Isolation and Characterization of 1α-Hydroxy-23-carboxytetranorvitamin D: A Major Metabolite of 1,25-Dihydroxyvitamin D_3†

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ABSTRACT: The in vivo side-chain oxidation of 1α,25-dihydroxyvitamin D_3 was investigated by using a double-label radiotracer technique. Rats dosed with 1α,25-dihydroxy-[3α-^{3}H]vitamin D_3 and 1α,25-dihydroxy[26,27-^{14}C]vitamin D_3 produced compounds with a high $^3H/^{14}C$ ratio. These compounds were found in sizable quantities in intestine and liver within 3 h after dosing. The major side-chain oxidized metabolite migrated as an acid on DEAE-Sephadex chromatography and contained no ^{14}C. Methyl esterification of this compound with diazomethane proceeded in good yield and rendered the compound more amenable to chromatographic purification. The metabolite was isolated in several steps from rats dosed with 1 μg of 1α,25-dihydroxy[3α-^{3}H]vitamin D_3. The metabolite was obtained in pure form as the methyl ester and was positively identified as 1α,3β-dihydroxy-24-nor-9,10-seco-5,7,10(19)cholatrien-23-oic acid. The trivial name calcitroic acid is proposed for this major side-chain oxidized metabolite of 1,25-dihydroxyvitamin D_3.

It is widely accepted that vitamin D must undergo metabolism in vivo to express its physiological activities (DeLuca & Schnoes, 1976). 1α,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$],[1] formed by a tightly controlled 1α-hydroxylation in the kidneys [for reviews, see Kodicek (1974), DeLuca (1974), and DeLuca & Schnoes (1976)], is thought to be the hormonal form of the vitamin responsible for physiological activities such as stimulation of intestinal calcium (Boyle et al., 1972) and phosphate (Chen et al., 1974) absorption and mobilization of bone calcium (Holick et al., 1972). The possibility remains that further metabolism of 1,25-$(OH)_2D_3$ is required to elicit any or all of these biological responses. In addition, metabolism of 1,25-$(OH)_2D_3$ is of interest from the standpoint of control by inactivation.

The above considerations have led to several studies on the metabolism of 1,25-$(OH)_2D_3$. 1α,24(*R*),25-Trihydroxyvitamin D_3 [1,24(*R*),25-$(OH)_3D_3$] has been identified as a natural metabolite of 24,25-$(OH)_2D_3$ (Holick et al., 1973), is found in the serum of the rats given 1,25-$(OH)_2D_3$ (Tanaka et al., 1977), and is formed in vitro by intestinal mucosa homogenates (Kumar et al., 1978). This metabolite was found to be less active than 1,25-$(OH)_2D_3$ (Holick et al., 1973, 1976).

Within the time course of intestinal transport and bone mobilization responses to 1,25-$(OH)_2D_3$, chloroform extracts of mucosa from chicks dosed with radiolabeled 1,25-$(OH)_2D_3$ contain 1,25-$(OH)_2D_3$ as the chief detectable radiolabeled component (Frolik & DeLuca, 1971; Tsai et al., 1972). In these experiments as much as 30% of the tritium in the 2 position or 10–20% of the tritium label at C-26,27 was found in the aqueous phase. Similar results were obtained for the rat (Frolik & DeLuca, 1972). These studies implied an unknown metabolism presumably involving side-chain oxidation. Side-chain metabolism of 1,25-$(OH)_2D_3$ was confirmed by using doses of 1,25-dihydroxy[26,27-^{14}C]vitamin D_3 [1,25-$(OH)_2$[26,27-^{14}C]D_3] (Kumar et al., 1976). These authors detected $^{14}CO_2$ expiration from animals dosed with 26,27-^{14}C-labeled vitamin D metabolites. $^{14}CO_2$ expiration from a dose of 1,25-$(OH)_2$[26,27-^{14}C]D_3 attained a maximal rate within 4 h and accounted for over 20% of the dosed radioactivity within 24 h. In 24 h, $^{14}CO_2$ expiration accounted for 7% of a dose of 25-hydroxy[26,27-^{14}C]vitamin D_3 (25-OH[26,27-^{14}C]D_3) and less than 5% of a dose of 24,25-dihydroxy[26,27-^{14}C]vitamin D_3 [24,25-$(OH)_2$[26,27-^{14}C]D_3]. This side-chain oxidation appeared to require 1α-hydroxylated metabolites since nephrectomy abolished $^{14}CO_2$ expiration from radiolabeled 25-hydroxyvitamin D_3 but did not greatly affect the amount of $^{14}CO_2$ expired from 1,25-$(OH)_2$[26,27-^{14}C]-D_3-dosed rats (Kumar et al., 1976). Removal of the jejunum, ileum, and colon drastically reduced the $^{14}CO_2$ expired by 1,25-$(OH)_2$[26,27-^{14}C]D_3-dosed rats, implicating an intestinal site of side-chain oxidation (Kumar & DeLuca, 1977).

The experiments described above clearly demonstrate that significant amounts of administered 1,25-$(OH)_2D_3$ undergo metabolic alterations within the time course of stimulation of intestinal calcium and phosphate absorption and bone mineral mobilization by the hormone. To further study the side-chain oxidation of the hormone, we carried out in vivo experiments using doses of 3α-^{3}H- and 26,27-^{14}C-labeled 1,25-$(OH)_2D_3$ with examination of tissue extracts for metabolites relatively enriched in tritium. These studies culminated in the isolation of the major side-chain oxidized metabolite of 1,25-$(OH)_2D_3$ and its identification as 1α,3β-dihydroxy-24-nor-9,10-seco-5,7,10(19)-cholatrien-23-oic acid, for which we propose the more convenient trivial name of calcitroic acid.

