

Enterohepatic Physiology of 1,25-Dihydroxyvitamin D₃

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ABSTRACT After intravenous administration of radiolabeled 1,25-dihydroxyvitamin D₃ to rats, ~25% of the administered radioactivity appeared in the bile within 24 h. Instillation of the biliary radioactivity into the duodena of other rats was followed by recovery of 15% of the radioactivity in newly secreted bile within 24 h. The process by which products of 1,25-dihydroxyvitamin D₃ were excreted in bile was not saturable in the dose range tested (0.275–650 ng). The metabolites of 1,25-dihydroxyvitamin D₃ present in bile were found to be much more polar than 1,25-dihydroxyvitamin D₃ and were resolved into three fractions on high performance liquid chromatography. 60% of the radioactivity present in bile was retained selectively by DEAE-cellulose; the radioactive material could be eluted from the gel at a low pH or at high salt concentrations. When bile containing the radiolabeled metabolites was incubated at 37°C and pH 5 with β-glucuronidase, there was an increase in the amount of radioactivity comigrating with 1,25-dihydroxyvitamin D₃. Treatment of the products of radiolabeled 1,25-dihydroxyvitamin D₃ in bile with diazomethane, an agent which converts acids into methyl esters, transformed one of the metabolites into a less polar compound. These results demonstrate that there is a quantitatively important enterohepatic circulation of the products of 1,25-dihydroxyvitamin D₃ in the rat.

INTRODUCTION

Metabolites of vitamin D₃ and 25-hydroxyvitamin D₃ appear in the bile after administration of these radiolabeled vitamins to man or to the rat (1–3). Furthermore, the metabolites are more polar than the administered compounds (2, 3). It is suspected that these more polar compounds are glucuronides, or other conjugates of the parent sterol, since treatment with β-glucuronidase results in a decrease in polarity of the biliary metabolites (1–3).

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1,25-dihydroxyvitamin D₃ is presently thought to be the active form of vitamin D and is known to be metabolized by side chain oxidation and 24-hydroxylation in the intestine and kidney (4–9). However, these processes account for only part of the metabolism of 1,25-dihydroxyvitamin D₃. A large fraction of the radioactivity is known to appear in the feces of both rat (unpublished observations) and man (10) after the intravenous administration of radiolabeled 1,25-dihydroxyvitamin D₃; these findings suggest that biliary excretion and possibly enterohepatic circulation of 1,25-dihydroxyvitamin D₃ may occur. The present studies were carried out to determine whether there was biliary excretion and enterohepatic circulation of 1,25-dihydroxyvitamin D₃, and if so, to partially characterize the metabolites excreted in bile.

METHODS

Animals

1-d-old white leghorn cockerels (Rochester Chicken Feed Supply, Rochester, Minn.) were raised on a 1% calcium rickets diet and sacrificed when 6 wk old to provide a source for renal mitochondria (11).

Male rats (200–210 g) were obtained from the Holtzman Co., (Madison, Wis.) and maintained on rat chow that contained 1.2% calcium, 0.86% phosphorus, and adequate amounts of vitamin D (5.31 IU/g feed). Experiments were conducted when the weight of the animals was between 200–220 g.

Anesthesia and surgery

Immediately before surgery, the rats were anaesthetized with sodium pentobarbital (30 mg/kg). A midline laparotomy incision was made, the bile duct was catheterized with polyethylene tubing, the tube was exteriorized, and the abdomen was closed with suture and metal clips. Immediately thereafter, the rats were injected intrajugularly with radiolabeled 1,25-dihydroxyvitamin D₃ and all bile was collected for 24 h.

In studies designed to detect the presence of an enterohepatic circulation of 1,25-dihydroxyvitamin D₃ metabolites, the bile ducts were catheterized in the manner described above. A small incision was made in the distal duodenum into which radiolabeled bile (~0.5 ml) obtained in the above experiments

was instilled with a syringe. The duodenal and abdominal incisions were then closed with suture, and bile was collected for 24 h. In some instances blood was collected immediately before termination of the experiment to test for the presence of circulating radioactivity.

Synthetic and radiolabeled compounds

Synthetic 1,25-dihydroxyvitamin D₃ was kindly donated by Dr. M. Uskokovic of Hoffman-LaRoche, Inc., Nutley, N. J.

[23,24-³H]25-hydroxyvitamin D₃ (110 Ci/mmol) and [26,27-³H]25-hydroxyvitamin D₃ (9 Ci/mmol) were obtained from the Amersham Corp. (Arlington Heights, Ill.). These compounds were hydroxylated enzymatically to give radiolabeled 1,25-dihydroxyvitamin D₃ by using kidney mitochondria from rachitic chicks (11). Radiochemical purity of each product was established by cochromatography with authentic 1,25-dihydroxyvitamin D₃ on high performance liquid chromatography. All radiolabeled materials were more than 98% pure by chromatography.

