OH)[^{3}H]D_3 (7 nM) and extracted with dichloromethane. The extracted vitamin D metabolites were separated on Sephadex LH-20 (1 × 56 cm; 15 g) columns by elution with hexane/chloroform/methanol (9:1:1). Roman numerals refer to unidentified metabolites. —, Representative metabolic profile from control cultures; - - -, differences that occur after an 8-hr preincubation with 10 nM 1,25(OH)$_2D_3$.

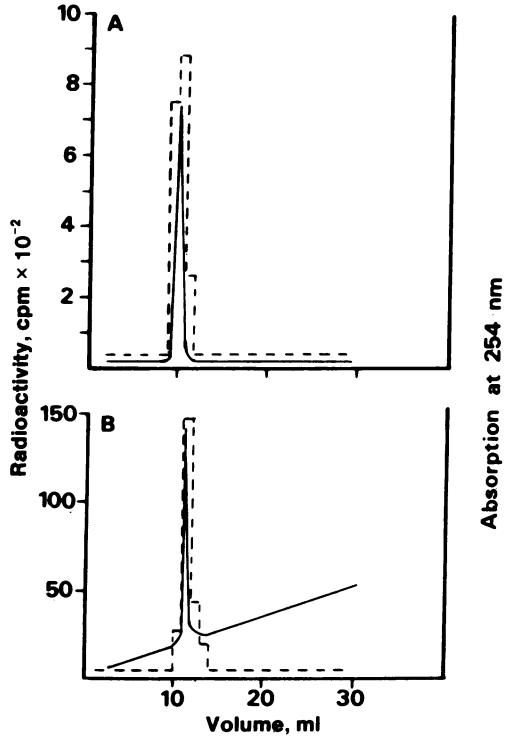

FIG. 3. HPLC of the putative 24,25(OH)$_2$[^{3}H]D_3 region after LH-20 column chromatography. Cultured calvarial cells were incubated with 25(OH)[^{3}H]D_3 for 2 hr. The vitamin D metabolites were extracted and chromatographed on Sephadex LH-20 (Fig. 2). The peak fractions in the appropriate region were then chromatographed. Elution profiles for synthetic 24,25(OH)$_2D_3$ (—) as followed by UV absorption at 254 nm and for the putative 24,25(OH)$_2$[^{3}H]D_3 (- - -) as followed by liquid scintillation counting are shown for two chromatographic systems: (*A*) Straight-phase HPLC using a hexane/isopropanol (90:10) solvent system at a flow rate of 1 ml/min and (*B*) reverse-phase HPLC using a mobile phase of methanol/water (gradient of 75:25 to 95:5 over 30 min) at a flow rate of 1 ml/min.

to bone (differentiated bone cells or their immediate precursors) rather than in endothelial cells or fibroblasts, because the latter, also common to liver, skin, and heart, did not produce either 1,25(OH)$_2D_3$ or 24,25(OH)$_2D_3$ *in vitro* (Table 1).

Peak IV was produced by cells from calvaria and kidney. Because the production of the metabolite was regulated by 1,25(OH)$_2D_3$, it might represent a specific product of biological significance. Peak IV has previously been shown to be produced by chicken, quail, and rat kidney cells (10, 13, 14) and has been identified in serum from rats (15) and quail (unpublished data). In contrast, metabolite V was produced by all cell types tested and did not appear to be regulated by 1,25(OH)$_2D_3$, suggesting that it may represent a nonspecific degradative product.

Effect of Incubation Time on Synthesis of 1,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$ by Cultured Calvarial Cells. 25(OH)[^{3}H]D_3 was hydroxylated by calvarial cells to 1,25(OH)$_2$[^{3}H]D_3 at a linear rate for incubations of up to 4 hr (Fig. 5). Similarly, 24,25(OH)$_2$[^{3}H]D_3 was produced in a linear manner. During our standard 2-hr incubation, 12–16% of the initial substrate was metabolized to more-polar products, of which approximately 15% was 1,25(OH)$_2D_3$ and approximately 60% was 24,25(OH)$_2D_3$.

Regulation of 25(OH)D_3:1- and 24-Hydroxylases in Cultured Calvarial Cells. Preincubation with 1,25(OH)$_2D_3$ altered the subsequent metabolism of 25(OH)[^{3}H]D_3 by cultured calvarial cells. The specific activity of the 25(OH)D_3:1-hydroxylase was reduced to 39% of the control value after cells from the same preparation had been preincubated with 10 nM 1,25(OH)$_2D_3$ for 8 hr (Table 2) and assayed at the same time. In contrast, the specific activity of the 24-hydroxylase was in-

Table 1. Distribution of 25(OH)D_3:1- and 24-hydroxylases in cultured chicken cells

Cell origin*	Specific activity, (fmol/min)/10^6 cells	
	24-Hydroxylase	1-Hydroxylase
Kidney	0.2†	7.2
Calvaria	3.8	1.2
Heart	<0.1‡	<0.1
Liver	<0.1	<0.1
Skin	<0.1	<0.1

* Cells were isolated from tissue pooled from two or three individuals.
† Two 60-mm dishes of cells were pooled for each determination.
‡ Limit of detection, 0.1 (fmol/min)/10^6 cells.