Experimental Procedures

Radiolabeled Compounds. 25-OH[26,27-^{14}C]D_3 (48 mCi/mmol) was synthesized by the method of Suda et al. (1971). 25-OH[3α-^{3}H]D_3 (28 Ci/mmol) was synthesized in this laboratory (S. Yamada, H. F. DeLuca, and H. K. Schnoes, unpublished experiments). Both of these compounds exhibited ultraviolet absorption spectra characteristic of the vitamin D triene and comigrated with crystalline 25-hydroxyvitamin D_3 on a high-pressure liquid chromatographic (LC) system

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[1] Abbreviations used: 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1,24(*R*),25-$(OH)_3D_3$, 1α,24(*R*),25-trihydroxyvitamin D_3; 1,25-$(OH)_2$-[26,27-^{14}C]D_3, 1,25-dihydroxy[26,27-^{14}C]vitamin D_3; 25-OH[26,27-^{14}C]D_3, 25-hydroxy[26,27-^{14}C]vitamin D_3; 24,25-$(OH)_2$[26,27-^{14}C]D_3, 24,25-dihydroxy[26,27-^{14}C]vitamin D_3; LC, high-pressure liquid chromatography.

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containing 2.5% 2-propanol in hexane on a 0.45 × 25 cm Zorbax-SIL column (Dupont Instruments, Fullerton, CA). 1,25-$(OH)_2$[3α-^{3}H]D_3 and 1,25-$(OH)_2$[26,27-^{14}C]D_3 were synthesized enzymatically by the method of Gray et al. (1972) as modified by Tanaka et al. (1975). The products were purified on Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ) eluted with 65:35 chloroform–hexane. In each case the radiolabeled 1,25-$(OH)_2D_3$ comigrated with crystalline 1,25-$(OH)_2D_3$ on an LC system consisting of 10% 2-propanol in hexane and a 0.46 × 25 cm Zorbax-SIL column, demonstrating better than 95% radiochemical purity.

Radioactivity was determined by using either a toluene–Triton counting solution containing 33% Triton X-100, 0.4% 2,5-diphenyloxazole, and 0.005% *p*-bis[2-(5-phenyloxazolyl)]benzene in toluene for aqueous samples or a toluene counting solution containing 0.2% diphenyloxazole and 0.01% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene for organic-soluble samples. Samples were counted in a Packard Model 3255 scintillation counter by using appropriate channel settings. Counting efficiencies were determined by using automatic external standardization, and ^{3}H and ^{14}C disintegrations per minute were calculated by using standard formulas for double-label counting. Solid samples were oxidized on a Packard Model B306 sample oxidizer.

Chemicals. Crystalline 1,25-$(OH)_2D_3$ was a generous gift from the Hoffmann-La Roche Co., Nutley, NJ. Diazomethane (CH_2N_2) was generated in ether by saponification of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide in a "diazald kit" (Aldrich Chemical Co., Milwaukee, WI). All solvents were redistilled before use.

General Procedures. Unless indicated otherwise, solvents were evaporated in a rotary evaporator under water pump vacuum. LC was performed on a Waters Model ALP/GPC 204 liquid chromatograph equipped with an ultraviolet absorbance detector monitoring at 254 nm. Columns and solvent systems used are noted elsewhere. LC-grade solvents purchased from Fisher Chemical Co. were used. Ultraviolet absorption spectra were recorded on a Beckman Model 24 recording spectrophotometer. Mass spectrometry was carried out on an A.E.I. Model MS9 mass spectrometer coupled to a DS-50 data system using direct probe sample introduction, at 110–120 °C above ambient, and electron impact ionization (70 eV).

Animals. Male albino rats were purchased from Holtzmann Co., Madison, WI, and were individually housed in overhanging wire cages and given free access to food and water. "Normal" rats were in the weight range of 200–210 g and were used within 24 h after receipt from the breeder. Rachitic rats were maintained for 3–4 weeks after weaning on a vitamin D deficient diet containing 1.2% calcium and 0.1% phosphorus (Tanaka & DeLuca, 1974). 1,25-$(OH)_2D_3$ and radiolabeled 1,25-$(OH)_2D_3$ were administered to the animals in 95% ethanol by intraperitoneal or intrajugular injection as noted.

Double-Label Experiments in Rachitic Rats. Rachitic rats were divided into two groups of seven animals. One group received 125 ng of 1,25-$(OH)_2D_3$ each intraperitoneally in 50 μL of ethanol, and the other group received ethanol vehicle alone. Six hours later the animals were dosed intrajugularly with a solution containing 98 000 dpm 1,25-$(OH)_2$[3α-^{3}H]D_3 and 38 500 dpm 1,25-$(OH)_2$[26,27-^{14}C]D_3, amounting to a total of 152 ng of the hormone. Three hours following the radioactive dose the animals were sacrificed by decapitation and their livers and entire small intestines (with contents) were removed and stored on ice. Appropriate tissues from each group were combined, and after addition of equivalent amounts of water (w/v) the tissues were homogenized on a Brinkman Polytron homogenizer. The homogenates were lyophilized to dryness and then twice extracted with 200 mL of 1:1 $CHCl_3$–MeOH by stirring at room temperature for 4 h. The solvents were removed by filtration. The residue was air-dried, and a portion was combusted in a simple oxidizer to determine unextracted ^{3}H and ^{14}C. The extract was aliquoted for scintillation counting, and then the solvents were removed on a rotary evaporator. The samples were filtered in 90% methanol, solvent was evaporated, and the residue was partitioned in a system containing 40 mL of methanol–40 mL of chloroform–20 mL of water (adjusted to pH 8 with NH_4OH).

