

## Metabolism of Vitamin D<sub>3</sub>-<sup>3</sup>H in Human Subjects: Distribution in Blood, Bile, Feces, and Urine \*

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**Summary.** Vitamin D<sub>3</sub>-<sup>3</sup>H has been administered intravenously to seven normal subjects, three patients with biliary fistulas, and four patients with cirrhosis. Plasma D<sub>3</sub>-<sup>3</sup>H half-times normally ranged from 20 to 30 hours. *in vivo* evidence that a metabolic transformation of vitamin D occurs was obtained, and a polar biologically active vitamin D metabolite was isolated from plasma.

Urinary radioactivity averaged 2.4% of the administered dose for the 48-hour period after infusion, and all the excreted radioactivity represented chemically altered metabolites of vitamin D. The metabolites in urine were mainly water-soluble, with 26% in conjugated form.

From 3 to 6% of the injected radioactivity was excreted in the bile of subjects with T-tube drainage and 5% in the feces of patients having no T-tube. The pattern of fecal and biliary radioactivity suggested that the passage of vitamin D and its metabolites from bile into the intestine represents an essential stage for the fecal excretion of vitamin D metabolites in man.

Abnormally slow plasma disappearance of vitamin D<sub>3</sub>-<sup>3</sup>H in patients with cirrhosis was associated with a significant decrease in the quantity and rate of glucuronide metabolite excretion in the urine.

### Introduction

The tissue distribution of radioactively labeled vitamins D<sub>2</sub> and D<sub>3</sub> administered orally or parenterally has been studied in the past, primarily in rachitic or normal rats and chicks, by DeLuca and co-workers (1-7), Callow, Kodicek, and Thompson (8), Bosmann and Chen (9), Schachter, Finkelstein, and Kowarski (10), and Nair and Bucana (11). Limited data are also available on the fate of an oral dose of radioactive vitamin D

in humans (12, 13), and normal parameters of vitamin D absorption, as well as abnormalities in patients with malabsorption syndromes, have been defined (13). Relatively little knowledge is available concerning the metabolism of vitamin D<sub>3</sub> in animals, and less in humans. Norman, Lund, and DeLuca have detected three radioactive compounds other than vitamin D<sub>3</sub> with antirachitic activity and at least one without vitamin activity in animal tissues after administration of vitamin D<sub>3</sub>-<sup>3</sup>H (3). Recently, Bell and Bryan (14) and Fraser and Kodicek (15) have commented on the absorption and biliary excretion of <sup>14</sup>C- and <sup>3</sup>H-labeled vitamin D in the rat; they concluded that the absorption and metabolism of vitamin D<sub>3</sub> and cholesterol in the rodent are similar (14).

The present paper presents the results of studies on the metabolism of vitamin D<sub>3</sub>-<sup>3</sup>H in 14 adult human subjects of both sexes. The group in-

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cludes seven normal individuals, three patients with bile fistulas, and four patients with cirrhosis of the liver. The results reported are of interest for the comparative physiology of vitamin D<sub>3</sub>, since the findings obtained in other species can now be related to the sequence of events observed in the human.

### Methods

Seven normal subjects (four male and three female) ranging in age from 23 to 36 years were used for these studies. All were studied during periods of hospitalization after 14 to 21 days of adaptation to diets containing 800 to 1,000 U of vitamin D per day. Randomly labeled vitamin D<sub>3</sub>-<sup>3</sup>H (specific activity 72  $\mu$ c per mg) was prepared according to previously published methods (1, 4). Each subject received 5 to 8  $\mu$ c of radiochemically pure D<sub>3</sub>-<sup>3</sup>H dissolved in 0.75 to 1.0 ml of absolute ethanol. The steroid was administered intravenously with the subject in the fasting state in the morning over a period of approximately 30 to 45 seconds. Fifteen- to 20-ml samples of heparinized blood were collected at 5, 15, 30, and 45 minutes and at 1, 2, 4, 8, 12, 16, and 24 hours. For the subsequent 24 hours plasma was obtained at 12-hour intervals.

During the first day cumulative 6-hour urine collections were made. Thereafter, urine was collected during 8- or 12-hour periods for the remainder of the study. All urine collections were refrigerated and subsequently lyophilized. Feces were collected for 4-day periods after the D<sub>3</sub>-<sup>3</sup>H injections and homogenized with water in a Waring blender; samples were lyophilized. All fecal collections were initiated and terminated with enemas to ensure complete recovery.

*Biliary excretion of radiometabolites of vitamin D<sub>3</sub>-<sup>3</sup>H.* Bile was collected for 48 hours in 8-hour periods from three patients who had undergone cholecystectomy for cholecystitis with insertion of a T-tube into the common bile duct. Complete bile collections were assured by a suction apparatus attached to the T-tube and by the appearance of acholic stools during the period of investigation. The patients had normal liver function preoperatively, and the D<sub>3</sub>-<sup>3</sup>H was injected in each case at least 5 days postoperatively.

*Studies in patients with liver disease.* Four patients with alcoholic cirrhosis confirmed by liver biopsy were subjected to the same dietary and experimental protocols as described above for the normal subjects. The cirrhotic patients had classical symptoms and laboratory findings of hepatocellular disease, viz., elevated serum bilirubin, abnormal thymol turbidity or cephalic flocculation tests, prolonged sulfobromophthalein retention, and low serum albumin. The hematocrits ranged from 39 to 43%, and in each case serum calcium, phosphorus, alkaline phosphatase, and skeletal roentgenograms were normal.

