The Metabolism of Vitamin D₃ in the Chick*

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SUMMARY

Physiological doses of radioactive vitamin D₃ are administered to vitamin D-deficient chicks 16 hours prior to the preparation of lipid extracts of small intestine, plasma, kidney, and bone. Analysis of the vitamin D metabolite pattern is carried out via column chromatography on silicic acid, Sephadex LH-20, and Celite (liquid-liquid partition), countercurrent distribution and treatment with periodic acid. The predominant form of the vitamin in the intestine is a polar metabolite (Peak 4B) which is homogeneous in all four separation systems, migrates in the area of known dihydroxy-D₃-vitamins, loses 40% of its tritium in the course of metabolic formation from [1α,2α-3H₂]vitamin D₃ and is insensitive to cleavage by periodate. These data are consistent with the recent identification of Peak 4B as 1,25-dihydroxy-vitamin D₃ by Holick, Schnoes, and DeLuca (1971) (Proc. Nat. Acad. Sci. U. S. A. 68, 803) and the original proposal by Haussler, Myrtle, and Norman (1968) (J. Biol. Chem. 243, 4055) that this metabolite is the active form of vitamin D in the intestine.

The major metabolite present in plasma, kidney, and bone is 25-hydroxy-vitamin D₃, but significant amounts of 1,25-dihydroxy-vitamin D₃ are detected in all three sites. The plasma dihydroxy-vitamin D₃-metabolite profile differs markedly from that of the kidney and bone in that the predominant species is 25,26-dihydroxy-vitamin D₃, with 21,25-dihydroxy-vitamin D₃ also being present in measurable quantities. Virtually all dihydroxy-vitamin D₃ in kidney and bone exists as the 1,25-dihydroxy derivative. The presence of 1,25-dihydroxy-vitamin D₃ in kidney strengthens the proposal that it is produced metabolically from 25-hydroxy-vitamin D₃ by a renal enzyme. The association of 1,25-dihydroxy-vitamin D₃ with bone and its considerable activity in promoting skeletal dissolution, raises the question as to whether 25-hydroxy-vitamin D₃ or the 1,25-dihydroxy-sterol represents the active form directing bone resorption.

Considerable effort has been devoted in the past 5 years to the study of the metabolism of vitamin D₃. Blunt et al. (1, 2) first identified the major metabolite of vitamin D₃ in plasma as 25-hydroxy-vitamin D₃ and indicated that it was 1½ times as active as vitamin D₃ in curing rickets and acted faster than the native vitamin in promoting intestinal calcium transport and bone resorption. Since 25-OH-D₃ acted directly on isolated intestine (3) and bone in tissue culture (4), and was incorrectly thought to represent the form associated with the intestinal nuclear fraction (5), DeLuca postulated that 25-OH-D₃ was the metabolically active form of vitamin D₃ (6-8). Haussler et al. (9, 10) showed that a sterol more polar than 25-OH-D₃ was specifically associated with the target intestinal mucosa. This more polar form, denoted Peak 4B, had activity greater than vitamin D and appeared in the gut prior to the onset of vitamin D-mediated intestinal absorption of calcium. Moreover, Peak 4B was found to be selectively associated with the chromatin fraction of the nucleus via a non-histone-protein receptor molecule (11). Since vitamin D had been hypothesized to function through the induction RNA and protein synthesis (12, 13), this localization of Peak 4B in the nuclear chromatin was consistent with the hypothesis that Peak 4B was the form of vitamin D initiating events at the molecular level in the intestine.

Kodieck and co-workers (14-16) confirmed the findings of Haussler et al. (9) and, in addition, discovered that [1α-3H]vitamin D₃ loses virtually all of its tritium during metabolism to Peak 4B (denoted Peak P by these workers). They also reported that 25-OH-D₃ was an intermediate in the conversion of vitamin D₃ to Peak 4B (17) and suggested that Peak 4B might contain an oxygen function at carbon 1 in addition to the hydroxyl function at carbon 25.