Table 2. Metabolism of 25(OH)D_3 by cultured embryonic calvarial cells

Treatment	*n*	Specific activity, (fmol/min)/10^6 cells		
		1-Hydroxylase	24-Hydroxylase	Metabolite IV
None	3*	0.81 ± 0.09†	3.14 ± 0.19	0.78 ± 0.10
Preincubation with 10 nM 1,25(OH)$_2D_3$	3	0.29 ± 0.02‡	7.69 ± 0.13‡	1.82 ± 0.02‡

* Two 60-mm dishes of cells were pooled for each determination.
† Mean ± SD.
‡ Differs from untreated, $P < 0.01$.

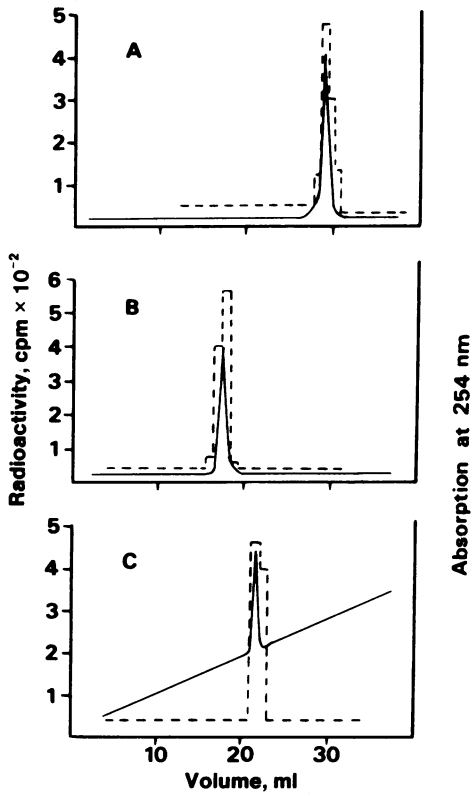

FIG. 4. HPLC of the putative $1,25(OH)_2[^3H]D_3$ region after Sephadex LH-20 column chromatography. Culture conditions were as for Fig. 3. Elution profiles for synthetic $1,25(OH)_2D_3$ (—) as followed by UV absorption at 254 nm and for the putative $1,25(OH)_2[^3H]D_3$ (---) as followed by liquid scintillation counting are shown for three chromatographic systems. (*A*) Straight-phase HPLC on a Zorbax-Sil column (4.6 mm × 25 cm) using a hexane/isopropanol (90:10) solvent system at a flow rate of 1 ml/min. (*B*) Straight-phase HPLC on the same column as in *A* using a hexane/isopropanol (87:13) solvent system at a flow rate of 1 ml/min; the $1,25(OH)_2D_3$ region had been recycled by rechromatographing it through the column four times. (*C*) Reverse-phase HPLC of the putative $1,25(OH)_2[^3H]D_3$ from the hexane/isopropanol (87:13) HPLC using a mobile phase of methanol/water (gradient of 75:25 to 95:5 over 30 min) at a flow rate of 1 ml/min.

creased by 245%, and the rate of production of metabolite IV was increased by 233%. Finally, the amount of metabolite V produced was not altered by $1,25(OH)_2D_3$.

Metabolites of $25(OH)[^3H]D_3$ Produced by Cells Isolated from Selected Chicken Embryo Organs. The tissue specificity of the $25(OH)D_3$:1- and 24-hydroxylases in 16-day embryos was determined by assaying enzyme activity in cells isolated from selected organs. The cells isolated from calvaria and kidney hydroxylated $25(OH)D_3$ to $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$, whereas the cells isolated from liver, skin, and heart did not appear to have this capacity (Table 1).

The specific activity of the $25(OH)D_3$:24-hydroxylase in calvarial cells was 3.8 (fmol/min)/10^6 cells, a value considerably larger than that for kidney cells [(0.2 fmol/min)/10^6 cells]. In contrast, the specific activity of the $25(OH)D_3$:1-hydroxylase in kidney cells was larger than that in calvarial cells [7.2 compared with 1.2 (fmol/min)/10^6 cells]. These results suggest that the distribution of the $25(OH)D_3$:1- and 24-hydroxylases within calvarial cells differs from that in kidney cells grown under identical conditions. However, the relative abundance of cells in either calvaria or kidney involved in metabolizing $25(OH)D_3$ is not known. Also, it is not known whether our cell culture conditions select for specific populations of cells, which could

