Metabolites of 1α,25-Dihydroxyvitamin D_3 in Rat Bile†

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ABSTRACT: Metabolites of 1α,25-dihydroxy[3α-^{3}H]vitamin D_3 in rat bile were studied. The water-soluble metabolites (77–91% of the total bile radioactivity) are predominantly acids (retained by DEAE-Sephadex chromatography) that become chloroform soluble after methylation with diazomethane. Calcitroic acid was isolated from this fraction (after conversion to the methyl ester) and unambiguously identified by chromatographic and mass spectrometric studies. The chloroform phase of extracted bile contains small amounts (1.1–8.1% of the metabolites in bile, dependent on dose given) of unchanged 1α,25-dihydroxyvitamin D_3 and no 1α,24(*R*),25-trihydroxyvitamin D_3. In addition, the chloroform phase contained a mixture of 24-dehydro- and 25-dehydro-1α-hydroxyvitamin D_3 and calcitroic acid methyl ester. The latter compound was shown to be an artifact resulting from the use of methanol as part of the solvent mixture for the initial bile extractions.

The induction of intestinal calcium transport, bone calcium mobilization, and intestinal phosphate transport by vitamin D_3 is known to require metabolism of vitamin D_3 to 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$][1] (DeLuca & Schnoes, 1976; DeLuca et al., 1979). Vitamin D_3 is hydroxylated at C-25 (primarily in the liver) to give 25-hydroxyvitamin D_3, which is subsequently converted to 1α,25-$(OH)_2D_3$ by the kidney as a response to hypocalcemia or hypophosphatemia. This two-step metabolic activation raises the question of the possible necessity for further metabolism of 1α, 25-$(OH)_2D_3$ for function. To examine this question, we have studied the metabolism of 1α,25-$(OH)_2D_3$.

Early examinations of the metabolism of 1α,25-$(OH)_2$-[26,27-^{3}H]D_3 showed that the chloroform phase of intestinal mucosa contained 1α,25-$(OH)_2D_3$ as the major component and that small amounts (20%) of the tissue radioactivity were aqueous soluble (Frolik & DeLuca, 1971, 1972). Using 1α,25-$(OH)_2$[26,27-^{14}C]D_3, however, Harnden et al. (1972) found substantial oxidative metabolism to $^{14}CO_2$. In rats, 21–35% of the dose was exhaled as $^{14}CO_2$ within 24 h of the dose (Kumar et al., 1976; Kumar & DeLuca, 1977). Metabolism of 1α,25-$(OH)_2$[26,27-^{14}C]D_3 to $^{14}CO_2$ appears to occur in chickens to a lesser extent (Kumar & DeLuca, 1976). The discovery of rapid and substantial oxidation of 1α,25-$(OH)_2$[26,27-^{14}C]D_3 to $^{14}CO_2$ stimulated the search for side chain cleaved metabolites of 1α,25-$(OH)_2D_3$. From the livers of rats dosed with 1α,25-$(OH)_2$[3α-^{3}H]D_3, Esvelt et al. (1979) have isolated 1α-hydroxy-23-carboxytetranorvitamin D (calcitroic acid). However, the amount of calcitroic acid found in liver and intestine accounts for at most 4–6% of the dose. Recently the bile has been shown to be a rich source of side chain cleaved metabolites of 1α,25-$(OH)_2D_3$ (B. L. Onisko and H. F. DeLuca, unpublished experiments).

We wish to report that the bile of rats given 1α,25-$(OH)_2$[3α-^{3}H]D_3 contains calcitroic acid. As much as 13% of the dose is present in bile (24-h collection) as calcitroic acid. In addition to calcitroic acid, the aqueous phase of bile contains other, as yet identified, side chain cleaved metabolites of 1α,25-$(OH)_2D_3$.

Materials and Methods

General. Mass spectra were obtained on an AEI Model MS-902 mass spectrometer (Associated Electrical Industries, Ltd., Manchester, England) at 70 eV using a direct probe for sample introduction and a source temperature of 110–130 °C above ambient. High-pressure liquid chromatography (HPLC) was performed with a Waters Model M6000A liquid chromatography (Waters, Inc., Melford, MA) equipped with a Model 450 variable-wavelength detector. For SP-HPLC (straight-phase high-pressure liquid chromatography), a Li-Chrosorb Si 60 (Merck & Co., Inc., Rahway, NJ) column [4.6 mm (i.d.) by 250 mm (*L*)] was used. For RP-HPLC (reversed-phase high-pressure liquid chromatography), a Zorbax-ODS (Du Pont Instruments, Wilmington, DE) column [4.6 mm (i.d.) by 250 mm (*L*)] was used. All solvents were redistilled before use with the exception of HPLC-grade hexane (Fischer Chemical Co., Itasca, IL). All solvents for HPLC were filtered through 0.2-μm filters (Millipore Corp., Bedford, MA) before use. Unless otherwise noted, solvents were removed by rotary evaporation with a water aspirator and a 25–35 °C bath. Synthetic 1α,25-$(OH)_2D_3$ was a gift from Hoffmann-La Roche (Nutley, NJ). Synthetic calcitroic acid methyl ester was prepared in this laboratory (R. P. Esvelt, H. F. DeLuca, and H. K. Schnoes, unpublished results). A mixture of Δ^{24}-1α-OH-D_3 and Δ^{25}-1α-OH-D_3 had been prepared previously in connection with another project (Napoli et al., 1978). The mixture was fractionated by RP-HPLC, and the identity of the two components was established by ^{1}H NMR (270 MHz) and mass spectrometry. Diazomethane was generated by saponification of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide.

Radiochemicals and Measurement of Radioactivity. 1α,25-$(OH)_2$[3α-^{3}H]D_3 (28 Ci/mmol) and 1α,25-$(OH)_2$-[26,27-^{14}C]D_3 (48 Ci/mmol) were prepared as previously described (Esvelt et al., 1979). Purity and authenticity of the radiolabeled compounds were checked by cochromatography with synthetic 1α,25-$(OH)_2D_3$ on SP-HPLC using 10% 2-propanol in hexane (Jones & DeLuca, 1975).

