

# Deposition in and Release of Vitamin D<sub>3</sub> from Body Fat: Evidence for a Storage Site in the Rat

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**ABSTRACT** Vitamin D in all body tissues was radio-labeled by supplementing completely vitamin D-deficient weanling rats with oral vitamin D<sub>3</sub>-4-<sup>14</sup>C for 2 wk. All vitamin D was then withheld, and radioactivity and vitamin D content in a variety of organs and tissues were measured. Adipose tissue was found to contain by far the greatest quantity of radioactivity throughout the 3 month experimental period. Immediately after supplementation, half of the total radioactivity in adipose tissue corresponded to unaltered vitamin D<sub>3</sub>, and the other half to polar metabolites and esters of vitamin D<sub>3</sub> and unidentified peak II. 1 month later there was approximately the same proportion but a decrease in the total quantity of each form. We conclude that adipose tissue is the major storage site for vitamin D<sub>3</sub> in its several forms. Unaltered vitamin D<sub>3</sub> was the principal storage form observed and presumably a source available for conversion to other metabolites during deprivation.

## INTRODUCTION

Revised concepts of vitamin D metabolism have recently emerged from studies by DeLuca and associates (1-4), who have identified a variety of its metabolites which appear in mammals. Of these, 25-hydroxycholecalciferol (25-HCC) has been demonstrated to be formed in the liver (4) and may be the compound which normally mediates the major biological effects of the vitamin. Storage phenomena, presumably of great importance in mammalian vitamin D-calcium physiology, have needed re-

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examination in the framework of this new information.

Indirect evidence that tissue storage does occur in mammals includes the following: (a) it may require many months to deplete a laboratory animal to the point of producing the deficiency state (5); (b) while the hepatic conversion of a pulse dose of vitamin D<sub>3</sub> to the active metabolite, 25-HCC, occurs within a very few hours, and the subsequent disappearance of the tracer vitamin D<sub>3</sub> from the serum occurs within 2 days (4), the biological effects of a modest single dose may persist for months (6, 7); and (c), the half-life of biological activity in plasma of humans after cessation of large doses has been estimated to be about 4 months (8).

Attempts to define a storage site of vitamin D in tissues of man and other mammals have previously been unsuccessful. Most of the early studies of tissue distribution after a single (usually very large) dose of the vitamin recovered only about 10% of that administered, and this was found to be distributed rather uniformly among a variety of tissues (8-11). Nevertheless, the liver has been considered by some to be the most likely site for storage in mammals (12).

In the present study, young rats with well established vitamin D deficiency were replenished with <sup>14</sup>C ring-labeled vitamin D<sub>3</sub>. Tissue levels of radioactivity were measured during repletion and also during a subsequent period of vitamin D deprivation. Our data show that adipose tissue acquired the greatest quantity of radioactivity and had the slowest rate of release. Chromatographic analysis showed that most of this radioactivity was associated with unaltered vitamin D<sub>3</sub>, and biological activity was found in the samples of adipose tissue. These results support the concept that body fat serves as a major site for storage of vitamin D in the rat.

## METHODS

*Experimental design.* 66 weanling Sprague-Dawley male rats, approximately 21 days old and weighing between 40 and 50 g, were housed in hanging cages in complete dark-