Further indication that Peak 4B represented the active form of the vitamin in the gut was provided when Haussler et al. (18, 19) demonstrated that Peak 4B acted 3 times faster than either 25-OH-D₃ or vitamin D₃ and was at least 5 times as active as the native vitamin in stimulating intestinal calcium transport. Positive identification of Peak 4B as 1,25-dihydroxy-vitamin D₃ (1,25-di-OH-D₃) was accomplished by Holick et al. (20) using extracts from 1450 chick guts. Lawson et al. (21) and Norman et al. (22) confirmed this identification employing metabolite generated in vitro using the renal enzyme capable of catalyzing the formation of Peak 4B from 25-OH-D₃ (23).

Although it is now apparent that 1,25-di-OH-D₃ is the active form which mediates intestinal calcium absorption, its exact

* The abbreviations used are: 25-OH-D₃, 25-hydroxy-vitamin D₃; 1,25-di-OH-D₃, 1,25-dihydroxy-vitamin D₃ (also referred to as Peak 4B); Peak P, and intestinal Peak V; 21,25-di-OH-D₃, 21,25-dihydroxy-vitamin D₃; 25,26-di-OH-D₃, 25,26-dihydroxy-vitamin D₃; CCD, countercurrent distribution; k, partition coefficient.
mechanism of action at the level of the intestine remains unclear. Previous studies with vitamin D₃ indicated that the vitamin (metabolite) functioned via induction of RNA and protein synthesis (12, 13). Vitamin D increases intestinal RNA synthesis (24) and the template efficiency of intestinal chromatin (25). Wasserman and co-workers (26-28) have isolated and extensively characterized a calcium-binding protein from intestine which is induced by vitamin D. In addition, vitamin D increases the activity of alkaline phosphatase (29) in the intestinal brush border membrane, and this enzyme is capable of hydrolyzing ATP and pyrophosphate (30-32). The functional involvement of these induced proteins in the transport of calcium and the exact site and mode of action of 1,25-di-OH-D₃ in this system remain to be elucidated. Direct experiments with 1,25-di-OH-D₃, preferably with isolated systems, should lead to the answers to these questions.

Unfortunately, previous studies of vitamin D metabolism have centered on one specific tissue. Investigations by Haussler et al. (9, 10) were concerned primarily with the physiologically important metabolites in the intestine. Extensive studies by DeLuca and co-workers were initially limited to characterization of plasma metabolites and led to the identification of 25-OH-D₃ (1), 21,25-dihydroxy-vitamin D₂ (21,25-di-OH-D₃) (33), and 25,26-dihydroxy-vitamin D₃ (25,26-di-OH-D₃) (34). These workers have not reported the unequivocal detection of the intestine's active metabolite (1,25-di-OH-D₃) in plasma or in tissues other than intestine. Another question which has not been answered is whether 21,25-di-OH-D₃ and 25,26-di-OH-D₃ are associated with tissues which carry out calcium translocation.

The purpose of the present report is to present a comprehensive study of the metabolites of vitamin D₃ in the tissues of the chick which play a role in calcium homeostasis. The significance of 1,25-di-OH-D₃ in the intestine is confirmed and extended and new evidence is provided which points to the importance of 1,25-di-OH-D₃ in bone resorption.

MATERIALS AND METHODS

Animals—White leghorn cockerels (Moyer's Chicks, Quakertown, Pa.) were utilized in all experiments. Vitamin D-deficient chicks were raised on a diet described elsewhere (13) and were used for investigation when they became rachitic during their fourth week of development.

Specialized Chemicals—[4,14C]Vitamin D₃ (specific activity 23.3 mCi per mmole) and [1α,2α-3H]vitamin D₃ (specific activity 577 mCi per mmole) were obtained from Amersham-Searle. [26,27-3H]25-OH-D₃ (specific activity 315 mCi per mmole) is a product of New England Nuclear, and nonradioactive 25-OH-D₃ was a gift from Dr. A. W. Norman. Crystalline vitamin D₃ was purchased from Calbiochem. Periodic acid (H₃IO₆) was secured from Mann Research Laboratories. α-Ecdysone was a kind gift from Dr. John Siddall. Silicie acid (Bio-Sil HA, minus 325 mesh) was purchased from Calbiochem, and Celite (Johns-Manville) was obtained from the A. H. Thomas Co., Philadelphia. All other chemicals and solvents used were reagent grade. Solvents employed in countercurrent distribution, Sephadex LH-20 chromatography, and liquid-liquid partition chromatography on Celite were glass-distilled just prior to the experiment.