The chloroform phases were evaporated, and the residue was chromatographed on 2 × 25 cm Sephadex LH-20 columns eluted with 400 mL of 65:35 chloroform–hexane and stripped with 1:1 chloroform–methanol; 5-mL fractions were collected, evaporated, and counted in toluene counting solution. Peak elution of 1,25-$(OH)_2D_3$ was between 240 and 250 mL.

DEAE-Sephadex A-25 anion-exchange columns were equilibrated in 4% NH_4OH in methanol and then washed extensively with methanol. The aqueous phases (~50 mL) from the partitioning step were placed on 2 × 15 cm columns. The samples were eluted into the column bed followed by 20 mL of methanol. A convex gradient of methanol (250 mL in a constant volume chamber) to 1 M ammonium acetate in methanol (250 mL, in the top chamber) was used to elute the metabolites. Ten-milliliter fractions were collected, and 1-mL aliquots were evaporated and counted in the toluene–Triton scintillation mixture. The uncharged metabolites eluted between 40 and 90 mL, and the charged components eluted between 150 and 230 mL (gradient calculated to be 025–0.3 M in ammonium acetate).

Double-Label Experiments in Normal Rats. Each of 25 "normal" Holtzmann rats (200–210 g) was dosed intrajugularly with 140 ng of 1,25-$(OH)_2D_3$ containing 2.8 × 10^5 dpm 3α-^{3}H label and 3.5 × 10^4 dpm 26,27-^{14}C label. Four hours after the dose the animals were sacrificed and blood, liver, and small intestine were collected. The tissues were homogenized, lyophilized, extracted, filtered, and partitioned as reported above, with appropriate scaling up. The aqueous phases were back-extracted with 0.3 part of chloroform. The resultant chloroform phase was combined with the original chloroform phase and washed by addition of 0.4 part of water and 0.6 part of methanol. The aqueous phases were combined and evaporated with the addition of ethanol to aid in evaporation of water. The aqueous-phase samples were then filtered in 90% methanol and chromatographed on 2 × 25 cm DEAE columns as reported above. Portions of the above chloroform phases were chromatographed as described earlier.

Fractions containing the uncharged compound from DEAE chromatography of the liver aqueous phase were evaporated, and the residue was saponified by stirring in 5 mL of 15% KOH in 95% methanol at room temperature for 1 h. This mixture was neutralized, diluted to 25 mL with water, and twice extracted with diethyl ether. The water phase was chromatographed on the DEAE system.

Fractions containing the charged metabolites from DEAE chromatography of the liver and intestinal aqueous phases were evaporated, filtered in 10 mL of ethanol, and placed on 2 × 58 cm Sephadex LH-20 columns equilibrated and eluted with methanol. The eluants containing the radioactive metabolites (100–170 mL) were combined, evaporated, and methylated in 1 mL of methanol at room temperature by the addition of excess diazomethane in ether. Solvent was then evaporated under a stream of nitrogen and the residue partitioned in 20

Table I[a]

group	tissue dry wt (g)	unextracted ^3H dpm	unextracted ^3H % of dose	extracted ^3H dpm	extracted ^3H % of dose	$CHCl_3$ soluble dpm ^3H	H_2O-MeOH soluble dpm ^3H	H_2O-MeOH soluble % of extract
+D liver	8.9	7 800	1.14	35 900	5.23	22 000	15 000	39.0
−D liver	8.4	8 070	1.17	34 700	5.05	21 000	13 500	38.5
+D intestine	6.8	30 060	4.46	65 800	9.58	24 000	29 500	44.8
−D intestine	6.9	25 100	3.66	72 800	10.60		35 000	48.1

[a] Extraction and partitioning data for the experiment with rachitic rats. +D refers to animals that received a dose of 125 ng of unlabeled 1,25-$(OH)_2D_3$ 6 h prior to the radiolabeled dose. −D animals received an ethanol vehicle 6 h before the radiolabeled dose. Tissues from seven animals were combined for each group.

mL of water (pH 8.0)–40 mL of methanol–40 mL of chloroform. The chloroform phases containing the methylated products were evaporated, and the residue was chromatographed on 2 × 24 cm Sephadex LH-20 columns eluted with 65:35 chloroform–hexane. Elution volumes of 36–50 mL were combined and evaporated, and the residue was chromatographed on an LC system consisting of a 10% 2-propanol–hexane solvent and a 0.7 × 25 cm Zorbax-SIL column operated at a flow rate of 4 mL/min at 1200 psi.

Isolation of Calcitroic Acid. Fifty-seven normal rats each received an intraperitoneal dose of 1 μg of 1,25-$(OH)_2D_3$ in 100 μL of ethanol containing 3.7 × 10^5 dpm 1,25-$(OH)_2$-[3α-^{3}H]D_3. Six hours after the dose, the animals were sacrificed and their livers were removed and stored on ice, yielding a total of 450 g of liver. Water (450 mL) was added, and the tissue was homogenized and lyophilized to yield 136.4 g of dry liver. This was twice extracted with 2 L of 1:1 chloroform–methanol for 24 h at 4 °C. The combined extracts were filtered with thorough washing of the residual solid (800 mL of methanol). The resulting filtrate was evaporated, and the residue was partitioned in 100 mL of water (pH 8.5) and 200 mL each of methanol and chloroform. The chloroform phase was back-extracted with water–methanol (90:140 mL). The aqueous phase was washed with 25 mL of chloroform. The two aqueous phases were combined and evaporated following the addition of ethanol for removal of water as an azeotrope.