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[1] Abbreviations used: 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1α,24*R*,25-$(OH)_3D_3$, 1α,24(*R*),25-trihydroxyvitamin D_3; Δ^{24}-1α-OH-D_3, 24-dehydro-1α-hydroxyvitamin D_3; Δ^{25}-1α-OH-D_3, 25-dehydro-1α-hydroxyvitamin D_3; SP-HPLC, straight-phase high-pressure liquid chromatography; RP-HPLC, reverse-phase high-pressure liquid chromatography; calcitroic acid, suggested trivial name for 1α,3β-dihydroxy-24-nor-9,10-seco-5,7,10(19)-cholatrien-23-oic acid; HPLC, high-pressure liquid chromatography.

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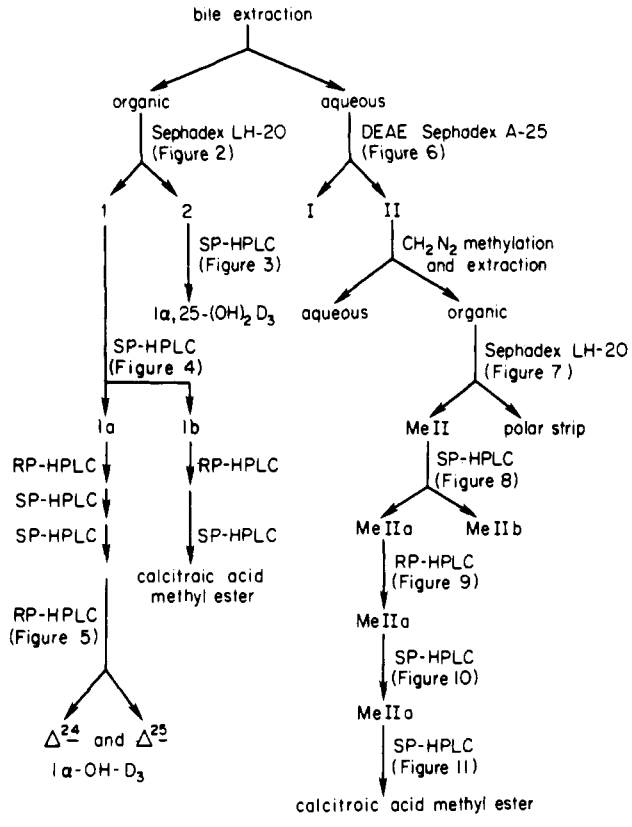

FIGURE 1: Chromatographic scheme detailing the methods used to fractionate the metabolites of 1α,25-$(OH)_2D_3$ in rat bile.

Organic-soluble samples were dissolved in 12 mL of a toluene counting solution containing 0.2% diphenyloxazole and 0.01% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene after solvent removal with an air stream. Aqueous methanol soluble samples were mixed with 12 mL of Aquasol (New England Nuclear, Boston, MA). Solid samples were combusted by use of a Packard Model B306 sample oxidizer. All samples were counted in a Packard Tri-Carb Model 3255 liquid scintillation spectrometer (Packard, Downers Grove, IL). Quenching was corrected for by use of automatic external standardization.

Animals, Bile Duct Cannulations, and Dose Protocol. Male weanling rats were obtained from the Holtzman Co. (Madison, WI) and housed individually in overhanging wire cages. They were fed ad libitum a vitamin D deficient diet containing 0.47% calcium and 0.3% phosphorus supplemented with vitamins A, E, and K (Suda et al., 1970). The animals were used after an average of 3 months on this diet when their body weights had reached 250–275 g.

Rats were anesthetized by intrajugular injection of phenobarbital (25 mg/kg of body weight). This injection was given under light ether anesthesia. The bile duct was then cannulated with polyethylene tubing. After the abdominal incision was closed with sutures and wound clips, the animals were kept in restraining cages and allowed 10% dextrose ad libitum.

For the isolation of calcitroic acid and the organic-soluble metabolites, 12 bile duct cannulated rats were dosed intrajugularly with 1α,25-$(OH)_2D_3$ (15 μg/rat) dissolved in 50 μL of ethanol. Only two of these animals received 1α,25-$(OH)_2$[3α-^{3}H]D_3 (7.5 μCi/rat). Bile samples from all rats were collected for 24 h after the dose and pooled.

For the distribution studies (see Table I), four dose levels of 1α,25-$(OH)_2D_3$ were used and three bile duct cannulated rats were used for each dose level. Doses for rats receiving 0.015, 0.15, and 15 μg of 1α,25-$(OH)_2D_3$ were prepared such that each animal received ~1.0 μCi of 1α,25-$(OH)_2$[3α-^{3}H]D_3. Synthetic 1α,25-$(OH)_2D_3$ was added as required. The animals given 1.5 μg of 1α,25-$(OH)_2D_3$ received 0.13 μCi of 1α,25-$(OH)_2$[3α-^{3}H]D_3 and 0.09 μCi of 1α,25-$(OH)_2$-[26,27-^{14}C]D_3. All doses were injected intrajugularly in 50 μL of ethanol. Bile was collected for 24 h after the dose.