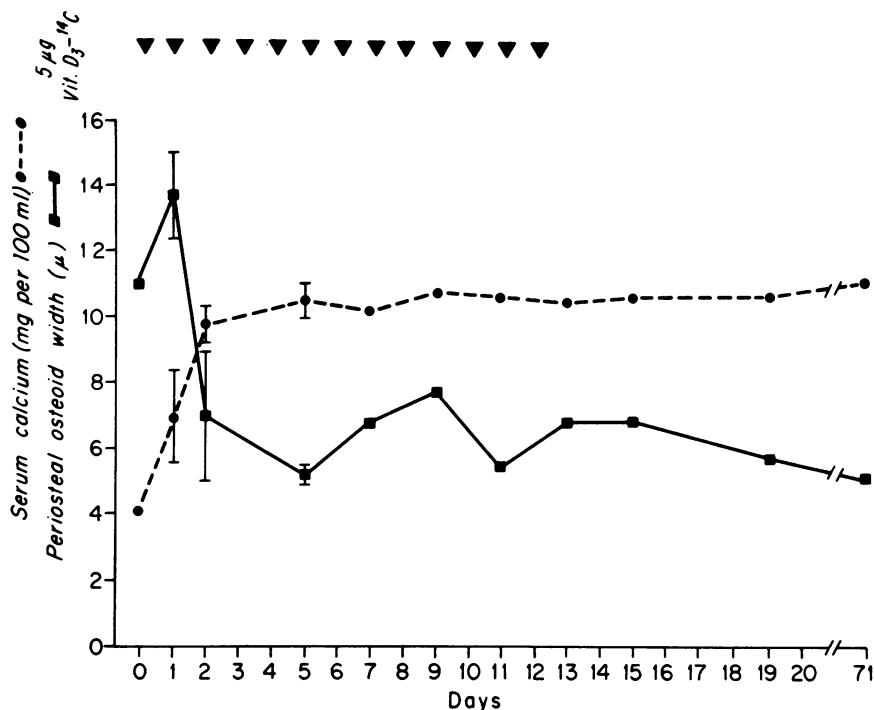


FIGURE 1 Effect of daily doses ( $\blacktriangledown$ ) of vitamin  $D_3$ - $4$ - $^{14}C$  on serum calcium and periosteal osteoid width of vitamin D-deficient rats of group B. Each point represents the mean values from three animals, and the brackets contain  $\pm 1$  SD.

ness and fed a semisynthetic diet devoid of all vitamin D, but complete in all other constituents including 0.6% calcium and 0.6% phosphate (13). The animals were vitamin D deficient after 1 month, as indicated by failure to gain weight, severe hypocalcemia ( $4.1 \text{ mg} \pm 1.4 \text{ mg}$  calcium per 100 ml serum), and marked increase in mean periosteal osteoid width ( $13.7 \mu \pm 1.3 \mu$  cf.  $\sim 6 \mu$  for normal animals of the same age; Fig. 1). The animals were then divided into three dose groups and fed purified vitamin  $D_3$ - $4$ - $^{14}C$  (Philips-Duphar, Amsterdam, The Netherlands; SA 19.2 mCi/mmoles per liter) dissolved in 0.1 ml corn oil (Mazola) daily for 12 or 14 days by direct instillation into the hypopharynx. The following doses were employed: group A (9 animals),  $0.5 \mu\text{g}$  (60,000 dpm)/day for 14 days; group B (45 animals),  $5.0 \mu\text{g}$  (240,000 dpm)/day for 12 days; and group C (9 animals),  $125 \mu\text{g}$  (120,000 dpm)/day for 14 days. All labeled vitamin was from the same manufactured lot but received in multiple ampoules. Each ampoule's vitamin  $D_3$  was  $> 90\%$  pure as determined by thin-layer chromatographic analysis when initially opened. It was stored at  $-20^\circ\text{C}$  under nitrogen and usually administered within 1 wk of the analysis. If used after more prolonged storage, the material was reanalyzed chromatographically and re-purified if necessary upon silicic acid columns to achieve the same quality.

After the period of supplementation, evidence of satisfactory repletion was found: all animals were normocalcemic, and in representative animals examined from all three dose groups, the vitamin D deficiency bone lesion was found completely healed (periosteal osteoid width  $\sim 6 \mu$ ; Fig. 1). Thereafter, the animals remained in the dark and received no further vitamin D from any source.

Groups of three animals were sacrificed by exsanguination during treatment (group B) and periodically during 3 subsequent months of vitamin D deprivation (groups A-C). Individual tissues from each animal were dissected out immediately after sacrifice and stored at  $-20^\circ\text{C}$  in separate vials until analyzed. Fat depots sampled included epididymal, subcutaneous, perirenal, mesenteric, and brown fat from the interscapular area. Epidermis, usually obtained from the back, was freed of hair and scraped to remove as much subcutaneous fat as possible. The bone usually examined was the humerus; it was freed of surrounding tissues, but the marrow was not removed. The small intestine was washed of contents with cold saline to remove as much contaminating materials as possible, and other tissues were similarly washed of blood. Serum was separated immediately from whole blood and similarly stored.