Extraction Techniques—Labeled sterols were dissolved in 0.2 ml of 1,2-propanediol and were administered either orally or intracardially. The chicks were killed by decapitation, the blood was collected with the aid of a funnel, and the small intestine, kidneys, and tibia were removed immediately. The small intestine was rinsed with isotonic sucrose by forcing the solution through the gut with a syringe; pairs of tibia and fibula were freed of adhering muscle and slit endwise to remove the bone marrow. Plasma was prepared from heparinized blood by centrifugation at 10,000 x g for 10 min.

Extraction of the small intestine, plasma, kidneys, or bone with acetonel-dichloroethane (2:1) was carried out in a 1-gallon Waring Blender at a setting of medium for 2 min. Four volumes of acetonel-dichloroethane per g wet weight of tissue were used in each extraction. The initial acetonel-dichloroethane extract was filtered, and, following a second identical extraction of the residue, the combined extracts were taken to dryness with a flash evaporator. The lipid residue was then solubilized in a minimal volume of diethyl ether, clarified by centrifugation for 10 min at 10,000 x g at 5°, and then used for chromatograph separations.

Sephadex LH-20 Column Chromatography—Sephadex was activated by heating to 120° for 24 hours just prior to use. Sephadex (25 g) was suspended in hexane and formed into a column (1.8 x 17.5 cm) with the aid of 7 pounds nitrogen pressure for packing. The sample was applied in a small volume of hexane-diethyl ether (1:1), and elution was carried out by the following schedule: 125 ml of hexane-ether (1:1), Fraction 1; 100 ml of diethyl ether, Fractions 2 to 5; 150 ml of diethyl ether-dichloroethane (1:1), Fractions 6 to 11; and 125 ml of acetone, Fraction 12. Each column was run under 4 pounds of nitrogen pressure and with a flow rate of 5 ml per min.

Countercurrent Distribution—Distribution studies were carried out on a H. O. Post, model 2B, 100-tube automated machine; 100 transfers were performed with a 10-ml mobile (upper) phase and a 10-ml stationary (lower) phase. A solvent system of ethyl acetate-hexane-ethanol-water (6:15:7:5:12.5) was utilized in all CCD runs.

Sephadex LH-20 Column Chromatography—Sephadex LH-20 chromatography on Sephadex LH-20 was performed using the method of Holick and DeLuca (35).

Celite Column Chromatography—Celite was washed with concentrated HCl and organic solvents, and the fine particles were removed prior to use (36). Each column contained 11 g of Celite prepared in the following fashion: 5 volumes of 10% ethyl acetate in hexane were equilibrated with 1 volume of 45% water in ethanol. The lower water-ethanol phase served as a stationary phase and 11 ml were mixed into the 11 g of Celite. The Celite was then suspended in excess ethyl acetate-hexane (upper phase) and packed with a rod into a homogeneous column (1 x 36 cm). Samples were applied in 0.4 ml of ethyl acetate-hexane and the column was eluted with ethyl acetate-hexane as a mobile phase. One to two pounds nitrogen pressure were applied to achieve a flow rate of 0.4 ml per min.

Periodate Oxidation—Reactions of metabolites with periodic acid were carried out by dissolving 12.5 mg of H₂IO₆ in 5 ml of lower phase of the CCD solvent system (water-ethanol). The metabolite was then added and allowed to react for 15 min at room temperature. The reaction was terminated by adding the reaction mixture to position "O" of the CCD machine and immediately initiating the transfer procedure. Periodate remained in the initial tube while metabolite migrated along the train to their appropriate positions after 100 transfers.
samples were counted to 2% error and counts in the intestine, plasma, kidney, and bone were 89%, 76%, and 71% in the intestine, plasma, kidney, and bone based mixture containing 4 g of 2,5-diphenyloxazole (PPO) and per min of tritium or 14C, or both, were converted to disintegrations per min by the dual-isotope, internal standardization method as previously described (9).

The samples were solubilized in 10 ml each of a toluene-liquid scintillation system. Entire fractions or appropriate aliquots of the sterols were dried in the vial under a stream of air. The samples were injected into the chromatography system. Extraction and chromatography were carried out as described in the text. Each column represents the chromatography of pooled extracts from 20 chicks. As is detailed in the text, Peaks 4B are eluted with 100% acetone. Recovery of applied radioactivity was 84%, 88%, 76%, and 71% in the intestine, plasma, kidney, and bone, respectively; further elution of the columns with increasing concentrations of methanol in acetone eluted two to three minor, more polar peaks. The more polar metabolites have been noted in other studies (9, 10, 33, 34), but they are quantitatively unimportant in the present context.