Dose of radiolabeled 1,25-dihydroxyvitamin D₃ administered

To test for excretion of radioactivity in bile, cannulated Holtzman rats were administered 160,000 dpm of [23,24-³H]1,25-dihydroxyvitamin D₃ or 160,000 dpm of [26,27-³H]1,25-dihydroxyvitamin D₃ with varying amounts (0.275–650 ng) of nonradiolabeled 1,25-dihydroxyvitamin D₃. The compounds were administered intrajugularly in 0.05 ml ethanol to 200–210 g rats.

In one series of experiments, the amount of biliary radioactivity appearing after the administration of [23,24-³H]1,25-dihydroxyvitamin D₃ was compared with the amount of radioactivity appearing after the administration of [26,27-³H]1,25-dihydroxyvitamin D₃. In these experiments, 300–310 g rats were used.

Estimation of radioactivity

Aliquots of bile or plasma were added to 15–20 ml of scintillation mixture (Aquasol II, New England Nuclear, Boston, Mass.). As significant quenching occurs with bile, quench curves were constructed and appropriate corrections were made. Counting efficiencies ranged between 15 and 50%. Window settings were optimized for different levels of quench. Solutions were monitored for fluorescence and chemi-luminescence by random coincidence monitoring. For determining radioactivity in nonaqueous samples, a scintillation mixture containing 2 g of 2,5-diphenoxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene per liter of toluene was used. A Beckman LS-9000 β-scintillation counter was used in all instances (Beckman Instruments, Inc., Fullerton, Calif.).

Ultraviolet spectroscopy

Ultraviolet spectra were obtained using a Beckman 25 ultraviolet spectrophotometer.

Chromatography

Solvents for use in high performance liquid chromatography were ultraviolet or spectral grade. Solvents for use in column chromatography were analytical grade or reagent grade and were redistilled before use.

High performance liquid chromatography. Chromatography was carried out with a liquid chromatograph equipped with 2 model 6000A pumps, a model U6K injector, a model 450 variable wavelength ultraviolet detector, and a model 660 gradient programmer (all from Waters Associates, Milford, Mass.) and a model 3380-A Hewlett Packard integrator (Hewlett-Packard Co., Palo Alto, Calif.).

To test the purity of the radiolabeled 1,25-dihydroxyvitamin D₃, 2000 cpm of the compound was mixed with 500 ng of authentic, nonradiolabeled 1,25-dihydroxyvitamin D₃ and chromatographed on a 0.4 × 30-cm column of μPorasil with 10 μm silica (Waters Associates), using 90:10 (vol/vol) *n*-hexane/2-propanol as the solvent system at a flow rate of 2 ml/min and a pressure of about 600 lb/in.². Radioactive 1,25-dihydroxyvitamin D₃ co-eluted with synthetic 1,25-dihydroxyvitamin D₃ in this system.

To determine the chromatographic mobility of the radioactive biliary metabolites of 1,25-dihydroxyvitamin D₃, an aliquot of bile (2000 dpm) to which 1 μg of cold 1,25-dihydroxyvitamin D₃ had been added was dried and resuspended in 100 μl of 50:50 (vol/vol) methanol/water and applied to a C-18 μBondapak column (Waters Associates). A linear gradient from 50% methanol/water to 100% methanol was developed over 35–40 min. The flow rate was 2 ml/min. Starting pressure (50% methanol/water) was 2,200 lb/in.²; final pressure was 600 lb/in.² (100% methanol); 2-ml (1 min) aliquots were collected. In other experiments, bile was treated with β-glucuronidase at pH 5, with buffer at pH 5 or with water alone for 16–24 h as described below. The reaction mixture was extracted three times with chloroform. The chloroform layers were combined and dried. The residue was resuspended in 300 μl 50:50 (vol/vol) methanol/water that contained 1 μg 1,25-dihydroxyvitamin D₃ and applied to the described high performance system. Alternatively, the reaction mixture was dried, resuspended in column solvent that contained 1 μg 1,25-dihydroxyvitamin D₃, and directly applied to the high performance system described. In both cases an aliquot of the sample was taken for determination of radioactivity.

Silicic acid chromatography. Aliquots of bile, 0.5–1.0 ml, obtained after the injection of radiolabeled 1,25-dihydroxyvitamin D₃ into rats, were chromatographed on a 10 g, 1 × 30-cm silicic acid column (Bio-Sil A, 100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.). The bile was applied by mixing it with ~0.5 g of dry silicic acid and applying it directly to the column. The column was developed successively with 100 ml of hexane, 100 ml of ethyl acetate, 250 ml of methanol, and 150 ml of 50:50 (vol/vol) methanol/water.

Sephadex LH-20 chromatography. (a) A 1 × 60-cm column of Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, N. J.) was employed with 65:35 (vol/vol) chloroform/*n*-hexane to purify the radiolabeled 1,25-dihydroxyvitamin D₃ before chromatographing the material on a high performance apparatus. (b) A Sephadex LH-20 column, 2 × 60 cm, was used with 65:35 (vol/vol) chloroform/*n*-hexane to determine the chromatographic mobility of the biliary metabolites of 1,25-dihydroxyvitamin D₃.