*Determination of total <sup>3</sup>H in biological samples.* One- to 2-ml samples of whole plasma, lyophilized feces and urine, bile, and water and chloroform extracts thereof

were combusted according to the procedure of Kelley, Peets, Gordon, and Buyske (16) with a Thomas Ogg safety igniter.<sup>1</sup> The resulting water vapor-<sup>3</sup>H was frozen on the bottom of a 2-L Erlenmeyer flask, which was placed in a dry ice-acetone bath for 45 minutes. Twenty ml of liquid scintillation counting solution B was added, and the flask was allowed to stand in the crushed ice bath for 45 minutes. Then, 18 ml of the solution was withdrawn and assayed for radioactivity. The efficiency of the tritium, which was strongly dependent upon the amount of water present in the sample, varied between 6 and 14%. Recoveries of D<sub>3</sub>-<sup>3</sup>H added to nonradioactive samples of bile, plasma, feces, and urine ranged between 95 and 101%.

*Extraction of radioactivity.* The lipid-soluble radioactivity present in plasma, urine, feces, and bile was extracted by the procedure of Bligh and Dyer (17). One part of the biological sample was homogenized in a solution of 2 parts methanol and 1 part chloroform in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. This monophasic solution was rehomogenized after the addition of 1 part chloroform and again after the addition of 1 part water. In some instances, 2 M KCl was added in place of the water after the second homogenization. After filtration to remove denatured protein, the phases were allowed to separate overnight at 12° C. The denatured protein was found to contain little or no tritium after this extraction procedure. The aqueous methanol layer was routinely re-extracted three times with additional volumes of chloroform and the combined chloroform extracts labeled "free radioactive D steroids." One- to 2-ml samples of aqueous and chloroform-soluble radioactivity were then combusted.

*Saponification.* For bile, plasma, and fecal samples the chloroform layers from the above extraction procedures containing free radioactive D steroids were combined, evaporated to dryness with a stream of nitrogen, and then placed in a solution of 10% KOH in methanol (vol/vol). The solution was refluxed under nitrogen for 2 hours. An equal volume of distilled water was added and the radioactivity extracted with small volumes of diethyl ether. The ether extracts were combined and prepared for chromatography.

*Chromatography.* The two types of chromatography used are described in detail in previous reports (3, 5, 18).

Thin-layer chromatography apparatus and Cab-O-Sil silica gel G<sup>2</sup> were used to prepare the thin-layer silicic plates. Ten  $\mu$ g of crystalline nonradioactive vitamin D<sub>3</sub> was placed at the origin on each side of the plate as a marker. The radioactive extract (ether or chloroform) was then applied across the origin of the plate between the marker vitamin D spots. The plates were chromatographed in a solvent of 10% acetone in *n*-hexane (vol/vol) (18). After drying the plates were sprayed with 0.20% KMnO<sub>4</sub> in 1.0% Na<sub>2</sub>CO<sub>3</sub> to locate the marker vitamin D. The radioactivity was located by scraping off successive 0.5-cm segments of the silicic acid with a

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microscopic slide and the scrapings placed in a counting vial containing liquid scintillation counting solution A. The radioactivity in the "vitamin D<sub>3</sub> band" was calculated either as a percentage of the total radioactivity in the chromatogram or as a percentage of the injected dose. The mean recovery of the radioactivity applied to the thin-layer plates was 91% (range 89 to 94). As noted previously (3, 18), the marker vitamin D always migrated the same distance in this system whether chromatographed alone or with a lipid extract of biological samples. The radioactivity migrating with the same R<sub>f</sub> as the stable vitamin D markers was labeled "free radioactive vitamin D."

Columns 58 × 1.5 cm were prepared with BioRad silicic acid, minus 325 mesh, in *n*-hexane according to previously published techniques (3, 5). Samples of lipids from the chloroform layer of the Bligh and Dyer (17) plasma extraction noted earlier were dissolved in *n*-hexane and applied to the column. The chromatogram was developed with a hyperbolic gradient elution of diethyl ether in *n*-hexane according to the technique of Lund and DeLuca (5). Ten-ml fractions were collected with a flow rate of 1 ml per minute, and radioactivity was monitored in 1.0-ml samples with counting solution A.

**Procedure for determination of vitamin D<sub>3</sub> conjugates in bile and urine.** To 4-ml samples of bile and lyophilized urine 2 to 4 ml of 0.05 M acetate buffer (pH 5.0) and 1,500 U of marine mollusk  $\beta$ -glucuronidase in 1 ml of water were added.<sup>3</sup> The mixtures were incubated with control samples (no  $\beta$ -glucuronidase added) for 48 hours at 37° C in a Dubnoff shaker, homogenized, and extracted according to the Bligh and Dyer technique outlined above. In some instances the amounts of radioactivity liberated from samples of urine by  $\beta$ -glucuronidase in the presence and absence of saccharo-1,4-lactone were determined. Saccharo-1,4-lactone was prepared from potassium hydrogen saccharate by Levvy's procedure (19); it was used in a 0.05 M concentration. The chloroform layers of control and enzyme-treated samples were evaporated by a stream of nitrogen and the residues combusted. The increment in radioactivity of the  $\beta$ -glucuronidase-treated samples (when compared to their respective controls) was called the "glucuronide fraction."