A 3 × 16 cm Sephadex-DEAE column was prepared by equilibrating and pouring the slurry in 0.3 M ammonium bicarbonate in 20% H_2O in methanol followed by extensive washing with 95% methanol. The filtered sample from the aqueous phase was placed onto this column in 40 mL of 95% methanol. The column was eluted by using a step gradient of 200 mL of 95% methanol, then 300 mL of 0.1 M ammonium bicarbonate in 90% methanol, and then 200 mL of 0.3 M ammonium bicarbonate in 80% methanol; 22-mL fractions were collected, and 100-μL aliquots were taken for detection of radioactivity. The charged metabolite was eluted with 0.1 M ammonium bicarbonate while most of the yellow contaminants eluted with 0.3 M salt. Fractions 17–27 were combined, the solvent was evaporated, and the residue was chromatographed on a 2 × 57 cm Sephadex LH-20 column equilibrated and eluted with methanol; 20-μL aliquots of 4.6-mL fractions were counted, and fractions 24–37 were combined and evaporated. The resulting residue was suspended in 6.4 mL of methanol, and an excess of diazomethane in ether (~40 mL) was added at room temperature. Solvent was evaporated and the residue partitioned between aqueous MeOH and $CHCl_3$ as reported above.

The chloroform-soluble methylated metabolite fraction was further purified on a 1 × 50 cm Sephadex LH-20 column eluted with 60:40 chloroform–hexane; 3.6-mL fractions were collected, 15-μL aliquots were counted, and fractions 26–35 were combined and evaporated for LC.

Reversed-phase LC was performed on a 0.45 × 21 cm Zorbax-ODS column eluted with 30% water in methanol and operated at 4300 psi with a 2 mL/min flow rate. The fractions collected between 36 and 48 mL contained all of the recovered radioactivity and were combined, and the solvent was evaporated for further chromatography.

Straight-phase LC was performed on a 0.46 × 21 cm Zorbax-SIL column by using a 7% 2-propanol–hexane solvent system and operating at 1100 psi with a 2 mL/min flow rate. The metabolite eluted between 40 and 45.7 mL (Figure 3); these fractions were combined, the solvent was evaporated, and the radioactive metabolite was dissolved in 0.5 mL of methanol to record its ultraviolet absorption spectrum. The sample was again chromatographed on the above straight-phase LC system, and ~400 ng of the material recovered was used for mass spectroscopy.

Results

Previous reports that the terminal carbons of the 1,25-$(OH)_2D_3$ side chain are metabolized to CO_2 (Kumar et al., 1976) suggested a double-labeling radiotracer technique for examining this side-chain oxidation. For this purpose, a mixture of 1,25-$(OH)_2$[3α-^{3}H]D_3 and 1,25-$(OH)_2$[26,27-^{14}C]D_3 ($^3H/^{14}C$ = 0.35) was administered to animals for detection of tritium-enriched metabolites. To determine whether 1,25-$(OH)_2D_3$ stimulates side-chain oxidation, we carried out the experiment with rachitic rats as described under Experimental Procedures.

Table I shows the distribution of radioactivity from each tissue for each group. In each group a significant amount of radioactivity was not extracted from the lyophilized tissue which was slightly enriched for tritium (1.1–1.5-fold, relative to the initial isotope ratio). The chloroform phase of the tissue extracts was very near the initial $^3H/^{14}C$ ratio and showed no metabolite fractions significantly enriched in tritium when chromatographed on Sephadex LH-20 (65:35 chloroform–hexane system). In this system ~75% of the chloroform-soluble radiolabeled material from the livers of both groups migrated in the 1,25-$(OH)_2D_3$ peak region. Approximately 50% of the intestinal chloroform-soluble radioactivity from animals receiving a predose of 1,25-$(OH)_2D_3$ and 60% of that from animals that had received the ethanol vehicle as the first dose migrated as 1,25-$(OH)_2D_3$. In all cases the radioactivity which did not migrate as 1,25-$(OH)_2D_3$ was about equally divided between components eluting before 1,25-$(OH)_2D_3$ and polar components eluting in the column strip. Since none of these chloroform-soluble components was tritium-enriched, they were not further examined.

The water–methanol phases accounted for about 39% of the tritium extracted from livers and 45% of the tritium extracted from the intestines plus contents (Table I), which represents ~34% of the total tritium label in each tissue. In each group the aqueous phase was about threefold enriched with tritium relative to the initial $^3H/^{14}C$ ratio. The aqueous phases were

Table II[a]

group	uncharged			charged				
	^{3}H dpm	x-fold ^{3}H enriched	% of extracted ^{3}H	^{3}H dpm	x-fold ^{3}H enriched	% of extracted ^{3}H	% of dose	dpm/g
+D livers	4300	5.0	12.0	11400	2.9	31.7	1.66	1280
−D livers	4000	4.7	11.0	9800	2.5	28.1	1.43	1170
+D intestines	5500	3.0	8.4	24400	2.7	37.1	3.55	3615
−D intestines	9700	2.7	13.3	25900	2.5	35.6	3.77	3750

[a] Results of DEAE chromatography of the water–methanol fractions from Table I. Charged refers to the radioactive components retained on the DEAE column, while uncharged refers to the radioactive components not retained on DEAE.