Lipidex 5000 chromatography. A 2 × 5.5-cm column of Lipidex 5000 (Packard Instrument Co., Inc., Downers Grove, Ill.) was used with 20:70:10 (vol/vol) chloroform/methanol/water or with 70:30 (vol/vol) methanol/water as the solvent system to determine the chromatographic mobility of the biliary products of 1,25-dihydroxyvitamin D₃; 5-ml aliquots were collected.

Use of DEAE-cellulose. DEAE-cellulose (DE52, Whatman Chemicals, Div. W&R Balston, Maidstone, Kent, England) was washed thoroughly with methanol. 3 g of the gel were mixed in a batch procedure with 1 ml of crude or methylated bile

that contained 700 cpm of radioactivity, the gel was filtered and washed with methanol (100 ml). The filtrate was evaporated and the radioactivity in the residue was determined in a β -scintillation counter. The gel was then washed with 100 ml of 1 M ammonium acetate in methanol; in a separate experiment the gel was washed with 50:50 (vol/vol) acetic acid/methanol to remove bound radioactivity. The eluates from each wash were dried and assayed in a scintillation counter.

In another experiment, a 2×30 -cm DEAE-cellulose column was packed with methanol. An aliquot of bile diluted with methanol was then applied. The column was eluted with 100 ml of methanol, followed by a linear gradient to 25% acetic acid in methanol over the next 250 ml; 5-ml aliquots were collected and assayed for radioactivity.

Paper chromatography. After treatment with diazomethane, the reaction mixture was chromatographed on paper with butyl acetate/n-butanol/water/acetic acid (9:1:9:1 vol/vol).

Chemical procedures

Treatment with β -glucuronidase or buffer at pH 5. Aliquots of bile that contained 1,000–2,000 cpm of radioactivity were incubated with sodium acetate buffer at pH 5, β -glucuronidase (8,000 U, type B-10) in a sodium acetate buffer at pH 5, or water alone at pH 7–8, for 16–24 h at 37°C. The final volume of the reaction mixture was 1.6 ml. β -glucuronidase was obtained from the Sigma Co., St. Louis, Mo. After the incubation was terminated, the aqueous reaction mixture was extracted three times with 5 ml of chloroform. The combined chloroform layers were dried over anhydrous sodium sulfate and the solvent was evaporated with a stream of nitrogen. The extract was resuspended in 300 μ l 50:50 (vol/vol) methanol/water that contained 1 μ g 1,25-dihydroxyvitamin D₃. An aliquot was taken for determination of radioactivity. The rest of the radioactivity was applied to a high performance liquid chromatography system described above. Alternatively, the entire reaction mixture was dried down without extraction with chloroform, resuspended in 300 μ l of 50:50 (vol/vol) methanol/water containing 1 μ g 1,25-dihydroxyvitamin D₃ and then applied directly to the high performance system described above. In either case the amount of radioactivity co-eluting with authentic 1,25-dihydroxyvitamin D₃ was determined and

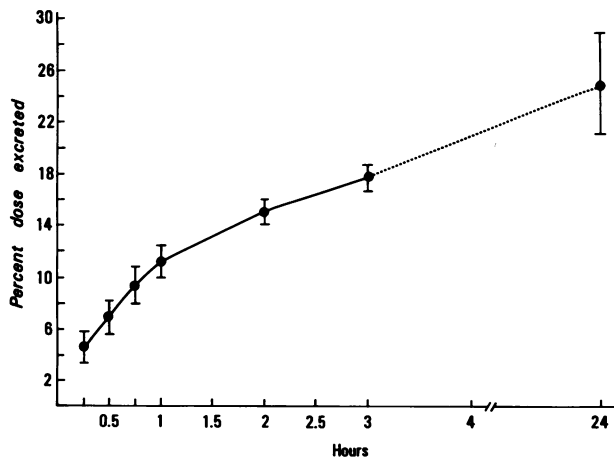


FIGURE 1 Cumulative amount of radioactivity (mean \pm SD) appearing in the bile of rats administered 0.275 ng of [23,24-³H]1,25-dihydroxyvitamin D₃ intravenously ($n = 5$).

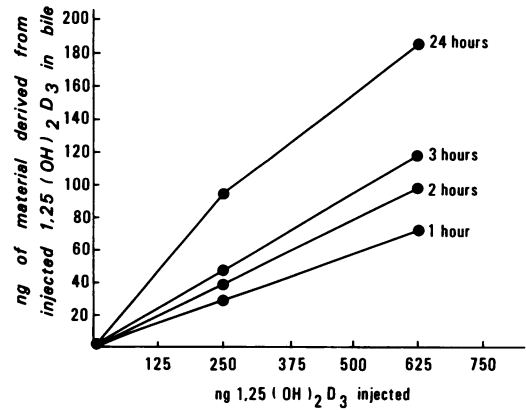


FIGURE 2 Amounts of 1,25-dihydroxyvitamin D₃ (mean) derived metabolites appearing in bile after the administration of increasing amounts of 1,25-dihydroxyvitamin D₃ ($n = 5$ per group).

expressed as a percentage of the total radioactivity in the bile sample. To determine if bile had β -glucuronidase activity, [³H]tetrahydroaldosterone β -D-glucosiduronate (12, 13) was incubated with sodium acetate at pH 5, with sodium acetate at pH 5 plus 1,500 U β -glucuronidase (type B-10) and with sodium acetate at pH 5 plus 150 μ l of bile obtained from a normal rat that received no 1,25-dihydroxyvitamin D₃.