Experiments with model compounds which were not expected to be altered by this treatment, such as 25-OH-D3, indicated that there was conservation of these steroids. Steroids with vicinal hydroxyl groups, such as α-ecdysone, were at least 80% cleaved under these conditions.

Radioactivity Determinations Tritium and 14C radioactivity were determined by counting samples in a Packard Tri-Carb liquid scintillation system. Entire fractions or appropriate aliquots of the sterols were dried in the vial under a stream of air. The samples were solubilized in 10 ml each of a toluene-based mixture containing 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) per liter of toluene. Samples were counted to 2% error and counts per min of tritium or 14C, or both, were converted to disintegrations per min by the dual-isotope, internal standardization method as previously described (9).

RESULTS

In order to assess the quantitative importance of metabolites of vitamin D3 after a physiological dose of the vitamin, a dose level of 20 i.u.1 was chosen. [4,14C]Vitamin D3 was employed

1 One international unit of vitamin D3 is equivalent to 65 pmoles

to eliminate misinterpretation of data because of tritium loss during metabolism of the various preparations of [3H]vitamin D3. In the experiments summarized in Fig. 1, chicks were given either oral or intracardial doses of 20 i.u. of [4,14C]vitamin D3 and were killed 10 hours later. Following extraction of the tissues in question, chromatographic evaluation of the metabolite pattern was performed with silicic acid chromatography. As previously reported (9, 10, 18, 19), the major metabolite in the intestine is peak 4Br. In contrast to the intestine, the plasma, kidney, and bone contain primarily 25-OH-D3. The 25-OH-D3 in all four sites has been identified by chromatography with standard preparations of radioactive and nonradioactive 25-OH-D3 (10, 18, 19). However, the plasma, kidney, and bone also have significant quantities of radioactivity migrating similarly to Peak 4Br. Pending further characterization by additional chromatography, these metabolites are designated Peak 4B with a subscript identifying the organ from which the metabolite(s) is obtained.

Further investigation of the Peaks 4B from the various tissues was accomplished by chromatography on CCD, Sephadex LH 20, and Celite. Fig. 2 illustrates distribution of Peak 4Br on CCD with added [26,27-3H2]25-OH-D3. The reference 25-OH-D3 migrates far along the train with a partition coefficient (k) of 15, while the more polar Peak 4Br forms a symmetrical peak near the center of the train with a k of 0.9. The shape of the Peak 4Br pattern is virtually identical with the theoretical distribution pattern predicted for one compound with a k of 0.9.

When Peak 4Br is analyzed in the identical CCD system, a significantly different pattern results (Fig. 3, upper portion). Peak 4Br yields a diffuse and skewed peak with an average k of 1.8, indicating that Peak 4Br is probably a composite of several metabolites. Since Peak 4Br has a larger k than Peak 4Bl, it is less polar than Peak 4Bl, and qualitatively different as previously described (9).

Fig. 3. CCD of 14C-labeled Peak 4Bl and standard [3H]25-OH-D3. 14C Labeled Peak 4Bl was secured following silicic acid chromatography in an experiment similar to that pictured in Fig. 1 and combined with commercially obtained [26,27-3H2]25-OH-D3. The metabolites were separated via CCD as described in the text; recovery was 84% for 14C and 95% for 3H.
Fig. 3. CCD of Peak 4Br before and after treatment with periodic acid. A sample of "H-labeled Peak 4Br secured in an experiment analogous to that pictured in Fig. 1 was divided into two equal parts. One portion was run without prior treatment (upper graph); the other was previously treated for 15 min with periodic acid as described in the text and then distributed in the CCD machine employing the standard solvent system (lower graph). Recovery of radioactivity was 86% in both runs.