Isolation of the Organic-Soluble Compounds (Figure 1). Pooled bile samples (1 volume) were extracted with chloroform–methanol (Bligh & Dyer, 1959). The chloroform phase was back-extracted once with MeOH (2.5 volumes) and H_2O (2.25 volumes), and the resulting chloroform solution was concentrated and chromatographed on Sephadex LH-20 (1 × 28 cm column) eluted with 75% chloroform in Skellysolve B to yield two components (Figure 2). Peak 2 was further

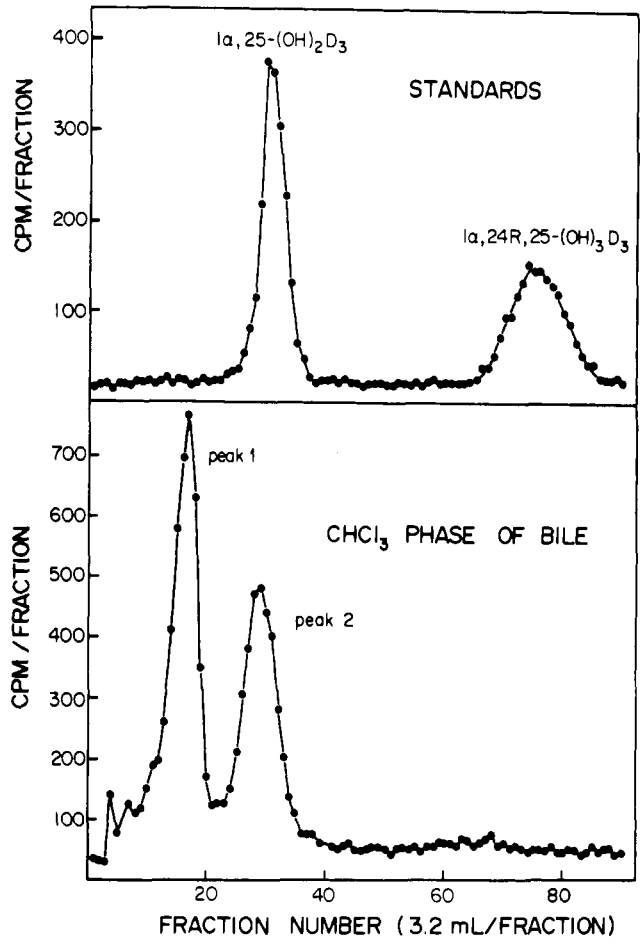

FIGURE 2: Chromatography of the chloroform-soluble metabolites from the bile of rats given 1α,25-$(OH)_2$[3α-^{3}H]D_3 on Sephadex LH-20 with 75% chloroform in Skellysolve B as the elution solvent (bottom panel). Chromatography of standard 1α,25-$(OH)_2D_3$ and 1α,24*R*,25-$(OH)_3D_3$ on the same chromatographic system (top panel).

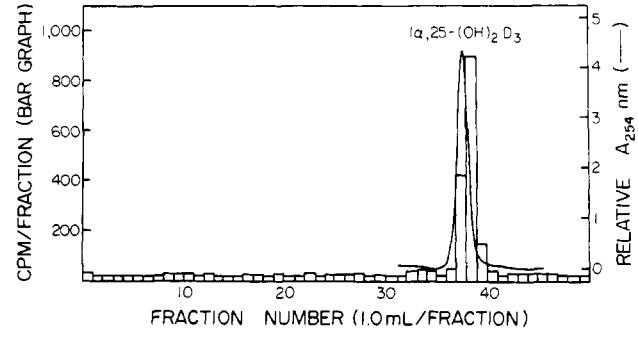

FIGURE 3: Cochromatography of synthetic 1α,25-$(OH)_2D_3$ with peak 2 (fractions 24–35 of Figure 2 were pooled) on Zorbax-SIL (HPLC) by using 10% 2-propanol in hexane as solvent.

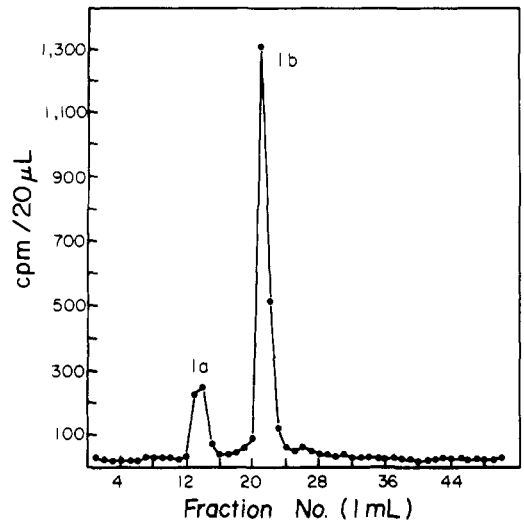

FIGURE 4: High-pressure liquid chromatography of peak 1 (Figure 2) on a straight-phase column eluted with 10% 2-propanol in hexane, at a flow rate of 2 mL/min (1100 psi); 1-mL fractions were collected and fractions 13–15 (peak 1a) and 20–23 (peak 1b) were pooled for further purification as described under Materials and Methods and summarized in Figure 1.

Table I: Distribution of Radioactivity of Bile in Various Chromatographic Fractions

fraction[a]		% of total bile ^3H[b] in the designated fraction from bile of rats given the following amount of 1α,25-$(OH)_2D_3$			
nonpolar	polar	0.015 μg	0.15 μg	1.5 μg	15 μg
peak 1		5.7	2.0	1.7	2.1
1α,25-$(OH)_2D_3$		8.1	2.0	1.1	1.6
1α,24,25-$(OH)_3D_3$		0.0[c]	0.0[c]	0.0[c]	0.0[c]
	peak I	11.0	20.0	18.0	18.0
	MeII	9.0	19.0	20.0	20.0
	aqueous	12.0	13.0	8.0	12.0
	polar strip	37.0	38.0	40.0	41.0
	unextracted bile solids	5.0	5.0	9.0	4.0

[a] For a description of the terms, see Figure 1. [b] Based on the disintegrations per minute recovered in the designated fractions; for recoveries of each chromatographic step, see Materials and Methods. [c] No peak was detected.