Treatment of bile with diazomethane. Aliquots of crude or partially purified bile (1 ml) were dried *in vacuo*, redissolved in 95% methanol (1 ml), and treated with an excess of freshly prepared diazomethane (0.96 mM) in ether. After 10 min at room temperature, the excess of diazomethane was distilled off or decomposed by the addition of 3–5% acetic acid in methanol. The reaction mixture was concentrated *in vacuo* and the residue was subjected to DEAE batch chromatography as described or to paper chromatography. 1-cm strips of the chromatogram were removed and the radioactivity was then determined by scintillation counting.

Statistical analysis. Statistical analyses were carried out with Student's *t* test (nonpaired).

RESULTS

In the present studies, $11.3 \pm 1.5\%$ (mean \pm SD) of the radioactivity from the [23,24-³H]1,25-dihydroxyvitamin D₃ injected, appeared in the bile within 1 h (Fig. 1). At 3 h, the amount of radioactivity excreted was $17.6 \pm 1.12\%$ and at 24 h the amount was $24.7 \pm 3.4\%$. There was a linear relationship at each sampling time between the amount of radioactivity excreted in bile and the dose of [23,24-³H]1,25-dihydroxyvitamin D₃ administered (Fig. 2).

The radioactivity recovered from bile after the intravenous injection of [³H]1,25-dihydroxyvitamin D₃ was instilled into the duodena of other rats; the amount of radioactivity subsequently recovered in newly excreted bile is shown in Fig. 3. Approximately 7, 12, and 15% of the radioactivity instilled into the duodenum was recovered from newly excreted bile at 2, 6, and 24 h, respectively.

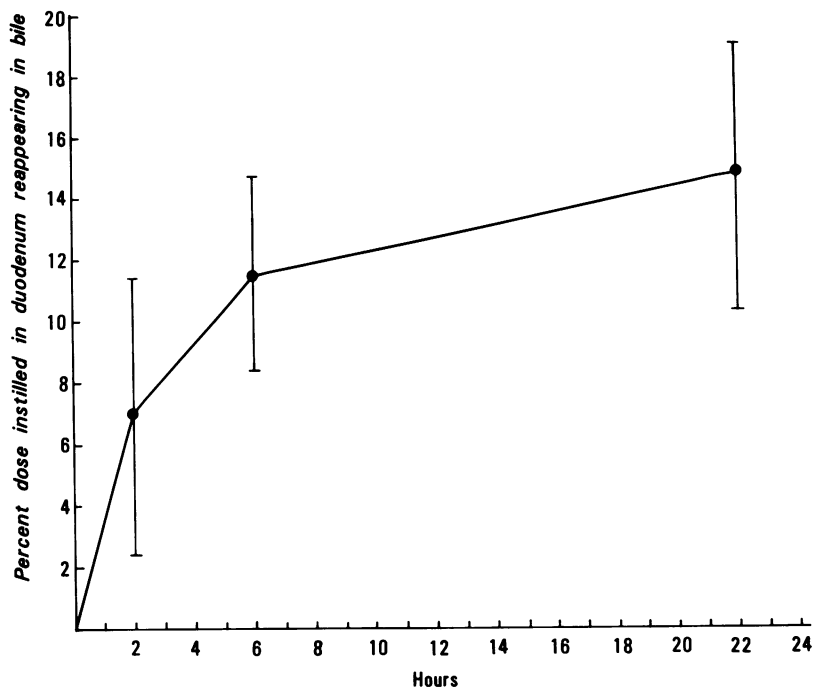


FIGURE 3 Cumulative amounts of radioactivity (mean \pm SD) appearing in bile after the intraduodenal administration of radiolabeled bile (obtained from rats administered radiolabeled 1,25-dihydroxyvitamin D_3). Rats ($n = 5$) underwent biliary catheterization followed by the intraduodenal administration of radiolabeled bile.

To determine if the position of the side chain label influenced the amount of radioactivity appearing in bile, [23,24- 3H]1,25-dihydroxyvitamin D_3 or [26,27- 3H]1,25-dihydroxyvitamin D_3 was administered to different groups of rats. The amount of radioactivity that appeared in the bile of animals administered [26,27- 3H]1,25-dihydroxyvitamin D_3 and in the bile of animals administered [23,24- 3H]1,25-dihydroxyvitamin D_3 , is given in Table I. There was no significant

difference in the amount of radioactivity excreted in bile with the label at the different positions in the side chain.