The aqueous methanol phase of the extracted control and enzyme-treated urine samples was adjusted to pH 1.0 with 65% sulfuric acid and extracted three times according to the Bligh and Dyer procedure. The chloroform extracts were combined, the chloroform was evaporated with a stream of nitrogen, and the residue was combusted. This residue was called the "acidic fraction."

**Measurement of radioactivity.** Counting solution A was used to measure the radioactivity of tritium samples containing only organic solvents; this solution consisted of 3.0 g PPO (2, 5-diphenyloxazole) and 100 mg di-

methyl-POPOP (1,4-bis [5-phenyl-2-oxazolyl] -benzene) per L of toluene. Counting solution B was used to measure the radioactivity of combusted samples; this solution consisted of 40 mg dimethyl-POPOP, 4.0 g PPO, 200 ml absolute ethanol, and 800 ml toluene. Tritium present in aqueous samples was measured with the counting solution of Bray (20). Interval standards of toluene-<sup>3</sup>H were added to all samples, and total disintegrations per minute were calculated. All samples were counted in an automatic Packard Tricarb liquid scintillation counter, model 314XE.

**Biological assays.** Various pooled fractions from the silicic acid column chromatography of lipid extracts from plasma samples obtained 24 hours after the vitamin D<sub>3</sub>-<sup>3</sup>H injection were concentrated to dryness in a flash evaporator or by a stream of nitrogen and then dissolved in diethyl ether. Ten-ml samples of the ether solution were evaporated on the ration of rachitic rats used for the assay. The biological assays for vitamin D activity were subsequently performed according to official USP procedures (21).

## Results

### Rate of disappearance of intravenously administered vitamin D<sub>3</sub> from plasma

When serial plasma D<sub>3</sub>-<sup>3</sup>H radioactivity values were plotted on semilogarithmic paper, a biphasic disappearance curve was obtained (Figure 1).

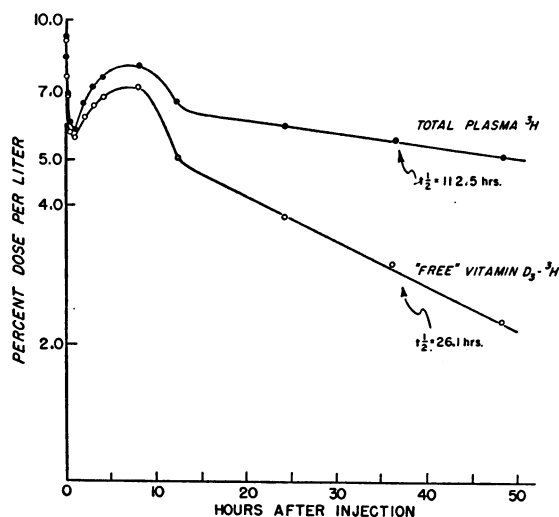


FIG. 1. TOTAL PLASMA <sup>3</sup>H AND DIRECT CHLOROFORM-EXTRACTABLE VITAMIN D<sub>3</sub>-<sup>3</sup>H ("FREE") IN PLASMA AFTER INFUSION OF VITAMIN D<sub>3</sub>-<sup>3</sup>H INTO NORMAL SUBJECTS. Each point on the curves represents the mean value obtained in seven normal subjects. Plasma D<sub>3</sub>-<sup>3</sup>H half-times calculated for each normal subject ranged from 20.3 to 29.5 hours, with a mean ± standard error of 26.1 ± 1.3 hours.

<sup>3</sup> The specificity of the  $\beta$ -glucuronidase used was initially established by control *in vitro* studies utilizing phenolphthalein-mono- $\beta$ -glucuronide as the substrate in the absence and presence of saccharo-1,4-lactone.

Plasma levels of  $D_3$ - $^3H$  fell rapidly for 3 hours, rose gradually during the subsequent 5 hours, and then decreased exponentially during the remainder of the study. As noted in Figure 1, the biological half-time of  $D_3$ - $^3H$  disappearance in the seven normal subjects as calculated from the mean slope values was 26.1 hours. Individual plasma  $D_3$ - $^3H$  half-times calculated for these same subjects ranged from 20.3 to 29.5 hours. When total  $^3H$  radioactivity of serial plasma samples was plotted semilogarithmically, a biphasic disappearance pattern similar to that of  $D_3$ - $^3H$  was observed, with a mean biological half-time for the seven normal subjects of 112.5 hours (Figure 1). In all of the plasma samples collected during the 48-hour experimental period and subjected to the Bligh and Dyer extraction procedure, over 96% of the radioactivity partitioned into the nonpolar chloroform phase. At 12, 24, and 48 hours after  $D_3$ - $^3H$  injection, an average of 77.6, 67.1, and 46.6%, respectively, of the plasma chloroform extracts was present in the region of the vitamin  $D_3$  marker on thin-layer chromatograms.