purified by SP-HPLC and identified as 1α,25-$(OH)_2D_3$ by high-pressure liquid cochromatography (Figure 3). Peak 1 (Figure 2), upon SP-HPLC (eluted with 10% 2-propanol in hexane), gave two components, **1a** and **1b** (Figure 4). Pooled fractions (20–23) of component **1b** were further purified by RP-HPLC (75% MeOH–H_2O) to give a sharp peak of radioactivity in fractions 27–29 (1-mL fractions, 91% recovery). Subsequent SP-HPLC of the pooled fractions (10% 2-propanol–hexane) eluted metabolite in fractions 24–27 (1-mL fractions, 72% recovery). An aliquot of the pooled sample was subjected to mass spectrometric analysis. Another aliquot of the sample was also silylated [with bis(trimethylsilyl)fluoroacetamide, at 60 °C for 40 min] and repurified on SP-HPLC (0.1% 2-propanol in hexane), and the collected material (fractions 12–13) was analyzed by mass spectrometry. Component **1a** (Figure 4) was further purified by RP-HPLC (90% MeOH in H_2O). Pooled fractions (1 mL) 26–28 (80% recovery) were then chromatographed on SP-HPLC (7% 2-propanol in hexane, 72% recovery). This gave a sharp, apparently homogeneous peak (eluting in fractions 24–27, 1-mL fractions) of which an aliquot was anlyzed by mass spectrometry. Another sample was cochromatographed on SP-HPLC (7% 2-propanol–hexane) with a mixture (1 μg) of synthetic Δ^{24}- and Δ^{25}-1α-OH-D_3. Radioactivity due to the sample coeluted as a sharp symmetrical peak with the peak of optical density (at 254 nm) due to the synthetic standards (no separation of isomers on SP-HPLC) in fractions 23–25 (1-mL fractions). The pooled fractions were then subjected to cochromatography on RP-HPLC (87.5% MeOH–H_2O) which, after recycling twice, accomplished the resolution of the synthetic dehydro isomers and established coelution of radioactivity with each isomer (Figure 5).

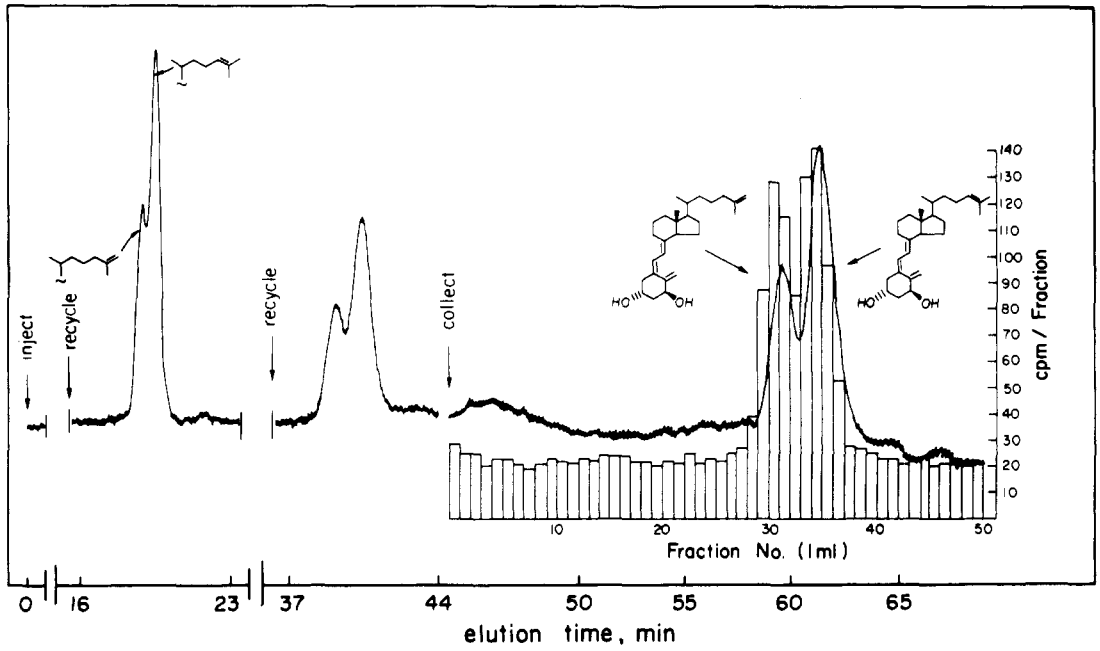

FIGURE 5: Cochromatography of purified **1a** (see Materials and Methods and Figure 1) with a mixture (1 μg) of synthetic Δ^{24}-1α-OH-D_3 and Δ^{25}-1α-OH-D_3 on a reverse-phase column eluted with an 87.5% MeOH–H_2O solvent. The material was recycled twice through the column, to achieve the necessary resolution of dehydro isomers. The bar graph represents radioactivity per fraction due to metabolites; the solid curve represents OD (at 254 nm) due to synthetic standards. UV monitor sensitivity was increased from 0.02 OD unit full scale to 0.01 OD unit full scale at 44 min. The identity of the synthetic standards was established by ^1H NMR (270 MHz) and mass spectrometry.

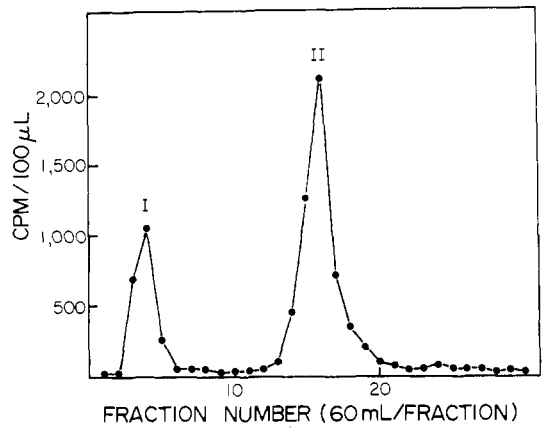

FIGURE 6: Chromatography on DEAE-Sephadex of components of the aqueous methanol phase of bile from rats dosed with 1α,25-$(OH)_2$[3α-^{3}H]D_3.