Chromatography of bile on Lipidex 5000 revealed that biliary radioactivity derived from 1,25-dihydroxyvitamin D_3 was eluted as a more polar peak (Fig. 4). Similarly, silicic acid chromatography demonstrated that ~ 85 – 90% of the radioactivity was present as a polar radioactive peak that eluted at about fraction 42 with 100% methanol (Fig. 5). Approximately 10% of the radioactivity was present as a nonpolar metabolite that co-eluted with 1,25-dihydroxyvitamin D_3 . The profile of radioactivity from high performance liquid chromatography of bile from rats injected with radiolabeled 1,25-dihydroxyvitamin D_3 is shown in Fig. 6. A small percentage of the radioactivity ($\sim 5\%$) was present as 1,25-dihydroxyvitamin D_3 , but most of the radioactivity was present as three polar peaks.

The effect of various types of treatment on the chloroform solubility of radioactivity from the bile of rats that had received radiolabeled 1,25-dihydroxyvitamin D_3 is presented in Table II. More radioactivity was extractable by chloroform after incubation. The addition of β -glucuronidase to bile did not significantly increase the amount of chloroform extractable radioactivity above the amount formed by incubating bile at pH 5. When chloroform extraction of the bile treated with β -glucuronidase, with buffer

TABLE I

Comparison of the Amount of Radioactivity Appearing in Bile after the Administration of either [23,24- 3H] 1,25-Dihydroxyvitamin D_3 or [26,27- 3H] 1,25-Dihydroxyvitamin D_3 to Rats

Time	Percent dose excreted*	
	[23,24- 3H]1,25(OH) $_2D_3$ †	[26,27- 3H]1,25(OH) $_2D_3$ †
<i>h</i>		
1	3.4 \pm 2.1	3.6 \pm 0.3
2	6.1 \pm 2.2	6.0 \pm 0.06
3	9.8 \pm 2.3	8.0 \pm 0.7
4	12.5 \pm 2.6	9.7 \pm 0.7
24	23.5 \pm 5.5	17.6 \pm 6.7

* Mean \pm SD.

† There were five rats per group.

at pH 5 or with water alone, was carried out before high performance liquid chromatography, the amount of radioactivity comigrating with 1,25-dihydroxyvitamin D₃ was 10.3, 6.2, and 3.7% of the total radioactivity in the reaction mixture, respectively. The remaining radioactivity present in the chloroform extracts was in the form of other more polar peaks. When the reaction mixture was dried and applied to the high performance liquid chromatography system without prior chloroform extraction, the amount of radioactivity comigrating with 1,25-dihydroxyvitamin D₃ after treatment with β -glucuronidase, with buffer at pH 5, or with water alone was 15.7 \pm 7.6, 6.7 \pm 3.4, and 5.7 \pm 0.3% of the total radioactivity in the reaction mixture, respectively (Table III). To demonstrate the presence of endogenous β -glucuronidase in bile, [³H]tetrahydroaldosterone 3 β -glucosiduronate was incubated with bile and appropriate controls as shown in Table IV. It is apparent that bile contains β -glucuronidase activity.

60–70% of the radioactivity excreted in the bile was selectively retained by DEAE-cellulose in the pres-

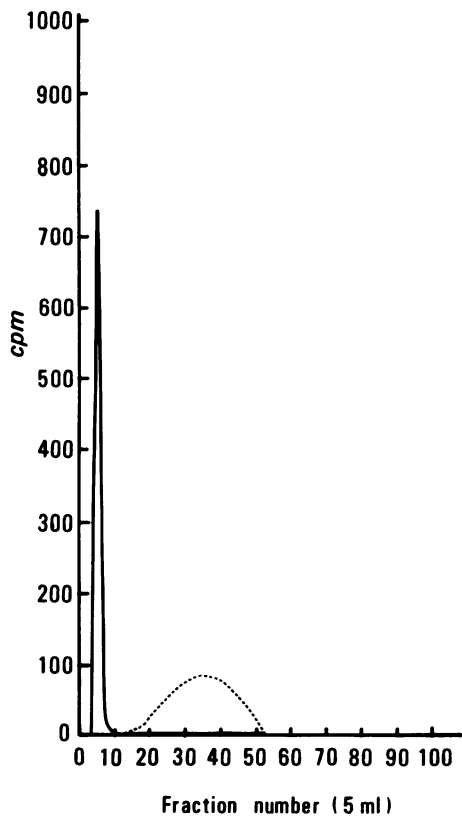


FIGURE 4 Profile of radioactivity present in bile in rats after administration of radiolabeled 1,25-dihydroxyvitamin D₃ (continuous line). Dotted line is 1,25-dihydroxyvitamin D₃ standard. 2 \times 15.5-cm Lipidex 5000 column, 70:30 (vol/vol) methanol/water system.