**Tissue radioactivity.** Radioactivity was extracted and measured according to the method of Schachter, Finkelstein, and Kowarski (14) with slight modifications. These workers found this method to extract  $> 95\%$  of the total radioactive vitamin  $D_3$ . We confirmed that further extraction of the residues by the same method or the method of Blich and Dyer (15) did not yield additional radioactivity. Weighed aliquots of wet tissue (usually 1 g) were homogenized in 25 volumes of acetone-ethanol (1:1) in a Virtis tissue homogenizer (14). Tissues for a given sampling time were analyzed separately except in the cases of brain, bone, and small intestine of group B animals. In these cases, equal weights of a tissue from three animals of a given time period were pooled and extracted together. The homogenate was filtered through Whatman filter paper No. 50, and the residue was washed with at least 25 volumes

of the extracting solution. The filtrate was evaporated to dryness at room temperature, and the residue was quantitatively transferred to counting vials, using three 5-ml aliquots of dioxane counting solution containing 5–17% water (16). It was found that, by varying the amount of water in the counting mixture, all residue could be dissolved. An internal standard (toluene-<sup>14</sup>C; Beckman Instruments, Inc., Fullerton, Calif.) was used in all samples to correct for quenching. All samples were counted in a Beckman LS 200 model liquid scintillation spectrometer.

*Thin-layer chromatography (TLC).* Samples of tissue were extracted as above except that they were shielded from light and kept in an atmosphere of nitrogen. Extracts in *n*-hexane were applied to silicic acid (Silica Gel H) or aluminum oxide (Aluminum Oxide H) thin-layer plates (250 or 750  $\mu$  thick) in a nitrogen atmosphere box (both from E. Merck, Darmstadt, Germany, distributed by Brinkmann Instruments, Inc., Westbury, N. Y.). A solvent system (17) used to separate neutral lipids (hexane-diethyl ether-water, 135:45:3) effectively separated vitamin D<sub>3</sub> from the other lipids present, with the exception of cholesterol. Chloroform was used to develop TLC plates when the purity of a vitamin D<sub>3</sub> preparation was being tested. Unlabeled vitamin D<sub>3</sub> (Mann Research Labs. Inc., New York), and a lipid standard (Hormel model mixture No. 1) were used as reference markers and located by charring or by UV light adsorption after spraying the plate with Rhodamine 6G (Allied Chemical Co.) in acetone. Radioactivity was localized and measured by scraping successive 1 cm segments of the test lanes into vials containing 15 ml dioxane counting solution (17% water) (16).

*Silicic acid column chromatography.* Extracts from up to 3 g of tissue were prepared as for TLC and chromatographed on columns of silicic acid (Bio-Sil-HA-325 mesh; Bio-Rad Labs, Richmond, Calif.); elution was carried out with a solvent gradient of increasing polarity, as described by DeLuca, Zile, and Neville (18). Ionol (1,5-di-*tert*-butyl-2-hydroxy-3-methyl-benzene) was used as an antioxidant (19). For tissues containing large amounts of lipid, it was necessary to use a layer of silicic acid and Hyflo-Supercel (1 g each) atop the silicic acid column as suggested by DeLuca et al. (18) and to load such extracts onto the column at room temperature. All columns were run at 4°C.

*Other methods.* To measure biological activity, extracts were prepared as described for TLC and fed to rachitic rats according to the standard USP line test (20). The concentration of calcium in serum was determined by atomic absorption spectroscopy (21). Periosteal osteoid width was determined on calcified sections of the midtibial diaphysis by means of a filar micrometer eye piece (22).

Total body fat was estimated by triplicate weighing of the shaved eviscerated carcass in air and in water (23). The weight of total body fat was then calculated by the following relationship, which is derived from specific gravity and chemically determined total body fat of rats of similar size, sex, and on a similar diet (24):

Total body fat = 340.6 - (294.3 × specific gravity of carcass)

$$\times \frac{\text{carcass weight}}{100}$$

## RESULTS

Evidence for vitamin D deficiency in all three groups of animals during the period of deprivation included failure to gain weight, hypocalcemia, and a marked increase in

periosteal osteoid width (Fig. 1). All of these manifestations of vitamin D deficiency were promptly reversed upon administering vitamin D<sub>3</sub>. Rats in all three groups, thereafter, remained normocalcemic and continued to gain weight throughout the entire subsequent period of vitamin D deprivation (36–88 days). The animals given 5  $\mu$ g vitamin D<sub>3</sub> per day (group B) were completely healed 24 hr after the second dose, and the serum calcium concentrations and bone histology remained normal for the ensuing 88 days of vitamin D deprivation.