Isolation of Calcitroic Acid. The pooled bile samples (1 volume) were extracted according to the method of Bligh & Dyer (1959). The chloroform phase was back-extracted once with methanol (2.5 volumes) and water (2.25 volumes). The combined aqueous phases were evaporated to a small volume then lyophilized. The resulting solids were extracted with methanol (3 times, 0.33 volumes each), and the methanol extract (after diluting to 5% water) was applied to a 2.5 × 37 cm column of DEAE-Sephadex A-25. The resin was prepared by treatment with 15% aqueous ammonium bicarbonate (ten 800-mL washes were required to free 100 g of resin of chloride), water (three 800-mL washes), and 5% water in methanol (three 800-mL washes). The column was eluted with 450 mL of 5% water in methanol and then with 1.35 L of 0.1 M ammonium bicarbonate in 10% water in methanol. Fractions (60 mL each) were collected and the radioactivity of 0.1-mL aliquots was measured (Figure 6). Recovery of ^{3}H was 116%. Fractions 13–19 were pooled, and solvents were evaporated to a small volume; then they were lyophilized, redissolved in methanol (60 mL), and treated with excess ethereal diazomethane. After standing at room temperature for 30 min, the ether and excess diazomethane were removed by evaporation with a nitrogen stream in the hood, and the remaining solvent was removed by rotary evaporation. The resulting oil was extracted according to the method of Bligh & Dyer (1959). Recovery of ^{3}H was 97%.

The metabolites which became chloroform soluble after methylation were applied to a 2 × 28 cm column of Sephadex LH-20 and eluted with 65% chloroform in Skellysolve B. Fractions (11.5 mL each) were collected and 0.1-mL aliquots were taken for tritium determination (see Figure 7). Column recovery of ^{3}H, including that in a methanol wash (150 mL), was 74%. Fractions 14–20 were pooled, solvents were removed, and the residue was further purified by SP-HPLC eluting with 10% 2-propanol in hexane. Fractions (1.0 mL) were collected and the radioactivity of 25-μL aliquots was determined (see Figure 8A). Recovery of ^{3}H was 86%. Fractions 20–25 were pooled, solvents were removed, and the residue was chromatographed on RP-HPLC eluting with 75% methanol in water. Fractions (1.0 mL) were collected and 25-μL aliquots were used for scintillation counting (see Figure 8B). Recovery of ^{3}H was 85%. Fractions 28–30 were pooled, solvents were removed, and the residue was again chromatographed on the SP-HPLC system described above. Recovery of ^{3}H was 92% (see Figure 8C). Fractions 17–19 were combined, providing 3.4 μg of pure material.

A portion of the pure metabolite (67 ng) was mixed with synthetic calcitroic acid methyl ester (1.5 μg), and the mixture

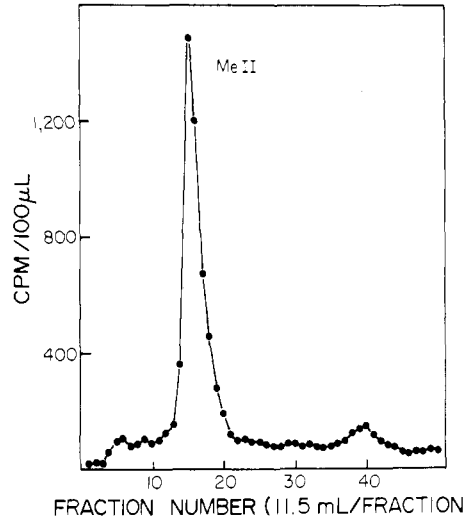

FIGURE 7: Chromatography of the chloroform-soluble metabolites after methylation of peak II (fractions 13–19 of Figure 6 were combined) on Sephadex LH-20 with elution by 65% chloroform in Skellysolve B.

was chromatographed on the same SP-HPLC system (see Figure 8D). Recovery of ^{3}H was 115%.

Distribution Studies. Bile samples were extracted as described above. The chloroform-soluble material was chromatographed on columns of Sephadex LH-20 (1 × 36 cm) eluted with 75% chloroform in Skellysolve B. Fractions (3.2 mL) were collected and aliquots were taken for measurement of ^{3}H content [see Figure 2 for the chromatogram of the sample from the bile of rats dosed with 15 μg of 1α,25-$(OH)_2D_3$]. The columns were washed with methanol. For the four samples recovery of ^{3}H was 75 ± 3% (mean ± SE). The percent of the radioactivity in the column wash was 21–34% of the recovered disintegrations per minute. Fractions containing peak 2 were combined, solvents were evaporated, and the residue, after addition of synthetic 1α,25-$(OH)_2D_3$, was chromatographed on a column (0.46 × 25 cm) of Zorbax-SIL (Du Pont Instruments). The column was eluted with 10% 2-propanol in hexane and 1.0-mL fractions were collected. The entire fraction was used for scintillation counting (see Figure 3). Recovery of ^{3}H was 72 ± 3% (mean ± SE, n = 3). The 1.5-μg sample was not analyzed by HPLC because of small amounts of radioactivity.

The aqueous phases of the bile extractions were chromatographed as described for the isolation of calcitroic acid with the exceptions in method noted below. Recovery of ^{3}H for the DEAE-Sephadex columns (1 × 25 cm) was 105 ± 17% (mean ± SE). Recovery of ^{3}H for methylation and extraction was 72 ± 12% (mean ± SE). Recovery of radioactivity for the Sephadex LH-20 columns (1 × 27 cm, 70% chloroform in Skellysolve B) was 66 ± 5% (mean ± SE).

Results

Bile duct cannulated rats were dosed with 1α,25-$(OH)_2$-[3α-^{3}H]D_3, and the collected bile was subjected to the chromatographic separations outlined in Figure 1. The bile was extracted according to the method of Bligh & Dyer (1959). The chloroform phase containing only small amounts of the total bile radioactivity was chromatographed on Sephadex LH-20 (see Figure 2). This column separated two components, named peak 1 and peak 2. Peak 2 migrated on Sephadex LH-20 similarly to standard 1α,25-$(OH)_2D_3$. Peak 2 was identified as 1α,25-$(OH)_2D_3$ by cochromatography with synthetic 1α,25-$(OH)_2D_3$ on SP-HPLC (see Figure 3); 1α,24R,25-$(OH)_3D_3$ was not detected in bile (Figure 2).