Because of the obvious discrepancy between total radioactivity of the chloroform-soluble fraction and that which migrated as vitamin  $D_3$  on thin-layer chromatograms, sera taken from all the nor-

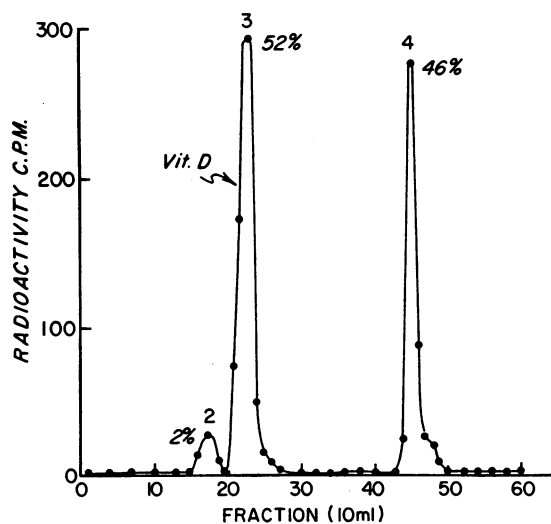


FIG. 2. COLUMN CHROMATOGRAPHIC PROFILE OF CHLOROFORM EXTRACTS OF SERA FROM SEVEN NORMAL SUBJECTS 24 HOURS AFTER AN INTRAVENOUS INJECTION OF VITAMIN  $D_3$ - $^3H$ . The chloroform extract of the serum was chromatographed on silicic acid columns as described previously, using gradient elution techniques (3, 5).

TABLE I

*Biological activity of fractions from normal sera 24 hours after intravenous vitamin  $D_3$ - $^3H$ \**

Fraction	No. rats in assay	Calcification score
Peak	8	$5 \pm 1.0$
Peak 4	8	$6 \pm 1.0$
Vitamin D standard (4.0 IU/rat)	8	$5 \pm 0.5$

\* Fractions refer to those shown in Figure 2. Fractions 3 and 4 were bioassayed by the line test technique (21). The amount of each compound was estimated on the basis of the specific activity of the parent vitamin  $D_3$ - $^3H$ , 160,000 dpm = 1  $\mu$ g.

mal subjects 24 hours after  $D_3$ - $^3H$  administration were pooled, extracted, and chromatographed on silicic acid columns by hyperbolic gradient elution. Three fractions were clearly separated (Figure 2), of which two (fractions 2 and 4) are still unidentified. The material in fraction 3 has been identified as unaltered vitamin  $D_3$  since, when pure  $D_3$ - $^3H$  was chromatographed in the gradient elution system, the only peak identified was present in fractions 20 to 26 (peak 3). Moreover, not only was the substance in peak 3 biologically active (Table I), but, when mixed with nonradioactive crystalline vitamin  $D_3$  and chromatographed on silicic acid plates and columns, the behavior of the radioactivity was identical to that of the crystalline vitamin  $D_3$  (3, 5).

Peak 4 (Figure 2) appears in large quantities in human serum. This fraction is currently under examination as potentially the metabolically active form of the vitamin. Chromatography of peak 4 with crystalline vitamin  $D_3$  has demonstrated that it is distinct from the parent vitamin (5). It does not appear to be a conjugated or esterified form of vitamin D, since saponification or  $\beta$ -glucuronidase hydrolysis of peak 4 did not yield the vitamin. Its behavior on silicic acid and alumina columns suggests that it is more polar than the parent vitamin. That both peak 3 and peak 4 fractions are biologically active is clearly demonstrated in Table I. The peak 4 metabolite fraction obtained from human plasma was fully able to cure rickets in rats as compared with the standard vitamin  $D_3$  or the peak 3 plasma fraction, which represents unaltered vitamin  $D_3$ . The polar metabolite in the peak 4 fraction is as yet uncharacterized, but in agreement with other results

from our laboratory (5), it comprises a large fraction of the biological activity attributed to normal human serum.

#### Excretion of vitamin D<sub>3</sub> and metabolites

*Urinary excretion of vitamin D and its metabolites in normal subjects.* After intravenous administration of D<sub>3</sub>-<sup>3</sup>H the cumulative 48-hour urinary excretion of tritiated material in the seven normal subjects ranged from 1.6 to 3.7% of the administered dose, with a group mean of 2.4 (Figure 3 and Table II). Of this, 0.9 to 2.2% (mean  $1.3 \pm 0.2$ ) was recovered within the first 24 hours. Attempts to trap <sup>3</sup>H-labeled water by lyophilizing frozen urine failed, indicating that all the tritium present in urine was organically bound. Direct extraction of the fresh untreated urine with chloroform recovered lipid-soluble material ranging from 8.1 to 16.9% (mean  $11.1 \pm 1.2$ ) of the total excreted radioactivity (Figure 4). Thin-layer chromatographic analysis of the chloroform extract of untreated urine revealed negligible amounts of free D<sub>3</sub>-<sup>3</sup>H. However, after glucuronidase hydrolysis and chloroform extraction of the urine, an additional 5.4 to 16.9% (mean  $10.4 \pm 1.7$ ) of the total excreted radioactivity was recovered (Table II), but only 2.3% (range 1.3 to 3.6) could be identified as free D<sub>3</sub>-<sup>3</sup>H by chro-

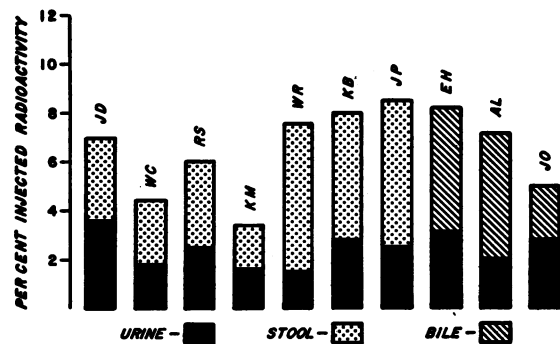


FIG. 3. TOTAL RADIOACTIVITY EXCRETED IN THE URINE, STOOL, AND BILE DURING THE 48-HOUR PERIOD AFTER VITAMIN D<sub>3</sub>-<sup>3</sup>H ADMINISTRATION. Bile fistula patients E.H., A.L., and J.O. had negligible radioactivity in their stools.