Previously established by tritium loss experiments (14). Additional information as to the nature of Peak 4Brp is provided by periodate oxidation prior to CCD. As is seen in the lower half of Fig. 3, the majority of Peak 4Brp is sensitive to cleavage by periodate. The migration pattern of the reaction products provides a fortuitous opportunity to identify the predominant metabolite present in Peak 4Brp. The product, which migrates with a $k$ of 15.5, is very similar to 25-OH-D$_3$ in its chromatographic behavior (see Fig. 2). The only sterol which will yield a 25 hydroxy-D$_3$-like compound upon periodate treatment is 25,26-di-OH-D$_3$, with the product being 25-keto, 26-normethyl-vitamin D$_3$. Thus, at this dose level, the major dihydroxy-vitamin D$_3$ in rachitic chick plasma is 25,26-di-OH-D$_3$.

Peak 4Br$_1$ was also examined after periodate oxidation, both to show that it differs from 25,26-di-OH-D$_3$ of plasma and to probe the possibility that vicinal hydroxyl formation at carbon 2 and carbon 3 could explain the loss of tritium (14). Fig. 4 pictures Peak 4Br$_1$ run on the standard CCD system before and after treatment with periodate. Peak 4Br$_1$ is virtually insensitive to cleavage except for a small fraction of about 4% which represents 25,26-di-OH-D$_3$. The insensitivity of Peak 4Br$_1$ to periodate oxidation indicates that carbon 2 does not contain an oxy-function and further points to carbon 1 as the site for a second additional hydroxyl function (14). These data are consistent with a structure of 1,25-di-OH-D$_3$ for Peak 4Br$_1$, and this assumption has been substantiated by the recent identification of Peak 4Br$_1$ as 1,25-di-OH-D$_3$ in three laboratories (20-22).

Additional comparison between Peak 4Br$_1$ and Peak 4Brp was accomplished via liquid-gel partition chromatography on Sephadex LH-20. Simultaneous chromatography of $^{14}$C-labeled Peak 4Br$_1$ and $^3$H-labeled Peak 4Brp obtained in experiments similar to the one pictured in Fig. 1 is shown in Fig. 5. Peak 4Br$_1$ again migrates as a single peak, whereas Peak 4Brp is resolved into at least three metabolites. 21,25-di-OH-D$_3$ and Peak Vi, as components of plasma Peak 4Br, are tentatively located by comparison with the results of Holick and DeLuca (35). Excellent recoveries and separations are obtained by this method with one significant exception. The majority of Peak 4Brp (25,26-di-OH-D$_3$) was not resolved from Peak 4Br$_1$ (1,25-di-OH-D$_3$). This fact puts severe limitations on this method for the detection of 1,25-di-OH-D$_3$ in plasma samples and precludes its use for unequivocally elucidating the species of di-OH-D$_3$ present in other tissues such as bone and kidney.

Celite rechromatography of Peak 4Br proved to be the most powerful tool in resolving and identifying the various dihydroxy-D$_3$-vitamins. Fig. 6 illustrates chromatography of $^{14}$C-labeled Peak 4Br$_1$ and $^3$H-labeled Peak 4Brp together on the same col-
Vitamin D Metabolites in Chick
Vol. 247, No. 8

Fig. 5. Liquid-gel partition chromatography of 3H-labeled Peak 4Br and 14C-labeled Peak 4Bp. Peaks 4Br and 4Bp were obtained by silicic acid column chromatography and then simultaneously chromatographed on Sephadex LH-20 according to the procedure of Holick and DeLuca (35). Recovery of 14C and tritium applied to the column was 95% and 92%, respectively. The small initial peak emerging in Fraction 7 represents slight contamination of both Peak 4Br and Peak 4Bp with 25-OH-D3.

Fig. 6. Liquid-liquid partition chromatography of 14C-labeled Peak 4Br and 3H-labeled Peak 4Bp. Peaks 4Br and 4Bp were harvested from silicic acid columns (Fig. 1) and then simultaneously chromatographed on Celite as described in the text. Portions of the 3H-labeled Peak 4Bp were saved for use as markers in the Celite columns illustrated in Figs. 7 and 8. The initial peak emerging in 1 hold-up volume (Fractions 3 and 4) is 25-OH-D3 contamination of the Peak 4Br and Peak 4Bp. Recoveries of radioactivity were 74% for 3H and 72% for 14C.