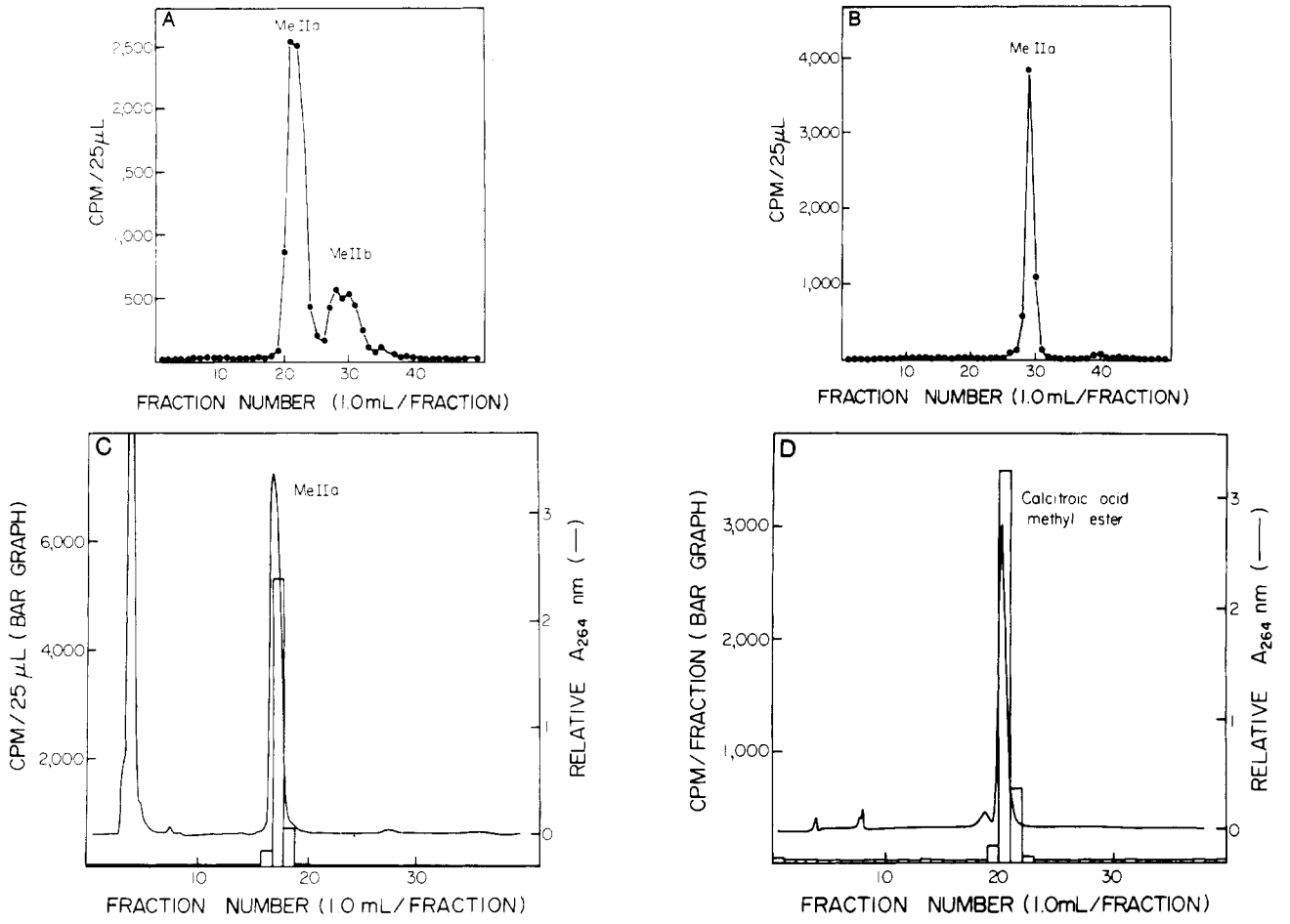

FIGURE 8: (A) Chromatography of MeII (fractions 14–20 of Figure 7 were pooled) on SP-HPLC with elution with 10% 2-propanol in hexane. (B) Chromatography of MeIIa [fractions 20–25 of (A) were pooled] on RP-HPLC using 75% methanol in water as the elution solvent. (C) Chromatography of MeIIa (fractions 28–30 of (B) were pooled) on SP-HPLC as described for (A). (D) Cochromatography of synthetic calcitroic acid methyl ester with MeIIa (fractions 17–19 of (C) were pooled and a portion was used) on SP-HPLC as described for (A).

The other bile component, peak 1, eluted prior to 1α,25-$(OH)_2D_3$ on the Sephadex LH-20 column (see Figure 2). By HPLC, peak 1 could be resolved into two components, **1a** (20%) and **1b** (80%) (Figure 4). Further purification of **1a** by a combination of RP- and SP-HPLC (see Materials and Methods and Figure 1) gave material eluting as a sharp and apparently homogeneous peak, which was analyzed by mass spectroscopy. A molecular ion of *m/e* 398, and peaks of *m/e* 380, 362 (M – H_2O and M – $2H_2O$), 269 (M – side chain – H_2O), and 251 (269 – H_2O) as well as the characteristic ring A peak pattern at *m/e* 152 and 134 (base) suggested that **1a** was a side chain dehydration product of 1α,25-$(OH)_2D_3$, i.e., either Δ^{24}-1α-OH-D_3 or Δ^{25}-1α-OH-D_3 or a mixture of both which would not have been resolved by the chromatographic purification steps.

Indeed, radioactive **1a** coeluted as a single sharp peak with a synthetic mixture of Δ^{24}-1α-OH-D_3 and Δ^{25}-1α-OH-D_3 (produced by chemical dehydration of 1α,25-$(OH)_2D_3$; Napoli et al., 1978) on SP-HPLC, and the mass spectrum of **1a** was nearly identical with that of the synthetic mixture. Subsequent cochromatography of **1a** with the mixture of synthetic Δ^{24} and Δ^{25} isomers on RP-HPLC resolved the isomers after recycling twice and conclusively established that isolated **1a** is a mixture (ca. 60:40) of the Δ^{24}- and Δ^{25}-1α-OH-D_3 compounds (Figure 5).