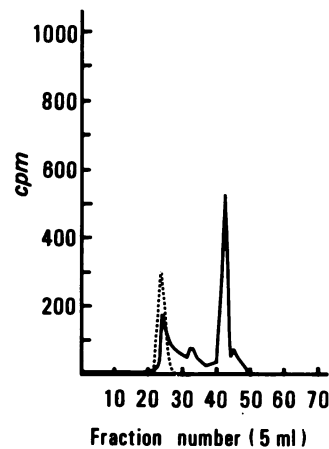


FIGURE 5 Profile of radioactivity present in bile of rats after the administration of radiolabeled 1,25-dihydroxyvitamin D₃ (continuous line). Dotted line is 1,25-dihydroxyvitamin D₃ standard. 1 \times 30-cm, Bio-Sil A column, solvents as indicated in text.

ence of methanol. The radioactivity could be eluted from the gel by adding acid or salt to the eluting solvent (methanol). Three radioactive peaks were eluted with increasing amounts of acetic acid in the methanol (Fig. 7). Treatment of the bile with diazomethane, a compound which esterifies acids, resulted in a decrease in polarity of one metabolite as assessed by paper chromatography. After treatment with diazomethane, 44% of the radioactivity of bile was present as nonpolar material ($R_f > 0.8$) on paper chromatography, whereas only 28% of the radioactivity was present in bile as nonpolar material before treatment with diazomethane. After treatment with diazomethane, 56 \pm 15% (mean \pm SD) of the total radioactivity applied to DEAE cellulose was eluted with methanol alone; before treatment with diazomethane 12 \pm 4% of the radioactivity applied to DEAE cellulose was eluted with methanol.

DISCUSSION

The present studies demonstrate that the excretion of products of 1,25-dihydroxyvitamin D₃ in bile occurs within 30 min of the injection of radiolabeled 1,25-dihydroxyvitamin D₃; 25% of an injected dose appears in bile within 24 h. A considerable portion of the radioactivity found in bile and instilled into the duodena of other rats, was subsequently absorbed from the intestine and reexcreted in newly formed bile. Thus, there is a quantitatively important enterohepatic circulation of metabolites of 1,25-dihydroxyvitamin D₃ in the rat. The liver appears to have the capacity to excrete increasing amounts of these metabolites in bile, even when pharmacological doses of the compound are administered intravenously. The excretory process is not saturable in the dose range tested (0.275–650 ng).

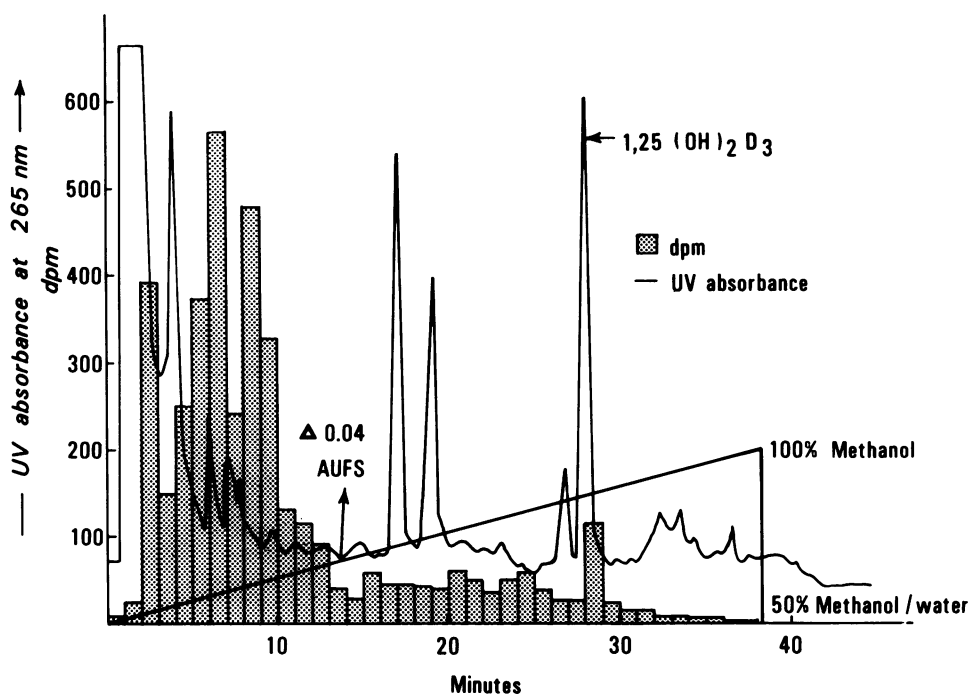


FIGURE 6 Profile of radioactivity present in the bile of rats administered radiolabeled 1,25-dihydroxyvitamin D₃. Continuous line represents UV (ultraviolet) absorbance at 265 nm. 0.4 × 30-cm μ Bondapak column, flow rate 2 ml/min, starting pressure 2200 lb/in.². Bars represent radioactivity.

There is no significant difference in the amount of radioactivity excreted in the bile when [23,24-³H]1,25-dihydroxyvitamin D₃ and [26,27-³H]1,25-dihydroxyvitamin D₃ are administered. However, the presence of the recently identified 1 α -OH-24,25,26,27-tetranorvitamin D₃-23 carboxylic acid (9) in bile cannot be definitely assessed with these two side chain labels.