matographic analysis. Insignificant quantities of radioactivity were liberated from urine samples by incubation at pH 5.0 and 37° C without added  $\beta$ -glucuronidase or by incubation in the presence of both  $\beta$ -glucuronidase and saccharo-1,4-lactone. As noted in Table II, an additional 9.8 to 28.1% (mean  $16.2 \pm 2.6$ ) was extractable with chloroform after treatment of urine with 65% sulfuric acid. In each instance the excretion of acid metabolites of vitamin D exceeded the conjugated glucuronide fraction (Table II). Chromatographic analysis of the radioactivity made chloroform-soluble by acid treatment failed to reveal

TABLE II  
Cumulative 48-hour urinary total <sup>3</sup>H, glucuronide-<sup>3</sup>H, and acid-conjugated <sup>3</sup>H in normal and cirrhotic subjects

Patient	Total <sup>3</sup> H	Glucuronide- <sup>3</sup> H		Acid-conjugated <sup>3</sup> H	
	% injected dose	% injected dose	% total urinary radioactivity	% injected dose	% total urinary radioactivity
Normal subjects					
J.D.	3.67	0.20	5.4	0.36	9.8
W.C.	1.90	0.17	8.9	0.23	12.1
R.S.	2.46	0.26	10.6	0.31	12.6
K.M.	1.63	0.27	16.9	0.46	28.1
W.R.	1.87	0.24	12.8	0.51	27.3
K.B.	2.76	0.20	7.3	0.34	12.3
J.B.	2.56	0.28	10.9	0.29	11.3
Mean	2.41	0.23	10.4	0.36	16.2
±SE	±0.29	±0.01	±1.7	±0.04	±2.6
Cirrhotic subjects					
T.C.	1.50	0.07	4.6	0.32	21.3
W.J.	1.87	0.11	5.9	0.39	20.8
S.C.	1.49	0.11	7.3	0.31	21.0
S.W.	1.35	0.09	6.7	0.35	25.9
Mean	1.55	0.09	6.1	0.34	22.2
±SE	±0.13	±0.01	±0.7	±0.02	±1.2

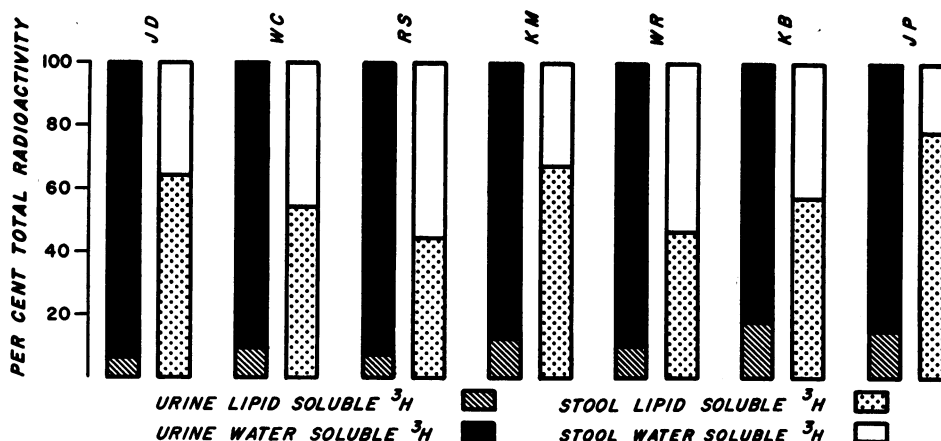


FIG. 4. RESULTS OF DIRECT CHLOROFORM-METHANOL EXTRACTION OF 48-HOUR URINE AND STOOL SPECIMENS OF SEVEN NORMAL SUBJECTS AFTER INFUSION OF VITAMIN  $\text{D}_3$ - $^3\text{H}$ . Lipid-soluble  $^3\text{H}$  refers to radioactivity extracted by chloroform; water-soluble  $^3\text{H}$  represents radioactivity remaining in the aqueous methanol layer.

any  $\text{D}_3$ - $^3\text{H}$ . A significant part of the radioactivity could not be extracted from urine, even after treatment of the urine residue with mammalian  $\beta$ -glucuronidase at pH 4.8,  $37^\circ\text{C}$ , for 4 additional days or with boiling 20% hydrochloric acid.

The terms "free" and "conjugated" refer to vitamin  $\text{D}_3$  or its metabolites in untreated urine easily extractable by chloroform and migrating on thin-layer chromatograms with an  $R_f$  identical to

that of crystalline vitamin  $\text{D}_3$  standard (free), and to methanol-water-soluble vitamin  $\text{D}_3$  metabolites or derivatives that were liberated by  $\beta$ -glucuronidase, acid treatment, or both (conjugated). It is recognized that these terms are not completely accurate in the strict chemical sense, since the products have not yet been isolated and characterized.