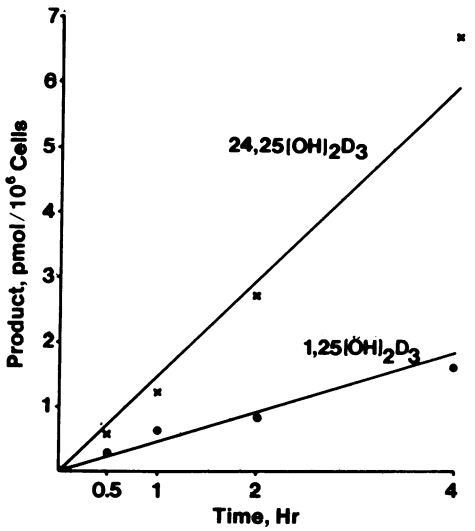

FIG. 5. Effect of incubation time on metabolism of $25(OH)[^3H]D_3$ by chicken embryo calvarial cells. $25(OH)[^3H]D_3$ (7 nM) was incubated with confluent cultures of cells, and the products were separated by chromatography.

be particularly important if the 1- and 24-hydroxylases are located in different cell populations. Based on these considerations, it will be difficult to evaluate the significance of differences in vitamin D metabolism in cells of renal and calvarial origin until specific populations of cells containing the $25(OH)D_3$:1- and 24-hydroxylases are isolated.

DISCUSSION

The results of this study demonstrate that cultured calvarial cells metabolize $25(OH)D_3$ to more-polar products, including $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$. The identity of these two metabolites is reasonably secure, based on the results of several complementary techniques: Sephadex LH-20 column chromatography, recycling on straight-phase HPLC, and straight-phase followed by reversed-phase HPLC.

A fundamental question raised by this study is whether the apparent participation of the skeleton in metabolism of vitamin D has a significant role in regulation of mineral homeostasis. Several lines of evidence suggest that this is the case: (*i*) $25(OH)D_3$, the principal circulating metabolite of vitamin D, is hydroxylated by calvarial cells to products that are of biologic importance [i.e., $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$]; (*ii*) the distribution of $25(OH)D_3$:1- and 24-hydroxylases in chicken embryos is organ-specific (i.e., the two enzymes are located in cells from kidney and calvaria but not in cells from liver, skin and heart); (*iii*) the *in vitro* metabolism of $25(OH)D_3$ by calvarial cells is altered dramatically by $1,25(OH)D_3$, a metabolite that has previously been shown to be a potent regulator of vitamin D metabolism *in vivo* (16) as well as by kidney cells in culture (10); and (*iv*) the specific activities of $25(OH)D_3$:1- and 24-hydroxylases in cultured calvarial cells are of the same order of magnitude as those in kidney cells.

Localization of $25(OH)D_3$:1-hydroxylase in calvarial cells appears to be discrepant with the fact that $1,25(OH)_2D_3$ has not been reported in plasma from either nephrectomized male rats or anephric humans (17, 18). There are, however, several possible explanations for this apparent discrepancy. These include the following: (*i*) calvarial cells produce $1,25(OH)_2D_3$ but do not release it into circulation; (*ii*) calvarial cells produce $1,25(OH)_2D_3$ and release it, but in quantities that are nondetectable by conventional assays; and (*iii*) synthesis of $1,25(OH)_2D_3$ by calvarial cells is species- or age-dependent (or

both). Regardless of which of these possibilities is correct, there is precedence for extrarenal 25(OH)D_3:1-hydroxylase activity in mammals and birds: 1,25$(OH)_2D_3$ is synthesized by rat placenta (5–7) and by chicken chorioallantoic membrane (19).

There are other *in vivo* observations that are consistent with the hypothesis that the skeleton is a site of 1,25$(OH)_2D_3$ production. 1,25$(OH)_2D_3$ and PTH are important physiological determinants of osteoclast number (reviewed in ref. 20). In vitamin D-deficient rats [in which serum 25(OH)D_3 and 1,25$(OH)_2D_3$ are markedly reduced], 7-fold increases in PTH are not associated with an increase in osteoclast number (20–22). In sharp contrast, in anephric patients [in whom 25(OH)D_3 levels are normal], PTH elevation no greater than that seen in vitamin D-deficient rats is associated with a marked increase in osteoclast number (23–25). Apart from reservations concerning species differences, these findings suggest that adequate 25(OH)D_3 is required for PTH-mediated increases in osteoclast number, possibly because of further metabolism to 1,25$(OH)_2D_3$ by the skeleton.

Production of active vitamin D metabolites in vitamin D target organs may provide a means of local regulation of the response to vitamin D. This duplication of synthetic activity may provide a more versatile control of calcium and phosphorus metabolism. For example, under certain physiological conditions there may be some advantage for a differential response by gut and bone, as mediated by different amounts or types of locally determined vitamin D metabolites. Moreover, it would not be surprising that other target organs, such as gut, participate in the metabolism of 25(OH)D_3. In keeping with this concept, the intestine has been shown to produce 24,25$(OH)_2D_3$ (26).

In summary, we have identified 25(OH)D_3:1-hydroxylase activity in cultured chicken calvarial cells. This finding constitutes evidence that production of 1,25$(OH)_2D_3$ is more widespread than previously believed. Furthermore, the local production of active metabolites of vitamin D by target organs such as bone raises interesting questions as to the role of these tissues in mediating vitamin D action. The answers to these questions may provide a new dimension to regulation of mineral metabolism by vitamin D.

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