Evidence presented shows that bile contains metabolites of 1,25-dihydroxyvitamin D₃ that are much

more polar than 1,25-dihydroxyvitamin D₃ itself. Moreover, a substantial amount of the material in bile binds to DEAE-cellulose under conditions in which neutral substances are not retained. At least one of the radioactive metabolites is methylated with diazomethane. These findings are compatible with the presence of an acid function in one of the biliary products. Treatment of bile with β -glucuronidase increases the amount of radioactivity comigrating with 1,25-dihydroxyvitamin

TABLE II
Metabolites [³H]1,25-Dihydroxyvitamin D₃ in Bile: Effect of Various Treatments on Solubility of Radioactive Products in Chloroform

	No.	Time	Temperature	pH	Enzyme	Percent*	P value
			C				
Experiment 1	1	5 min	22°	7-8	0	7.4±1.6	
	2	16 h	37°	7-8	0	9.7±0.7	P < 0.01 1 vs. 2
	3	16 h	37°	5	0	37.0±4.4	P < 0.001 3 vs. 2
	4	16 h	37°	5	β glucuronidase	40.7±2.0	P < 0.001 4 vs. 2 P > 0.05 < 0.1 4 vs. 3
Experiment 2	1	10 min	22°	7-8	0	7.9±1.0	
	2	24 h	37°	7-8	0	10.9±1.1	P < 0.05 1 vs. 2
	3	24 h	37°	5	0	25.5±8.0	P < 0.01 2 vs. 3

* Percent radioactivity extractable into chloroform after appropriate incubation. (Mean±SD of five incubation flasks).

TABLE III
Bile that Contained Radiolabeled Metabolites of 1,25-Dihydroxyvitamin D₃ after Incubation with β -Glucuronidase at pH 5, Buffer Alone at pH 5, or with Water for 16 h at 37°C

No.	Temp	pH	Enzyme	Percent	P value
	C			%	
1	37°	7-8	0	5.7±0.3	
2	37°	5	0	6.7±3.4	<0.1 >0.05 1 vs. 2
3	37°	5	β -glucuronidase	15.7±7.5	<0.01 1 vs. 3 <0.05 2 vs. 3

* Amount of radioactivity comigrating with 1,25-hydroxyvitamin D₃ present in bile expressed as a percentage of total radioactivity applied to the column (mean±SD of five incubations in each group) as assessed by high performance liquid chromatography.

D₃ from 5.7 to 15.7% ($P < 0.01$). This suggests that one of the compounds present in bile is possibly a glucosiduronate. However, the majority of radioactivity present in bile treated with β -glucuronidase and buffer at pH 5 is in the form of the same polar peaks that are present in untreated bile. If these radioactive compounds are negatively charged as is suggested by their retention on DEAE-cellulose under neutral conditions, then the acidic incubation conditions used in the reaction mixture might suppress ionization and increase their solubility in chloroform. There is a small, but statistically insignificant increase in the amount of radioactivity comigrating with 1,25-dihydroxyvitamin D₃ present in bile samples treated with buffer at pH 5 when compared with bile treated with water alone, that may be caused by endogenous biliary β -glucuronidase activity. All of the polar metabolites in bile are not hydrolyzed by β -glucuronidase under the conditions used. This suggests that metabolites other than 1,25-dihydroxyvitamin D₃ glucosiduronate may be present in bile. Alternatively, the incubation conditions used may not have been ideal to convert all glucosiduronates to free compounds. Therefore, there are metabolites derived from 1,25-dihydroxyvitamin D₃ in bile that are more

polar than 1,25-dihydroxyvitamin D₃ itself. These metabolites are retained by DEAE-cellulose in neutral methanol, are susceptible in part to methylation with diazomethane, and are partially hydrolyzed to radioactivity comigrating with 1,25-dihydroxyvitamin D₃ by β -glucuronidase.

The present findings may be of importance in elucidating the pathogenesis of hepatic osteodystrophy in man. It is thought that defects in vitamin D metabolism are responsible for hepatic osteodystrophy (14). It seems possible that the reabsorption of metabolites of vitamin D₃ from the intestine may be altered in conditions such as coeliac disease, or that fecal excretion may be enhanced in conditions such as primary biliary

TABLE IV
Incubation of [³H]Tetrahydroaldosterone 3- β -D-Glucosiduronic Acid with Either β -Glucuronidase with Buffer, Bile with Buffer, or Buffer Alone (0.1 M Sodium Acetate, pH 5) at 37°C

No.	Hydrolyzing agent	Incubation time	Percent radioactivity recovered in chloroform
		h	%
1	Sodium acetate buffer	72	1
2	β -Glucuronidase*	72	76
3	Bile (150 μ l)	72	54

* 1500 U.