**Biliary and fecal excretion of vitamin  $\text{D}_3$ - $^3\text{H}$  and metabolites.** The amount and nature of the radioactivity excreted in the bile is illustrated in Figures 3, 5, and 6. The three patients (E.H., A.L., and J.O.) with constant 48-hour T-tube drainage excreted 3 to 6% of the radioactivity of the injected  $\text{D}_3$ - $^3\text{H}$  in the bile (Figure 3), with 40 to 60% of the excretion occurring within the first 24 hours. As noted in Figure 5, an average of 8.7% (range 8.4 to 9.3) of the radioactivity of untreated bile was removed by direct chloroform extraction. The biliary excretion of free vitamin  $\text{D}_3$  was negligible. After  $\beta$ -glucuronidase hydrolysis, an additional 38.7, 40.3, and 40.1% of the biliary radioactivity was extractable with chloroform in patients E.H., A.L., and J.O., respectively (Figures 5 and 6). Of the glucuronidase-liberated radioactivity in bile, 4.5, 5.7, and 6.1% (mean 5.4) was tentatively identified as free vitamin  $\text{D}_3$  (Figure 6). As noted in Figure 6, an additional 5% (range 4.3 to 5.2) of the total biliary radioactivity was recovered in the chloroform solvent after sulfuric acid treatment.

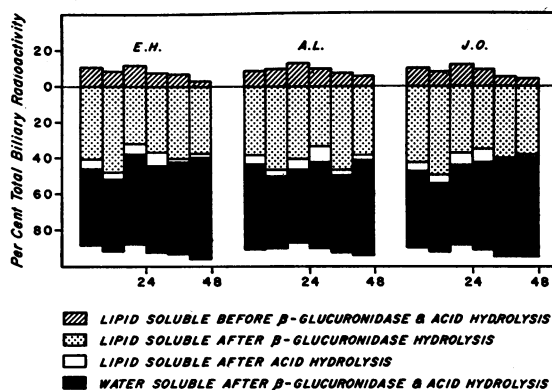


FIG. 5. EXCRETION OF RADIOACTIVITY IN THE BILE AFTER VITAMIN  $\text{D}_3$ - $^3\text{H}$  INFUSION IN THREE PATIENTS WITH BILIARY FISTULAS. Bars above the center line represent chloroform-soluble radioactivity in untreated bile; bars below the line represent the amounts of aqueous methanol radioactivity rendered chloroform-soluble by various methods of incubation. Each bar represents an 8-hour collection period. Note the absence of free vitamin  $\text{D}_3$ - $^3\text{H}$  in bile.

The plasma disappearance of D<sub>3</sub>-<sup>3</sup>H in the three bile fistula patients was also biphasic, and calculated plasma D<sub>3</sub>-<sup>3</sup>H half-times for E.H., A.L., and J.O. were 24.3, 27.5, and 28 hours, respectively. The amount of radioactivity excreted in the urine in these patients was similar to that observed in the normal subjects with 2.3 to 3.5% of the administered dose recovered in 48 hours (Figure 3).

Of the administered radioactivity, 4.9% (range 1.8 to 6.4) was recovered in the feces of the seven normal subjects (Figure 3). Of the recoverable stool radioactivity, 50.3% was removed after direct chloroform extraction (Figure 4). Only 6.5% of this chloroform-soluble radioactivity migrated as D<sub>3</sub>-<sup>3</sup>H on thin-layer chromatograms.

In all three patients with bile fistulas, fecal excretion of the injected radioactivity was negligible, ranging from 0.2 to 0.5% of the injected dose.

#### Results in patients with liver disease

In four patients with cirrhosis the plasma disappearance of D<sub>3</sub>-<sup>3</sup>H was considerably slower than normal, and biological half-times of 36.0, 37.5, 39.5, and 42.5 hours were observed for cirrhotic patients T.C., W.J., S.C., and S.W. (Table II). In each case the D<sub>3</sub>-<sup>3</sup>H disappearance curve followed the identical biphasic pattern observed for the normal subjects and illustrated in Figure 1. As noted in Table II, a 36% decrease in total urinary radioactivity to  $1.55 \pm 0.13\%$  of the injected dose was observed in the cirrhotic group. Whereas no significant difference was noted in the chloroform-soluble radioactivity and acid-conjugated urinary metabolites of cirrhotic patients when compared with normal subjects, there was a two- to threefold reduction in chloroform-soluble radioactivity after glucuronide hydrolysis (Figure 6 and Table II). None of the radioactivity liberated by glucuronidase in these patients could be identified as D<sub>3</sub>-<sup>3</sup>H.

#### Discussion

Our findings on the plasma disappearance of vitamin D<sub>3</sub> in man are at variance with results obtained by Thompson, Lewis, and Booth (13) and Scott and co-workers (22), where mean plasma half-lives of 54 hours and 3 days, respectively, were obtained in normal subjects after oral doses of vitamin D<sub>3</sub>-<sup>3</sup>H of relatively low spe-