Table III[a]

tissue	dry wt. (g)	unextracted		extracted		$CHCl_3$ soluble	H_2O–MeOH soluble	
		dpm ^{3}H	% of dose	dpm ^{3}H	% of dose	dpm ^{3}H	dpm ^{3}H	% of extract ^{3}H
blood (75 mL)	28.5	3.5×10^4	0.49	3.12×10^5	4.4	2.01×10^5	2.4×10^4	7.8
livers	48.6	1.4×10^5	1.99	6.18×10^5	8.7	3.88×10^5	2.56×10^5	41.3
intestine	41.5	2.7×10^5	3.80	1.00×10^6	14.1	5.96×10^5	1.8×10^5[b]	18.0[b]

[a] Extraction and partitioning data from the double-label dosing experiment in normal rats. Tissues from 25 rats were combined. [b] An undefined portion of the intestinal water–methanol phase was lost; the numbers refer to the amount remaining.

Table IV[a]

tissue	uncharged			charged				
	^{3}H dpm	x-fold ^{3}H enriched	% of extract ^{3}H	^{3}H dpm	x-fold ^{3}H enriched	% of extract ^{3}H	% of dose	dpm/g
blood	18 500	1.24	5.9	5 730	0.6	1.8	0.08	201
liver	20 600	1.2	3.3	220 000	>10	35.6	3.1	4526
intestine				175 000[b]	3.5[b]	17.2[b]	2.4[b]	4145[b]

[a] Results from DEAE chromatography of the water–methanol phases in Table III. [b] See footnote *b* in Table III.

resolved into "uncharged" and "charged" components on DEAE chromatography (Table II). The majority of the side-chain oxidized components in both tissues was in the charged fraction, although both the uncharged and charged components contain tritium-enriched metabolites.

As indicated in Tables I and II, there was little difference in tissue radioactivity levels between rats receiving a dose of nonradioactive 1,25-$(OH)_2D_3$ 6 h prior to the labeled dose and those receiving the ethanol vehicle. Under the conditions of this experiment the intestines plus contents contain higher levels of side-chain oxidized metabolites than do the livers. Just 3 h following the double-labeled dose, 3–4% of the dosed tritium was recovered from the intestines plus contents as charged tritium-enriched components.

The next experiment was carried out on normal rats as described under Experimental Procedures. Table III gives the extraction data for this experiment. The percent of the dose in various samples was similar to what was found at 3 h in the rachitic animals. Direct comparison of the intestinal aqueous phase is difficult since an undefined portion of the sample was lost before aliquoting. However, it can be inferred from the radioactivity in the chloroform phase that 30–40% of the extracted tritium would have been in the water–methanol-soluble fraction, in agreement with the earlier experiment.

In each of the tissues, the chloroform-soluble radioactivity had the same $^3H/^{14}C$ ratio as the initial dose and showed no components with significant tritium enrichment upon chromatography.

The blood extract contained a lower proportion of water–methanol-soluble radioactivity, and most of it was found in the uncharged fraction (Table IV) that was slightly enriched in tritium. The charged components from blood were ^{14}C enriched presumably because the side-chain fragments, possibly including [^{14}C]bicarbonate, were being cleared from the site of side-chain oxidation via the blood. This conclusion is supported by a partial resolution of tritium-enriched and ^{14}C-enriched components of the charged components on DEAE (data not shown).

The aqueous methanol-soluble radioactivity from the liver extract was found to migrate primarily as a charged component on DEAE (Table IV). This charged component contained almost exclusively tritium radioactivity. A sample was saponified and partitioned between ether and water to determine if the uncharged material contained esters of the charged component. Thirty percent of the saponified product was recovered in the ether phase and was not enriched for tritium. The water-soluble products showed tritium enrichment (1.5-fold enriched), but over 80% of the material in the phase represents uncharged components. These results suggest a mixture of metabolites in the uncharged fraction.

The charged material from liver was chromatographed on Sephadex LH-20 by using methanol as the solvent. This gel filtration column achieved partial separation of the radioactive material from yellow impurities and salts from the DEAE elution step; 83% of the radioactivity was recovered in the peak. A portion of the sample was methylated with an excess of diazomethane in ether. Ninety-six percent of the radioactivity in the reaction mixture partitioned into the chloroform phase in a water–methanol–chloroform system, indicating nearly quantitative conversion to methylated products. Eighty-three percent of this methylated material migrated as a single substance containing only tritium by using the 65:35 chloroform–hexane Sephadex LH-20 column (Figure 1). The elution position of the ^{3}H on this system suggested a compound less polar than 1,25-$(OH)_2D_3$. Of the recovered radioactivity, 5% migrated as a tritium-enriched substance in the column strip. The major methylated component from the LH-20

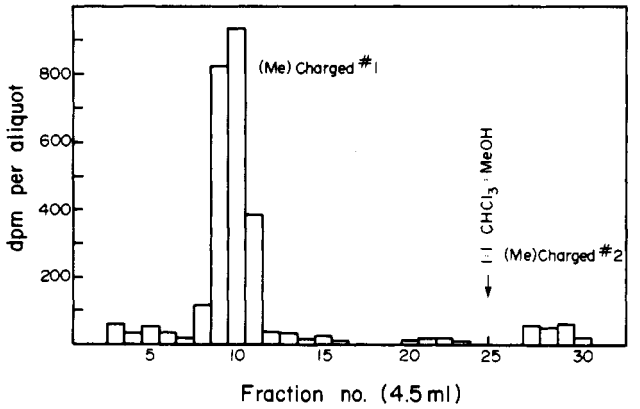


FIGURE 1: Chromatography of the radiolabeled, methylated, charged compounds from livers of normal rats given 1,25-$(OH)_2$[3α-^{3}H]D_3 and 1,25-$(OH)_2$[26,27-^{14}C]D_3. Chromatography was carried out with a 2 × 24 cm column of Sephadex LH-20 eluted with 65:35 chloroform–hexane.