Fig. 7. Liquid-liquid partition chromatography of 14C-labeled Peak 4Br along with added 3H-labeled Peak 4Bp to serve as a marker. Clearly the majority of kidney Peak 4B exists as 1,25-di-OH-D3, a small amount of 25,26-di-OH-D3 is also found in the kidney. Since 1,25-di-OH-D3 is postulated to be produced exclusively by a renal enzyme system (23), this result supports the concept that 1,25-di-OH-D3 is present in the kidney in vivo. Because the skeleton is considered a second target organ for vitamin D, it was of interest to examine the nature of the dihydroxy-D3-vitamins present at this site. Fig. 8 illustrates data which indicate that virtually all of the bone Peak 4B consists of 1,25-di-OH-D3. Very small amounts of 25,26-di-OH-D3 and 21,25-di-OH-D3 are present in the bone. The finding of significant quantities of 1,25-di-OH-D3 in bone raises the question as to whether the intestinally active steroid might also represent the active form of vitamin D3 at the level of the skeleton. Biological assays on 1,25-di-OH-D3 in terms of bone dissolution were carried out in order to answer this question. As can be seen in Table 1, 1,25-di-OH-D3 has considerable activity in promoting bone resorption. 1,25-di-OH-D3, isolated from the guts of 400 rachitic chicks which had previously received 50 i.u. of [3H]vitamin D3 was purified by solvent partitions as previously described (19) and then via silicic acid and Celite chromatography as in Figs. 1 and 6. The purified 1,25-di-OH-D3 was tested initially at a level of 65 pmoles with chicks maintained on a standard rachitogenic diet (Table 1). Only 10 hours after administration, this low dose produced a significant rise in plasma Ca++. However, since the chicks were maintained on a normal diet with respect to calcium, it is not possible

![Image of chromatography](https://example.com/figure5)

![Image of chromatography](https://example.com/figure6)

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![Image of chromatography](https://example.com/figure8)

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1 M. R. Haussler, unpublished results.
was determined 24 hours later with a Technicon Auto-Analyzer. Vitamin D$_3$ and metabolites were administered orally in 0.2 ml of 1,2-propanediol, and plasma calcium was measured.

Bone resorption (2, 38) was promoted by Vitamin D$_3$ and metabolites. Peak 4Br, and 3H-labeled Peak 4Bp on Celite. Recoveries of radioactivity were 73% for 3H and 68% for 1%.

**TABLE I**

**Biological activity of 1,25-di-OH-D$_3$ in promoting bone resorption in chicks**

Animals employed in this experiment were grown for 3 weeks on the standard rachitogenic diet and then either used or transferred for 3 days to a similar diet containing <0.1% calcium. Chicks transferred to the low calcium diet showed a further drop in plasma calcium concentration below the rachitic value. In all of these chicks grown on the low calcium diet is regarded as a true measure of sterol-induced skeletal resorption. Coupled with a recent report by Tanaka and DeLuca (40), that 1,25-di-OH-D$_3$ proved to be more active than vitamin D$_3$ in mediating skeletal resorption, these data suggest that 1,25-di-OH-D$_3$ plays a metabolic role in calcium translocation at the level of bone.

An over-all summary of the amounts of the various physiologically active metabolites of vitamin D$_3$ present in intestine, plasma, kidneys, and bone is provided in Table II. 1,25-di-OH-D$_3$ dominates the intestinal pattern and is concentrated in this organ in relation to other sites. The plasma carries primarily 25-OH-D$_3$, but considerable amounts of all other metabolites are present. The plasma is unique in that it is the only location where 21,25-di-OH-D$_3$ and 25,26-di-OH-D$_3$ are found in appreciable amounts. The kidney contains primarily 25-OH-D$_3$, presumably as a precursor for the production of 1,25-di-OH-D$_3$ at this site. It is not known whether 25-OH-D$_3$ or 1,25-di-OH-D$_3$, or both, have physiological effects on the calcium-transporting system in the kidney itself. The bone metabolite pattern is characterized by a predominance of 25-OH-D$_3$ and substantial amounts of 1,25-di-OH-D$_3$. It is possible that both metabolites regulate bone function.