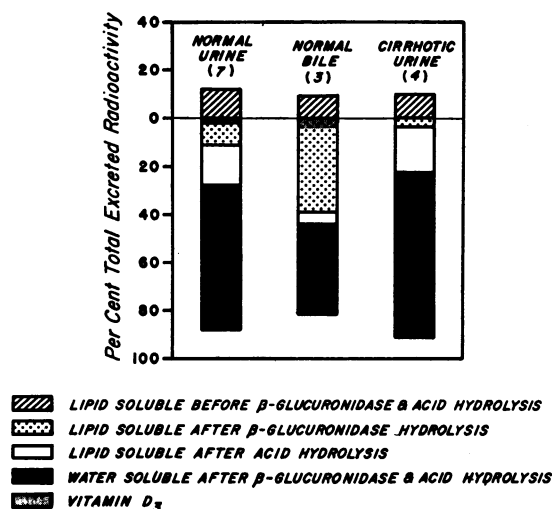


FIG. 6. COMPARISON OF URINARY AND BILIARY RADIOACTIVITY IN NORMAL SUBJECTS AND URINARY RADIOACTIVITY IN PATIENTS WITH CIRRHOSIS AFTER VITAMIN D<sub>3</sub>-<sup>3</sup>H INFUSION. Bars above the line represent chloroform-soluble radioactivity; bars below the line represent the amounts of aqueous methanol radioactivity rendered chloroform-soluble by various methods of incubation. The height of the bar in each case represents the mean value for the group. The number of patients is noted in parentheses above the bar. Note the identification of free vitamin D<sub>3</sub>-<sup>3</sup>H in the glucuronide fraction of normal urine and bile.

cific activity. This may possibly be due to the variability in the amount of carrier vitamin D<sub>3</sub> in plasma after oral and intravenous administration, since the aforementioned investigators administered carrier doses of vitamin D<sub>3</sub> of 40,000 (13) to 100,000 (22) U.

Moreover, the calculated plasma half-times of vitamin D in the aforementioned studies (13, 22) may actually represent the disappearance of total plasma radioactivity rather than plasma D<sub>3</sub>-<sup>3</sup>H activity. As noted in Figure 1, the biological half-time of total plasma <sup>3</sup>H activity in the present study was much longer than that of D<sub>3</sub>-<sup>3</sup>H, averaging 4 to 5 days. Our results are in accord with the recent observations of Chen and Lane, who reported a vitamin D-4-<sup>14</sup>C plasma half-time in dogs of 20 hours during the first 2 to 3 days after isotope injection (23). The significance of the biphasic disappearance curve obtained for "free vitamin D<sub>3</sub>-<sup>3</sup>H" in the first 4 to 8 hours after infusion has yet to be established (Figure 1). The results of Norman and DeLuca (1), Neville and

DeLuca (4), and Nair and Bucana (11) suggest that the biphasic curve may relate to the hepatic metabolism and release of vitamin D<sub>3</sub>, since the rodent liver is known to accumulate vitamin D rapidly after its administration (11), reaching peak levels at 40 minutes and declining gradually during the subsequent 24-hour period (1, 4). However, this explanation appears unlikely since, despite an increase in plasma D<sub>3</sub>-<sup>3</sup>H half-times and a decrease in urinary D<sub>3</sub>-<sup>3</sup>H glucuronide conjugates in subjects with hepatic disease (Table II), the biphasic plasma curve persisted. It also seems unlikely that the biphasic curve represents the intestinal absorption of biliary radioactivity, since it was observed in the three patients with complete biliary fistulas. Since a 4- to 6-hour lag obtains between the administration of vitamin D and the appearance of its earliest physiological effect (24), the biphasic plasma curve may represent different metabolic pools with varied rates of turnover or certain physiological processes essential for the transport and localization of the vitamin at its functional sites.

From the present and previous data (5, 8), it is obvious that it is no longer correct to assume that antirachitic activity of plasma is synonymous with unaltered vitamin D<sub>3</sub>. Clearly, much of the antirachitic effectiveness must be attributed to a polar metabolite of vitamin D (peak 4, Figure 2) that is easily separated on silicic acid from the unaltered vitamin. This material appears in large proportions in bone, liver, and blood of rats (5). *In vivo* evidence that a metabolic transformation of vitamin D occurs with the gradual accumulation of polar metabolites in plasma has also been obtained in dogs by Chen and Lane (23). The proportion of this polar metabolite that appears in the tissues of rats increases as the vitamin D level administered approaches a minimal daily dose (5). The chemical nature of the polar material in peak 4 is presently unknown. Preliminary experiments reveal that it is not an esterified or conjugated form of vitamin D. The fact that it induces all the known physiologic effects of the vitamin, in some cases much more rapidly than the parent vitamin, adds further support that it may be the metabolically active form of the vitamin.

Another metabolite of vitamin D isolated from the chloroform extract of plasma in the present

study is represented by peak 2 in Figure 2. This substance, which accounts for 2% of the chloroform-soluble plasma radioactivity 24 hours after D<sub>3</sub>-<sup>3</sup>H injection, is still uncharacterized. A less polar substance previously identified as a vitamin D ester (6) in animal tissues and designated peak 1 (3, 5) was characteristically absent from human plasma. This substance has not been found in any appreciable quantities in rat serum as well (5).

Despite a normal biphasic plasma D<sub>3</sub>-<sup>3</sup>H disappearance curve, the four patients with liver disease evidenced a decreased clearance rate of infused D<sub>3</sub>-<sup>3</sup>H from plasma and a decrease in the urinary glucuronide fraction. A possible source of error when employing per cent dose per volume to describe the disappearance of D<sub>3</sub>-<sup>3</sup>H from plasma is the variability of individual hematocrits. It is unlikely that this contributed significantly to the abnormal increments in plasma D<sub>3</sub>-<sup>3</sup>H half-times of the cirrhotic patients, since their hematocrits (39 to 43%) were well within the range of 39 to 46% obtained for the seven normal subjects. It is tentatively concluded that the abnormal clearance and decreased glucuronide conjugation of D<sub>3</sub>-<sup>3</sup>H observed in the cirrhotic patients (Table II) reflect the importance of the liver in the regulation and metabolism of circulating vitamin D and its metabolites in man. This defect in the capacity of the cirrhotic liver to metabolize sterols appears relatively specific, since abnormal metabolic schemes have also been documented for testosterone (25) and cortisol (26) but not for corticosterone, cortisone, and aldosterone (26). Since the rate of disappearance of vitamin D<sub>3</sub> from plasma is presumably also a measure of its rate of metabolism, reports of osteomalacia and rickets (27) in patients with liver disease may relate to a defect in the biological transformation of free vitamin D<sub>3</sub> to an "active" metabolic derivative. Certainly other proposed factors such as intestinal malabsorption associated with biliary obstruction (10, 13) and steatorrhea may also play an essential role in the reported instances of bone disease complicating hepatic dysfunction.