*Distribution of radioactivity in tissues.* The findings in all three groups of rats were similar. Major emphasis is focused on group B, which received an intermediate dose of vitamin D<sub>3</sub> and was studied in greatest detail. During the period of vitamin D<sub>3</sub> administration, the concentration of radioactivity in various tissues steadily increased, always being highest in kidney (Table I). The total body mass of adipose tissue contained the greatest quantity of radioactivity at all periods of sampling, corresponding to an almost constant 10–12% of the cumulative dose. However, in other tissues this fraction decreased from about 6% of the first dose to 2–3% by the time the animal had received 12 doses (Table I).

The concentration of radioactivity at the end of the 12 days of treatment varied from tissue to tissue, being highest in kidney (> 28,000 dpm/g), intermediate in fat from several different sources, blood, ileum and liver (4000–8000 dpm/g), and lowest in skin, muscle, bone, brain, and duodenum (< 3000 dpm/g) (Table I). There was a marked difference in the rate of fall of radioactivity in the different tissues examined. This is evident from the results in group B in which tissue radioactivity was monitored at more frequent time periods (Fig. 2). In most tissues (cf. blood, liver, and kidney), radioactivity disappeared at a rapid rate for the first 3 wk and, thereafter, more slowly. This slower component very approximately could be fitted to an exponential function with a half time of 21 days for blood and 45–50 days for liver and kidney. In contrast, the rate of disappearance of radioactivity from fat was slow at all times, approximating an exponential rate with a half time of about 81 days during most of the period of observation.

The derived values for total radioactivity per organ or tissue are expressed as per cent of total dose per tissue in Table I. Adipose tissue retained the largest amount of radioactivity at the end of the period of vitamin D<sub>3</sub> administration, as well as during the entire subsequent 88 day period of Vitamin D deprivation. Thus, 8 wk (day 71) after vitamin D<sub>3</sub> had been discontinued, the concentration of radioactivity in adipose tissue (995–1835 dpm/g) far exceeded that in any other of the tissues examined (43–359 dpm/g) (Table II): the fraction of the total administered dose represented by this radioactivity was still 4.9%, equivalent to 2.9  $\mu$ g. Skin ac-

TABLE I  
<sup>14</sup>C Content of Tissues and Organs, Group B\*

Day	Fat, organ†			Blood, organ‡			Kidney, organ			Liver, organ			Brain, organ		
	dpm/g	wt. in g	% dose	dpm/g	wt. in g	% dose	dpm/g	wt. in g	% dose	dpm/g	wt. in g	% dose	dpm/g	wt. in g	% dose
Supplementation															
1	800	37.4	12.5	2099	7.4	6.5	12,196	1.20	6.1	2047	6.05	5.2	94	1.13	0.04
2	1653	39.0	13.4	2398	8.1	4.0	16,085	1.30	4.6	2660	6.14	3.4	—	—	—
4	2675	38.5	10.7	3613	8.5	3.2	18,996	1.38	2.7	4224	6.09	3.0	—	—	—
8	3285	55.4	9.5	4141	10.0	2.2	22,489	1.66	1.9	3728	9.78	1.9	194	1.51	0.02
12	5696	53.5	10.6	6179	11.0	2.3	28,502	1.55	1.5	4579	7.88	1.2	233	1.49	0.02
Deprivation															
14	4909	55.2	9.5	4927	12.0	2.1	17,676	1.86	1.1	3083	9.49	1.0	239	1.44	0.01
18	4245	59.6	8.8	2933	13.0	1.3	10,521	1.75	0.64	1376	9.56	0.46	118	1.37	0.00
21	4520	63.0	9.9	2010	15.0	1.0	4,712	2.01	0.33	782	10.80	0.29	—	—	—
27	3841	63.2	8.4	1442	14.0	0.73	2,567	1.96	0.17	469	10.72	0.17	—	—	—
35	2567	77.7	6.9	782	18.0	0.48	1,258	2.33	0.10	280	11.92	0.12	—	—	—
55	2271	87.1	6.9	492	20.0	0.35	650	2.64	0.06	210	12.99	0.10	—	—	—
71	1835	76.6	4.9	265	19.0	0.18	497	2.42	0.04	114	11.23	0.04	—	—	—
99	1368	100.0	4.8	88	21.0	0.06	359	3.85	0.05	113	11.34	0.04	—	—	—
Skin¶															
Muscle**															
Skeleton‡‡															
"Duodenum"															
"Ileum"															
Supplementation															
1	593	30.7	7.4	265	36.8	4.1	357	18.4	2.7	1536	4.34	2.8	5874	2.66	6.5
12	2552	44.7	4.0	1100	53.6	2.0	780	26.8	0.73	1643	2.15	0.13	4462	1.16	0.18
Deprivation															
27	804	60.4	1.7	167	72.5	0.42	155	36.2	0.20	349	1.81	0.02	552	0.995	0.02
71	218	79.4	0.60	43	95.2	0.14	56	7.6	0.08	92	2.11	0.01	188	1.02	0.01