Component **1b** (Figure 4), after further purification by HPLC (see Materials and Methods and Figure 1), was identified as the methyl ester of calcitroic acid by direct comparison of its mass spectrum with that of authentic methyl calcitroate (Esvelt et al., 1979). The assignment was further confirmed by preparing the 1,3-bis(trimethylsilyl) ether derivative of **1b** which gave a mass spectrum matching that of authentic methyl calcitroate bis(trimethylsilyl) ether. Component **1b**, however, appears to be an artifact of the extraction procedure, based on results of a subsequent experiment in which an aliquot of the same bile sample was extracted with chloroform only (the methanol was omitted). The resulting chloroform phase, when chromatographed on Sephadex LH-20 by using the conditions illustrated in Figure 2, contained the expected amount of 1α,25-$(OH)_2D_3$ (125% of that recovered in the regular extraction procedure), but the amount of peak 1 was decreased dramatically (ca. 5×) compared to that found by using the chloroform/methanol extraction. Furthermore, HPLC of peak 1 (conditions as in Figure 4) revealed the absence of component **1b**, whereas the abundance of peak **1a** was essentially unchanged (96% of that obtained with the chloroform/methanol procedure). We conclude, therefore, that peak **1b** (methyl calcitroate) is a product of the standard methanol/chloroform extraction procedure, possibly arising by transesterification of an unstable calcitroic acid conjugate.

The aqueous methanol phase of bile, containing the majority of the total bile radioactivity, was chromatographed on DEAE-Sephadex (Figure 6). This gave two peaks: peak I (minor) which eluted with 95% methanol in water and peak II (major) which required 0.1 M ammonium bicarbonate in 90% methanol in water for elution. To check if the column was overloaded, we rechromatographed peak I on the same column system. No peak II was detected. Peak II was

Table II: Ratio of ^3H to ^{14}C in Bile Fractions from Rats Given 1α,25-$(OH)_2$[3α-^{3}H,26,27-^{14}C]D_3

fraction[a]		(fraction $^3H/^{14}C$)/(dose $^3H/^{14}C$)
nonpolar	polar	
peak 1		0.72
1α,25-$(OH)_2D_3$		0.94
	peak I	2.0
	MeII	6.7
	aqueous	3.1
	polar strip	1.6

[a] For a description of the terms, see Figure 1.

methylated with excess diazomethane, and the majority of the radioactive metabolites became organic soluble. The metabolites which remained aqueous soluble mostly migrated like peak II on DEAE-Sephadex chromatography, and reaction of the reisolated peak II with additional diazomethane did not alter its chromatographic properties on the ion-exchange resin. It thus appears that the aqueous methanol phase of bile contains acidic metabolites that either methylate very slowly (if at all) or hydrolyze rapidly in mixtures of methanol and water to give back acidic compounds.

The water-soluble, charged metabolites which became chloroform soluble after methylation were chromatographed on Sephadex LH-20 (Figure 7). Elution with 65% chloroform in Skellysolve B gave a single peak (named MeII), but the majority of the radioactivity did not elute with this solvent mixture but did elute with methanol. MeII was further purified on SP-HPLC (Figure 8A). This yielded MeIIb (minor) and MeIIa (major). MeIIa was then chromatographed on RP-HPLC (Figure 8B) and again on SP-HPLC (Figure 8C). In this last chromatogram, the radioactivity migrated with a single UV-absorbing component and was considered pure. Metabolite MeIIa was identified as calcitroic acid methyl ester by cochromatography with the authentic substance on SP-HPLC (Figure 8D) and by its mass spectrum (not shown to conserve space), identical with that of the natural product isolated from rat liver by Esvelt et al. (1979). It had the following ions (intensity): *m/e* 388 (22), 370 (20), 352 (7), 314 (2), 287 (4), 269 (5), 152 (42), 134 (100).

The distribution of total bile radioactivity in the various chromatographic fractions is summarized in Table I. The data found for 0.15-, 1.5-, and 15-μg doses at 1α,25-$(OH)_2D_3$ are quite similar. Bile from rats given the lowest dose of 1α,25-$(OH)_2D_3$ (0.015 μg) showed substantially larger amounts (as a percentage of the total dose) of 1α,25-$(OH)_2D_3$ and peak 1 and smaller amounts of calcitroic acid.

The enrichment in ^3H of the various chromatographic fractions of bile from rats dosed with 1α,25-$(OH)_2$[3α-^{3}H]D_3 and 1α,25-$(OH)_2$[26,27-^{14}C]D_3 is shown in Table II. Neither of the chloroform-soluble components was enriched in ^3H. All of the aqueous fractions were ^3H enriched, indicating the presence of side chain cleaved metabolites. The highest enrichment was found for MeII.

Discussion

Calcitroic acid was found in substantial quantities in the bile of rats given 1α,25-$(OH)_2D_3$. Over a wide range of 1α,25-$(OH)_2D_3$ dose levels (0.15-15 μg, see Table I), 19–20% of the total bile radioactivity was found to be calcitroic acid. Since ~65% of a dose of 1α,25-$(OH)_2D_3$ appears in bile within 24 h of injection (B. L. Onisko and H. F. DeLuca, unpublished results), it can be estimated that calcitroic acid secretion in bile amounts to ~13% of the administered dose. The function of this signficant pathway of 1α,25-$(OH)_2D_3$ metabolism is not known. One possibility is that side chain oxidation and biliary secretion are a route for inactivation and excretion of 1α,25-$(OH)_2D_3$. Alternatively, metabolites of 1α,25-$(OH)_2D_3$ in bile may be reabsorbed by the intestine and have biological functions there. The report of Webling & Holdsworth (1966) that bile duct ligation in normal rats diminishes calcium transport supports the latter possibility. Bile duct ligation did not decrease the intestinal calcium transport in chickens, however (Webling & Holdsworth, 1965).

There remain substantial amounts of unknown metabolites of 1α,25-$(OH)_2D_3$ in bile. The fraction eluting with methanol from the Sephadex LH-20 column (see Figure 1) amounts to 37–41% of the total bile radioactivity. This fraction consists of metabolites which are water soluble, negatively charged, rendered chloroform soluble after methylation and thereafter adsorb much more strongly to Sephadex LH-20 than does the methyl ester of calcitroic acid. Esvelt et al. found much less of this material (named Me charged no. 2 by these workers) in either the livers or intestines plus contents of rats.