As noted in Table II, the mean 48-hour urinary excretion of radioactivity of the seven normal subjects after D<sub>3</sub>-<sup>3</sup>H infusion was 2.4% of the administered dose. Of this, an average of 54% was excreted within the first 24 hours after infusion.



These results are in good agreement with those of Thompson and his associates (13) and Kodicek (12) who measured urinary radioactivity in humans after an oral dose of radioactive vitamin D. Since approximately as much radioactivity derived from radioactive vitamin D appears as metabolites in urine during the first 24 hours after oral administration as after intravenous administration, it appears likely that absorptive processes in humans do not delay the chemical transformation and subsequent excretion of vitamin D metabolites. Urinary radioactivity was mainly water-soluble, only 11% being extractable with chloroform (Figure 4). A negligible amount of this radioactivity could be identified as unaltered vitamin D<sub>3</sub>-<sup>3</sup>H. However, an additional 6 to 17% was rendered chloroform-soluble after  $\beta$ -glucuronidase hydrolysis (Table II). It is theoretically possible that the enzyme preparations used for these studies contained some enzyme other than  $\beta$ -glucuronidase that cleaved the conjugate. This possibility seems remote, because  $\beta$ -glucuronidase released an insignificant amount of additional chloroform-soluble radioactivity in the presence of saccharo-1,4-lactone, which is a relatively specific inhibitor of this enzyme (28). Moreover, it was subsequently shown that after  $\beta$ -glucuronidase hydrolysis an average of 2.3% of the urinary radioactivity migrated on thin-layer chromatograms with the same  $R_f$  as crystalline vitamin D<sub>3</sub> standards (Figure 6). These findings taken collectively make it probable that a portion of vitamin D<sub>3</sub> and its metabolites is excreted in man as glucuronide conjugates. In addition to the glucuronide conjugates, 9 to 28% of the urinary radioactivity was released and soluble in chloroform after treatment with 65% sulfuric acid. Whether this fraction represents glucuronides resisting enzymatic hydrolysis, sulfate, or other conjugation products remains to be determined as does the nature of the residual water-soluble metabolites, which completely resist extraction and account for an average of approximately 73% of the total urinary radioactivity.

In view of animal evidence which indicated that intravenously administered D<sub>3</sub>-<sup>3</sup>H localized primarily in the liver within minutes after injection (1, 4, 11) and other studies which revealed that absorbed D<sub>3</sub>-<sup>3</sup>H is taken up mainly by the liver and excreted into bile (12), a study of the possible enterohepatic circulation of vitamin D<sub>3</sub> or its

metabolic products was undertaken in three subjects with complete bile fistulas. The presence of glucuronide and acid conjugates of D<sub>3</sub>-<sup>3</sup>H was established by the 40 and 5% increments in chloroform-soluble material after glucuronidase and acid hydrolysis, respectively. Approximately 47% of the biliary radioactivity could not be extracted after the hydrolytic procedures and remained soluble in aqueous methanol (Figure 6). This material was unidentified but may represent taurine and glycine bile acid conjugates of vitamin D<sub>3</sub> or its metabolites, as recently demonstrated in the rat by Bell and Bryan (14). This latter assumption is tentative in view of reports wherein species differences in hepatic conjugation and biliary excretion of other steroids have been noted for man and rodents (29).

Five per cent of the biliary radioactivity rendered chloroform-soluble by  $\beta$ -glucuronidase was identified on thin-layer chromatograms as D<sub>3</sub>-<sup>3</sup>H, the remainder represented mainly material which was less polar than vitamin D<sub>3</sub>. The fecal excretion of radioactivity in the normal subjects averaged 49% of administered radioactivity, 50% of which represented water-soluble metabolites. 6.5% of the chloroform-soluble fecal radioactivity migrated in the vitamin D<sub>3</sub> band on thin-layer chromatograms.

Cumulative biliary radioactivity approximated 5% of the injected dose, but, in contrast to feces, over 90% of this material represented water-soluble metabolites. Since it has been demonstrated that human feces contain  $\beta$ -glucuronidase and phenosulfatase activities (30), the role of fecal enzymes in the transformation of biliary D<sub>3</sub>-<sup>3</sup>H water-soluble conjugates must be considered, as well as the enterohepatic circulation of water-soluble metabolites derived from the biliary tract. The small amounts of radioactivity present in the feces of patients with T-tubes may be due to either excretion of radioactivity in gastrointestinal juices or the escape of small amounts of bile. Comparable amounts of fecal radioactivity in normal subjects and biliary radioactivity in patients with T-tubes suggest that the passage of vitamin D and its metabolites through the bile and hence into the intestine represents an obligatory stage for the enterohepatic circulation and fecal excretion of vitamin D metabolites by man.

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