**DISCUSSION**

The present communication reports the combined use of highly refined chromatographic systems for the separation of vitamin D$_3$ metabolites and physiological doses of the vitamin to examine the metabolism of vitamin D in the rachitic chick. Special attention is directed toward vitamin D target organs as well as other sites of calcium regulation. It is concluded that there are at least four biologically significant metabolites of vitamin D$_3$. Fig. 9 details the sequence of formation of these metabolites from the parent vitamin. The initial step involves hydroxylation at carbon 25. This reaction is catalyzed primarily by a liver enzyme (41), but we have obtained recent evidence that the kidney is another site of 25-OH-D$_3$ formation from vitamin D$_3$. The kidney is therefore capable of converting native vitamin D$_3$ completely to 1,25-di-OH-D$_3$. Although 25-OH-D$_3$ was previously thought to be the active form of the vitamin, it is now generally accepted that this
metabolite represents an intermediate in the synthesis of the final functional sterol, 1,25-dihydroxy-cholecalciferol (25-OH-D3). Production of 1,25-di-OH-D3 occurs exclusively in the kidney (23), qualifying the kidney as an endocrine organ which produces the sterol hormone that controls calcium transport.

The site(s) of production of the 21,25- and 25,26-dihydroxyvitamin D metabolites is not known, and these metabolites are found chiefly in the plasma. The present report documents the fact that neither form is present in significant amounts in any of the tissues involved in calcium transport (Figs. 6 to 8). Yet they do appear in plasma even after low physiological doses of vitamin D3, indicating that they probably play some part in the overall metabolic mechanism of vitamin D action. Their submaximal biological activity may explain the observation that patients with chronic renal failure and those on hemodialysis show decreased absorption of calcium from the gut and suffer from secondary hyperparathyroidism and bone demineralization (45-47). It is likely that 1,25-dihydroxy-D3 will become a valuable supplement to maintain renal concentration of this metabolite in vivo (Fig. 7). This observation explains the observation that patients with chronic renal failure and those on hemodialysis show decreased absorption of calcium from the gut and suffer from secondary hyperparathyroidism and bone demineralization (45-47). It is likely that 1,25-dihydroxy-D3 will become a valuable supplement to maintain renal concentration of this metabolite in vivo (Fig. 7).

The detection of 1,25-dihydroxyvitamin D3 in plasma (Fig. 6) has several important implications. The metabolite is presumably in transit from the kidney to the target intestine and possibly to the bone. The fact that 1,25-dihydroxyvitamin D3 circulates via the blood argues that it is both the active form and the circulating active form. The later designation has been put on 25-OH-D3 by DeLuca and co-workers (20, 42) primarily because 25-OH-D3 is synthesized in the kidney is supported by our finding of a high renal concentration of this metabolite in vivo (Fig. 7). This concept explains the observation that patients with chronic renal failure and those on hemodialysis show decreased absorption of calcium from the gut and suffer from secondary hyperparathyroidism and bone demineralization (45-47). It is likely that 1,25-dihydroxy-D3 will become a valuable supplement to maintain renal concentration of this metabolite in vivo (Fig. 7).

The use of Sephadex LH-20 columns (35) has been employed as an effective means of separating vitamin D3, 25-OH-D3, and several of the more polar metabolites. But as has been pointed out in the present study (Fig. 5), resolution of 25,26-dihydroxyvitamin D from 1,25-dihydroxyvitamin D3 cannot be achieved with this system. Combination of this technique and a preliminary separation of vitamin D3, 25-OH-D3, and the dihydroxy-D3-fraction on silicic acid or Sephadex LH-20, this method permits the complete chromatographic evaluation of vitamin D3 and its known metabolites. Based on our initial studies with countercurrent distribution, we have devised a liquid-liquid partition separation procedure on columns of Celite which is capable of separating all of the heretofore identified dihydroxy-D3-vitamins. A solvent system similar to that employed for CCD (Figs. 2 and 3) is utilized, with the Celite column increasing strikingly the number of "theoretical plates." Once the routine homogeneous packing of the columns is mastered, this technique performs perfectly reproducibly. It has an advantage over previous Celite systems (9, 33) in that the metabolites emerge (earlier) within 2 to 6 hold-up volumes without considerable loss of resolving power. Combined with a preliminary separation of vitamin D3, 25-OH-D3, and the dihydroxy-D3-fraction on silicic acid or Sephadex LH-20, this method permits the complete chromatographic evaluation of a given metabolite mixture.