It is of interest that no 1α,24*R*,25-$(OH)_3D_3$ was found in bile (Table I and Figure 2). Vitamin D deficient rats given 1α,25-$(OH)_2D_3$ are known to produce 1α,24*R*-25-$(OH)_3D_3$ (Tanaka et al., 1977). It thus appears that the 1α,24*R*,25-$(OH)_3D_3$ is further metabolized before biliary excretion or, less likely, that the 1α,24*R*,25-$(OH)_3D_3$ is excreted by some other route. The synthesis of 1α,24*R*,25-$(OH)_3D_3$ from 1α,25-$(OH)_2D_3$ is known to be extrarenal in part since nephrectomy does not diminish its concentration in blood (Tanaka et al., 1977). The site of the extrarenal 24-hydroxylation of 1α,25-$(OH)_2D_3$ is probably intestinal since homogenates of this organ have been shown to carry out the reaction (Kumar et al., 1978). Whether or not 1α,24*R*,25-$(OH)_3D_3$ is an intermediate in the oxidation of 1α,25-$(OH)_2D_3$ to calcitroic acid is unknown. $^{14}CO_2$ is formed from a dose of 24*R*,25-$(OH)_2$[26,27-^{14}C]D_3 but at a much slower rate than that found for 1α,25-$(OH)_2$[26,27-^{14}C]D_3 (Kumar et al., 1976). This suggests that 1-hydroxylation of 24*R*,25-$(OH)_2D_3$ is necessary for the observed side chain cleavage of 24*R*,25-$(OH)_2D_3$.

The two dehydro isomers (Δ^{24}- and Δ^{25}-dehydro-1α-hydroxyvitamin D_3) isolated from bile very likely originate by direct dehydration of 1α,25-$(OH)_2D_3$. Since the two compounds occur in very nearly the same ratio that is obtained by standard chemical dehydrations of the 25-hydroxy group (Figure 5), we conclude that the isomers arise via a nonenzymatic elmination process in bile.

The methyl ester of calcitroic acid, a significant component of peak 1 using our standard extraction procedure, was not isolated when methanol was omitted from the extraction solvent mixture and therefore appears to be an artifact of the customary Bligh and Dyer extraction process. One should note also that peak 1 does not show the tritium enrichment (Table II) expected if the two dehydro isomers and methyl calcitroate were its sole components. An explanation of this discrepancy is that the nonpolar peak 1 eluting very early from the Sephadex LH-20 column (Figure 2) very likely includes various small ^{14}C-containing side chain fragments which would alter the expected $^3H/^{14}C$ ratio but would, of course, go undetected in our isolation experiments, where tritium-labeled 1α,25-$(OH)_2D_3$ was used exclusively.

This report provides positive identification of the first biliary metabolite of vitamin D in general and 1,25-$(OH)_2D_3$ in particular. It also illustrates, however, the complexity of the metabolite pattern in bile and the rather large number of significant components that remain to be isolated and identified.

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Factors Affecting the Binding of Chick Oviduct Progesterone Receptor to Deoxyribonucleic Acid: Evidence That Deoxyribonucleic Acid Alone Is Not the Nuclear Acceptor Site†

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ABSTRACT: Studies on the interaction of the chick oviduct progesterone-receptor complex (P-R) with various nuclear components revealed a variable, nonsaturable binding of P-R to pure deoxyribonucleic acid (DNA). In contrast, a receptor-dependent, saturable, high level of binding of P-R was observed with a nonhistone protein–DNA complex called nucleoacidic protein (NAP). Three categories of factors were identified which affected the binding of P-R to the DNA. These were (1) the conditions of the binding assay, (2) the properties of the receptor, and (3) the state of the DNA. The conditions in the binding assay which affect DNA binding are the choice of the blanks, the salt concentration, and the pH of the assay. The receptor preparations display their own characteristic levels of binding to native DNA. The basis of this DNA binding capacity by each preparation is unknown. Lastly, the purity and the integrity of the DNA itself determine the level of binding of the P-R. Protein impurities, moderate degradation of the DNA by enzymatic or physical fragmentation, and ultraviolet (UV) light treatment greatly enhance the receptor binding to the DNA. The extent of binding to DNA depends on the degree of damage. Interestingly, totally denatured (single-stranded) DNA displays little or no binding of the P-R. Seasonal differences which are observed for the binding of P-R to chromatin in vivo and in vitro and to NAP in vitro do not occur with DNA whether it is undamaged or damaged. It is concluded from these studies that under controlled conditions and by using DNA preparations as native as possible, minimal binding of P-R to pure DNA occurs. The numerous reports in the literature describing marked binding of the steroid-receptor complex to DNA may well be due to conditions described in this paper. Further, it is concluded that native or partially degraded DNA alone does not appear to represent the native nuclear acceptor sites for the chick oviduct P-R. In contrast, the DNA–nonhistone protein (acceptor protein) complexes do show characteristics of the native-like acceptor sites.

A primary action of steroid hormones is the regulation of specific gene expression in target cells. The alteration in gene expression has been attributed to the direct interaction of steroid receptors with the chromosomal material of several tissue systems (Mueller et al., 1958; Means & Hamilton, 1966; Hamilton et al., 1968; O'Malley et al., 1969, 1972; Glasser et al., 1972; Baxter & Tomkins, 1971; Knowler & Smellie, 1971; Palmiter, 1972; Kurtz et al., 1976; Tata, 1976; Spelsberg, 1976; Spelsberg et al., 1971, 1972; Martial et al., 1977; Thrall et al., 1978). Since the discovery of steroid receptors, their translocation to the nucleus, and their binding to chromatin, a growing interest has arisen as to the nature of their nuclear binding sites. One of the ultimate goals in such studies has been to determine if the steroid receptor binds directly to deoxyribonucleic acid (DNA) or whether other nuclear components are involved in the high-affinity interaction.

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