\* All values are means of tissues from three animals.

† As calculated from specific gravity of carcass; see Methods and Table II.

‡ 6% body weight (25).

|| Based on analysis of subcutaneous fat.

¶ 25% body weight (25).

\*\* 30% body weight (25).

‡‡ 15% body weight (25).

counted for 0.6% of dose, and all other tissues examined contained only 0.4% of the dose. Therefore, at this time adipose tissue contained 5 times more radioactivity than all of the other tissues combined.

**Recovery of radioactivity.** At the end of the period of vitamin D<sub>3</sub> treatment, 10% of the dose was then in fat, 4% in skin, 2.3% in blood, 2.0% in muscle, 1.5% in kidney, 1.2% in liver, and 1.2% in the other organs examined, accounting altogether for 22.2% of the total amount of radioactivity administered to the animals in group B (Table I).

Two rats were placed in metabolic cages for collection of excreta. During the 12 day period of treatment, one rat excreted 24% and the other 53% of the total dose in stools and urine combined. If these values are assumed to be the limits of the range from all the animals, a total of 46-75% of the cumulative dose was recovered from tissues analyzed and excreta. Radioactivity not accounted for may be attributed to that contained in the intestinal lumen and organs not analyzed (lung, spleen, endocrine organs, etc.) and, possibly, to incomplete extraction from tissues.

**Identification of radioactivity.** Chromatographic methods to identify further the chemical form of the radioactive molecules were applied to extracts of subcutaneous fat, blood plasma, liver, and kidney. To determine if the label were associated with lipids such as triglyceride (which might occur if the A ring containing the <sup>14</sup>C had been split and the resulting 2-carbon-labeled fragment reutilized by the different tissues), thin-layer chromatography with silicic acid or neutral alumina as adsorbant and a developing system to separate different neutral lipids and sterols were used (17). Vitamin D<sub>3</sub> was thereby always separated effectively from mono-, di-, and triglycerides, free fatty acids, and phospholipids, more polar lipid, nonlipid material, and, often, also cholesterol. We found that in adipose tissue (subcutaneous and epididymal) obtained on days 7 and 14, 60% of the radioactivity migrated to the same position as reference non-radioactive vitamin D<sub>3</sub>; the other 40% remained at the origin. In blood, liver, and kidney samples obtained on the 14th day, 24%, 39%, and 22% of the radioactivity migrated to the vitamin D<sub>3</sub> area. Radioactivity was never found associated with the triglycerides extracted from

these tissues. However, only those adipose tissue samples with the highest concentrations of radioactivity could be analyzed by means of TLC because the large amount of triglycerides present overloaded the adsorbant.

Because TLC, as performed in this study, does not separate the various metabolites of vitamin D<sub>3</sub>, gradient elution column chromatography on silicic acid was per-

formed on extracts of pooled subcutaneous fat and also pooled plasma obtained from groups of three animals sacrificed on days 12 and 55. A representative chromatogram showed separation of radioactivity into four peaks which closely resembled the patterns reported by DeLuca and coworkers (1, 18) for other tissues (Fig. 3). Recovery by this method was about 90% of that applied.