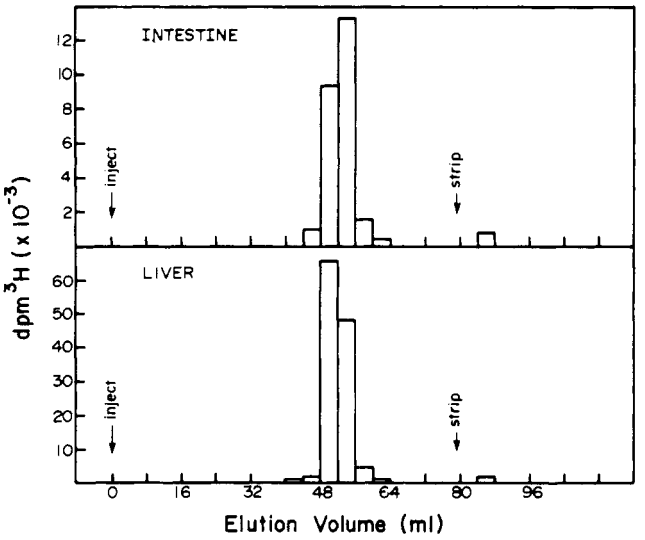


FIGURE 2: LC of the major, methylated, charged component from livers and intestines of normal rats given 1,25-$(OH)_2$[3α-^{3}H]D_3. The charged components from LH-20 chromatography as illustrated in Figure 1 were chromatographed as described under Experimental Procedures.

column migrated as a single peak on straight-phase LC (Figure 2). On the assumption of similar recoveries for all radioactive components through transfers and chromatography, these results indicate that ~80% of the charged peak from liver is a single compound.

It was noted above that losses of a portion of the intestinal aqueous phase preclude calculating recoveries of specific components. The amounts of uncharged components were small, were not completely resolved from the leading edge of the charged component, and thus are not reported (Table IV). The charged component was subjected to LH-20–methanol chromatography followed by methylation with diazomethane. The peak was methylated in 87.5% yield, as calculated from chloroform-soluble radioactivity, with the product being fivefold enriched in tritium. The unreacted, water-soluble, radioactivity had essentially the same $^3H/^{14}C$ ratio as the 1,25-$(OH)_2D_3$ originally administered. The methylated product exhibited the same elution as the liver component on Sephadex LH-20 chromatography (65:35 $CHCl_3$–hexane) with the peak containing over 60% of the radioactivity from the chloroform phase of the reaction mixture. This substance also migrated as a single peak on straight-phase LC, with the same elution volume as the methylated compound from liver (Figure 2). Thus, the major side-chain oxidized products in liver and intestine appear to be identical.

Table V[a]

	dpm	% of dose	% recovery
aqueous phase	1.68×10^6	7.7	
uncharged peak	2.2×10^5	1.06	
charged peak	1.21×10^6	5.8	
after LH-20-MeOH	7.4×10^5	3.57	61
methyl ester	7.28×10^5	3.52	98
after LH-20 (60:40)	5.16×10^5	2.49	71
after LC (reversed phase)	4.59×10^5	2.22	89
after LC (straight phase)	4.23×10^5	2.04	92

[a] Yields and recoveries in the isolation of the major side-chain oxidized metabolite.

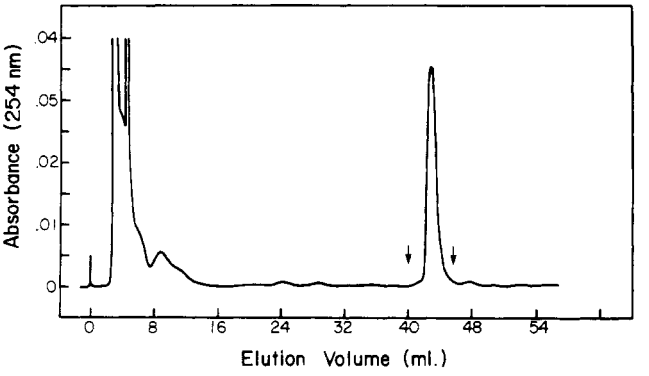


FIGURE 3: Straight-phase LC of the methyl-esterified metabolite recovered from the reversed-phase column. This material was used for the identification of calcitroic acid. The arrows bracket the region that was collected for spectral analysis.

The intestinal extracts contained much more contaminating material than the liver extracts, and as a result the recoveries in filtration and chromatographic steps were low. For this reason the liver was chosen as the tissue for the isolation and identification of the major side-chain oxidized peak, despite the higher content of the charged side-chain oxidized compound found in intestines. The side-chain cleaved product was isolated as described under Experimental Procedures. Yields and recoveries during isolation are shown in Table V. The low recovery from Sephadex LH-20–methanol chromatography was largely the result of losses in filtration of the sample before loading onto the column. The sample was methylated in excellent yield, and 71% of the methyl ester was recovered in a single peak on subsequent chromatography.

Final purification of the methylated metabolite was achieved on reversed-phase and straight-phase (Figure 3) LC. The single sharp peak of absorbance at 254 nm on the straight-phase column attested to the purity of the preparation. This UV-absorbing peak contained all of the radioactivity recovered from the column. A total of 2.8 nmol of the metabolite was recovered from this column as calculated from the specific activity of the dosed 1,25-$(OH)_2D_3$. This peak accounts for 2.04% of the tritium administered.