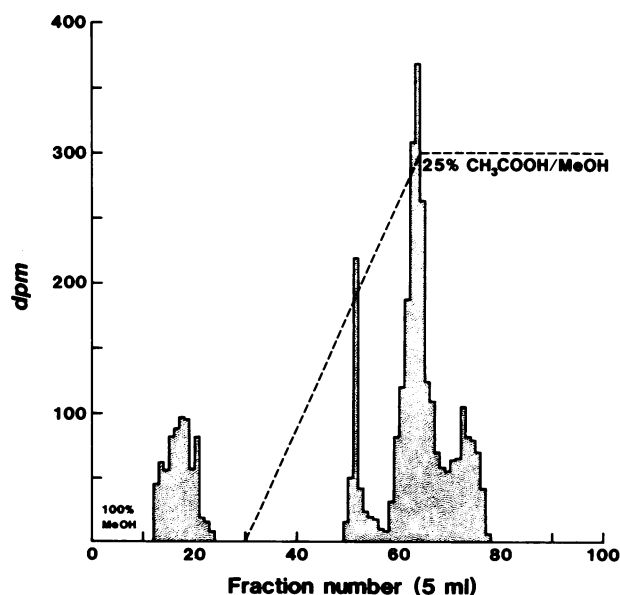


FIGURE 7 Profile radioactivity present in bile of rats after the administration of radiolabeled 1,25-dihydroxyvitamin D₃. 2 × 30-cm DEAE-cellulose column. Authentic 1,25-dihydroxyvitamin D₃ elutes between fractions 15 and 35 on this system.

cirrhosis. A study of these defects, if present, must await a description of the enterohepatic physiology of 1,25-dihydroxyvitamin D₃ in man.

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REFERENCES

1. Avioli, L. V., S. W. Lee, J. E. McDonald, J. Lund, and H. F. DeLuca. 1967. Metabolism of vitamin D₃-³H in human subjects: Distribution in blood, bile, feces, and urine. *J. Clin. Invest.* **46**: 983-992.
2. Arnaud, S. B., R. S. Goldsmith, P. W. Lambert, and Vay L. W. Go. 1975. 25-hydroxyvitamin D₃: evidence of an enterohepatic circulation in man. *Proc. Soc. Exp. Biol. Med.* **149**: 570-572.
3. Bell, P. A., and E. Kodicek. 1969. Investigations on the metabolites of vitamin D in rat bile. Separation and partial identification of a major metabolite. *Biochem. J.* **115**: 663-669.
4. Kumar, R., D. H. Harnden, and H. F. DeLuca. 1976. Metabolism of 1,25-dihydroxyvitamin D₃: evidence for side chain oxidation. *Biochemistry.* **15**: 2420-2423.
5. Harnden, D. H., R. Kumar, M. F. Hollick, and H. F. DeLuca. 1976. "Side chain oxidation of 25-hydroxy (26,27-¹⁴C) vitamin D₃ and 1,25-dihydroxy (26,27-¹⁴C) vitamin D₃ in vivo. *Science (Wash. D. C.)*. **193**: 493-494.
6. Kumar, R., and H. F. DeLuca. 1976. Side chain oxidation of 25-hydroxy [26,27-¹⁴C]vitamin D₃ and 1,25-dihydroxy-[26,27-¹⁴C]vitamin D₃ in vivo by chickens *Biochem. Biophys. Res. Commun.* **69**: 197-200.
7. Kumar, R., H. K. Schnoes, and H. F. DeLuca. 1978. Rat intestinal 25-hydroxyvitamin D₃ and 1 α ,25-dihydroxyvitamin D₃-24-hydroxylase. *J. Biol. Chem.* **253**: 3804-3809.
8. Esvelt, R. P., H. K. Schnoes, and H. F. DeLuca. 1979. Isolation of a side chain oxidized metabolite of 1 α ,25-dihydroxyvitamin D₃. *Fed. Proc.* **38**: 482.
9. DeLuca, H. F. 1979. Vitamin D system in the regulation of calcium and phosphorus metabolism. W. O. Atwater Memorial Lecture. *Nutr. Rev.* **37**: 161-193.
10. Gray, R. W., A. E. Caldas, D. R. Wilz, J. Lemann, Jr., G. A. Smith, and H. F. DeLuca. 1978. Metabolism and excretion of ³H 1,25(OH)²-vitamin D₃ in healthy adults. *J. Clin. Endocrinol. Metab.* **46**: 756-765.
11. Gray, R. W., J. L. Omdahl, J. G. Ghazarian, and H. F. DeLuca. 1972. 25-hydroxycholecalciferol-1-hydroxylase. *J. Biol. Chem.* **247**: 7528-7532.
12. Carlson, G. L., and V. R. Mattox. 1979. Glucuronidation of 3-hydroxysteroids in vitro. *J. Steroid Biochem.* **10**: 161-165.
13. Cooper, B., M. N. Eakins, and R. F. Slater. 1976. The effect of various anaesthetic techniques on the flow rate, constituents and enzymic composition of rat bile. *Biochem. Pharmacol.* **25**: 1711-1718.
14. Rasmussen, H. 1974. Textbook of Endocrinology. R. H. Williams, editor. W. B. Saunders Co., Philadelphia. 660-770.