We have substantiated our identification of the metabolites in the Celite chromatography system (Figs. 6 to 8) by additional chromatography not detailed under "Results." The initial peak emerging in Fractions 3 and 4 on Celite migrates identically with 25-OH-D3 in three chromatography systems (1, 18, 35). The second peak is resolved into 21,25-dihydroxyvitamin D3 and Peak VB either by chromatography on Sephadex LH-20 as in Fig. 5 or on a Celite system devised for 21,25-dihydroxyvitamin D3 (37). The isolated 25,26-dihydroxyvitamin D3 peak is the only peak susceptible to periodate oxidation prior to rechromatography on either Sephadex LH-20 or Celite. Furthermore when the 4Bp fraction from an experiment such as that pictured in Fig. 5 is isolated and chromatographed in Celite, it is resolved into 26,25-dihydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 as expected. The cross-checks provided by this additional chromatography strengthen the assignment of structures to the various peaks.

The detection of 1,25-dihydroxyvitamin D3 in plasma (Fig. 6) has several important implications. The metabolite is presumably in transit from the kidney to the target intestine and possibly to the bone. The fact that 1,25-dihydroxyvitamin D3 circulates via the blood argues that it is both the active form and the circulating active form. The later designation has been put on 25-OH-D3 by DeLuca and co-workers (20, 42) primarily because 25-OH-D3 is quantitatively more dominant in the plasma. In addition, the detection of significant amounts of 1,25-dihydroxyvitamin D3 in plasma suggests that a competitive binding assay or radioimmunoassay can be devised for this metabolite in blood samples. Such an assay would be of great utility in diagnosing defects in vitamin D metabolism in patients with clinical disorders in calcium homeostasis.

The proposal by Fraser and Kodicek (23) that 1,25-dihydroxyvitamin D3 is synthesized in the kidney is supported by our finding of a high renal concentration of this metabolite in vivo (Fig. 7). This concept explains the observation that patients with chronic renal failure and those on hemodialysis show decreased absorption of calcium from the gut and suffer from secondary hyperparathyroidism and bone demineralization (45-47). It is likely that 1,25-dihydroxyvitamin D3 will become a valuable supplement to main-
tain a normal calcium balance in such cases where the metabolic formation of 1,25-di-OH-D$_3$ is disrupted by kidney disease and uremia.

Detection of 1,25-di-OH-D$_3$ in bone implies that the intestinal active metabolite may also function at the skeleton. The localization of 1,25-di-OH-D$_3$ in bone is not as striking as is seen in the intestine (Table II), but sufficient quantities are present to warrant a search for specific receptors as has been done in the intestine (9, 11). Preliminary data indicate that specific receptor systems may exist in bone for both 25-OH-D$_3$ and 1,25-di-OH-D$_3$ (48); this perhaps represents one receptor with a low level of specificity (i.e., binds both forms). Further binding studies are required before a conclusion can be reached. The other approach which led to acceptance of 1,25-di-OH-D$_3$ as the active form in the intestine is the assessment of the relative biological potency and kinetics of action of the metabolites in question. As can be seen in Table I, 1,25-di-OH-D$_3$ has considerable activity in promoting bone resorption and is more active than 25-OH-D$_3$ in this single point assay. Recent findings of Tanaka and DeLuca (40) indicate that 1,25-di-OH-D$_3$ acts more rapidly than 25-OH-D$_3$, but both metabolites have approximately equal potency. Additional experiments with isolated bone in culture (4) are necessary to elucidate the active metabolite in the skeleton. Perhaps 25-OH-D$_3$ is able to function as a more efficient substitute for 1,25-di-OH-D$_3$ in the bone system than it is in the gut. Thus, a mass-action effect of 25-OH-D$_3$ to simulate 1,25-di-OH-D$_3$ may explain its direct activity in bone (4) and in the isolated intestine (3).

A general picture of the metabolism of vitamin D now exists, but a complete understanding of this vitamin awaits the elucidation of numerous details related to the production and function of the various metabolite forms. Little information is available concerning the enzymes which catalyze metabolic alterations of the native vitamin. The discovery of physiological and pharmacological factors which control these enzyme reactions could be of great importance to our over-all knowledge of calcium metabolism. Finally, the biochemical mechanisms of action of the hormonal form(s) of vitamin D remain as poorly understood phenomena and will be the topic of considerable future research.

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