The sample from straight-phase LC had an ultraviolet absorption spectrum (Figure 4) characteristic of the vitamin D *cis*-triene chromophore. After recording the ultraviolet spectrum, we again chromatographed the sample on the straight-phase LC system to ensure purity for mass spectral analysis.

The mass spectrum (Figure 5) together with the ultraviolet absorbance spectrum of the methylated metabolite positively identified this compound as the methyl ester of 1α,3β-dihydroxy-24-nor-9,10-seco-5,7,10(19)cholatrien-23-oic acid

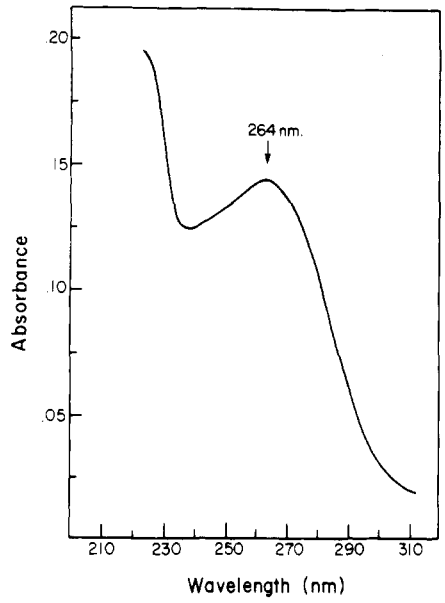


FIGURE 4: Ultraviolet absorbance spectrum of the methylated metabolite (methyl calcitroate).

(Figure 6). This conclusion is based on the following observations.

The ultraviolet absorption spectrum (Figure 4) demonstrated an intact *cis*-triene, unaltered from the precursor 1,25-$(OH)_2D_3$. The mass spectrum contains a prominent molecular ion at *m/e* 388 and peaks at *m/e* 370 and 352, representing loss of one and two molecules of H_2O, respectively. The presence of two hydroxyl substituents in ring A of the molecule is established by the peaks at *m/e* 152 and 134 (152 – H_2O). These fragments, which comprise ring A plus C-6 and C-7, are characteristic of 1α,3β-dihydroxylated vitamin D compounds (Holick et al., 1972, 1973; Kumar et al., 1978). Loss of the entire side chain (C-17,20 bond cleavage) accounts for the peak at *m/e* 287, and the ions of *m/e* 269 and 251 arise by subsequent elimination of one or both A ring hydroxy groups from that fragment. The presence of a side-chain methyl ester function is confirmed by the peak at *m/e* 357 (M – OCH_3), by the loss of $COOCH_3$, yielding *m/e* 329, and by the elimination of 74 mass units (CH_2COOCH_3 + H) via a McLafferty-type rearrangement to give the ion of *m/e* 314. The occurrence of this rearrangement fragmentation also specifies a C-23 position for the carbomethoxy function. These data define the structure of the isolated methyl ester product (structure III, Figure 6) and establish that the in vivo metabolite is the corresponding carboxylic acid as depicted by structure II (Figure 6). The acid can be regenerated from the isolated ester by hydrolysis in dilute methanolic base. Since the metabolite arises directly from 1α,25-$(OH)_2D_3$, it is inferred that the A ring hydroxyls are in the 1α and 3β stereochemical orientations. The similarity of A ring fragment intensities in its mass spectrum to that of other 1α,3β-dihydroxylated vitamin D compounds strongly supports this conclusion since the orientation of the hydroxyls on the A ring greatly influences the A ring fragmentation pattern (H. E. Paaren, H. K. Schnoes, and H. F. DeLuca, unpublished experiments). In addition, epimerization at the 3 position presumably would have resulted in the loss of the 3α-tritium.

Discussion

The oxidation of the terminal carbons of the side chain of 1,25-$(OH)_2D_3$ was investigated by using a double-labeling radiotracer technique. Rats dosed with 1,25-$(OH)_2$[3α-^{3}H]D_3 and 1,25-$(OH)_2$[26,27-^{14}C]D_3 produced tritium-enriched metabolites which partition into the basic aqueous phase in a water–methanol–chloroform system. These metabolites had not been detected previously since most workers in the vitamin D field examine only the chloroform phase of lipid extracts and use side-chain labeled radiotracers. The use of methanol–chloroform extraction of lyophilized tissue prior to partitioning minimized contamination of the aqueous-phase preparations with proteins and other water-soluble materials. This procedure may have resulted in lowered recoveries of some of the side-chain oxidized metabolites as evidenced by significant amounts of unextracted radioactivity.

Until recently, most of the work on the metabolism of 1,25-$(OH)_2D_3$ involved the use of side-chain tritiated radiotracers. Frolik & DeLuca (1972) examined metabolism of 1,25-$(OH)_2$[26,27-^{3}H]D_3 in the rat. These workers recovered only 3% of the tissue radioactivity in intestinal mucosa as water-soluble components 7 h after the dose and found ~20% of the radioactivity in liver to be water-soluble. Their tritium label would be expected to be oxidized to tritiated water during side-chain oxidation and would be rapidly lost from the tissues. Indeed, Frolik & DeLuca (1971) found that a dose of 1,25-$(OH)_2$[2-^{3}H]D_3 gave higher yields of tritiated water-soluble products in chick tissue extracted than a dose of 26,27-labeled 1,25-$(OH)_2D_3$.

In the above studies and in a study by Tsai et al. (1972) 1,25-$(OH)_2D_3$ accounted for a larger fraction of the chloroform-soluble radioactivity in intestines than was found in the experiments here. This can be explained by the fact that those authors were using intestinal mucosa while the present experiments were performed on whole intestine plus contents. Indeed, the results found for the chloroform phase from liver in this study closely agree with the results reported for 1,25-$(OH)_2D_3$ metabolism in rats (Frolik & DeLuca, 1972).

The major portion of the tritium-enriched material in the aqueous phases migrated as acidic compounds on DEAE chromatography. This charged material reached relatively

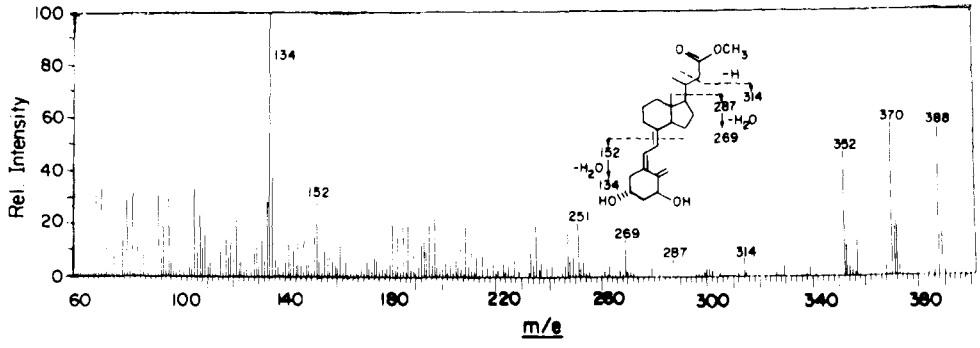
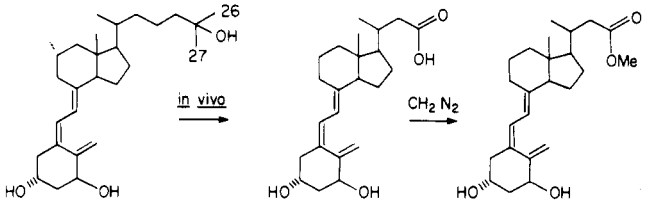


FIGURE 5: Mass spectrum of the methyl-esterified metabolite (methyl calcitroate) isolated from 57 rat livers. Peak heights above *m/e* 180 have been expanded fivefold to facilitate examination.



I II III

FIGURE 6: Structures of 1,25-$(OH)_2D_3$ (I), of the new metabolite, calcitroic acid (II), and of methyl calcitroate (III). Calcitroic acid (II) is produced in vivo by side-chain oxidation of 1,25-$(OH)_2D_3$ and was isolated as its methyl ester (III) prepared from the acid by treatment with diazomethane.

high levels in both intestines and livers as early as 3 h after an intrajugular dose, accounting for 3.5% of the dose and 37% of the extracted tritium in the intestine. This material contained more of the tissue tritium radioactivity than did the 1,25-$(OH)_2D_3$ fraction. Thus, the side-chain oxidized metabolites achieve significant concentrations in this target tissue within the time required for onset of tissue response to the hormone (Tanaka & DeLuca, 1971). These findings indicate that the side-chain oxidized compounds could be performing important functions in the tissue response to 1,25-$(OH)_2D_3$.

In the studies with rachitic rats, little if any differences were found in levels of tritium-enriched metabolites between rats that had received a prior dose of 1,25-$(OH)_2D_3$ and those that had not. Thus, within 6 h, 1,25-$(OH)_2D_3$ does not seem to alter the activity of the side-chain metabolizing enzymes. It is not known whether side-chain oxidation is altered by the physiological calcium and phosphate status of the animal. It is possible that the enzyme systems catalyzing the synthesis of these metabolites are not highly regulated, depending instead on the tightly controlled 1-hydroxylation step to modulate the amount of side-chain oxidized metabolites formed. Indeed, Kumar et al. (1976) presented strong evidence that only 1α-hydroxylated metabolites of vitamin D can undergo side-chain oxidation.

In normal rats, greater than 95% of the tritium-enriched charged material from liver was methyl esterified with diazomethane. Over 80% of this methyl-esterified material migrated as a discrete peak on subsequent chromatography. A similar material from intestines was also methylated in good yield and eluted in the same solvent volume on Sephadex LH-20 and LC chromatography, indicating that the intestinal compound is the same as the liver metabolite. Since the intestinal preparation contained much more contaminating material, the liver was chosen as the tissue from which sufficient quantities of the metabolite could be isolated for identification. The blood contained very little of this side-chain oxidized metabolite.

The major side-chain oxidized metabolite was isolated and purified as the methyl ester in five chromatographic steps. The structure of the compound was elucidated by using ultraviolet spectrophotometry and mass spectrometry. The compound was identified as the methyl ester of 1,3-dihydroxy-24-nor-9,10-seco-5,7,10(19)-cholatrien-23-oic acid. Thus, the metabolite occurring in vivo (Figure 6) is the corresponding 23-carboxyvitamin D, produced by oxidative cleavage of carbons 24–27 of 1,25-$(OH)_2D_3$. The mechanism of this novel side-chain oxidation pathway is unknown at present, but the data of Kumar et al. (1976) indicate that the terminal carbons are oxidized to CO_2. In accordance with the proposed new nomenclature in the vitamin D field, we suggest the trivial name of calcitroic acid for this metabolite since it is formed from 1,25-$(OH)_2D_3$, or calcitriol.

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