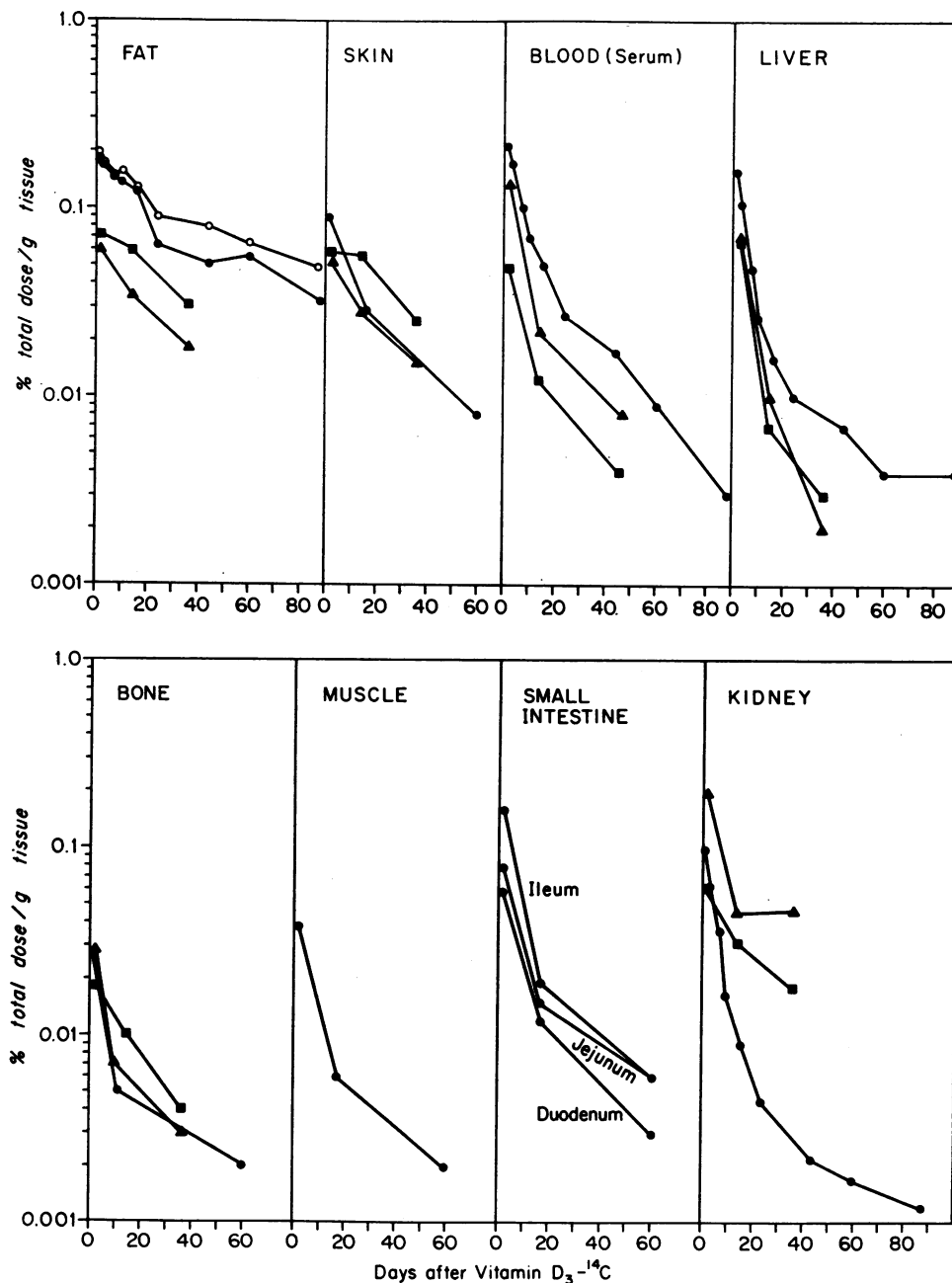


FIGURE 2 Decline in tissue radioactivity after completion of vitamin D<sub>3</sub>-<sup>14</sup>C supplementation. Group A = ▲; group B = ●; group C = ■. For Fat, group B animals, epididymal = ●, subcutaneous = ○.

After mild alkaline hydrolysis (26) of peak I, 40% of its radioactivity cochromatographed with unlabeled vitamin D<sub>3</sub> (Fig. 3B). This indicated that at least 40% of peak I material was present as esters of vitamin D<sub>3</sub>. The notch in peak I was found in all runs and may indicate esters with different fatty acid composition. Peak II was identified by means of its chromatographic location (1, 18). Its composition is unknown. As shown in Fig. 3B and 3C, the material in peak III migrated to areas corresponding to unlabeled marker vitamin D<sub>3</sub> on both the silicic acid columns and thin-layer chromatography. Furthermore, peak III was shown to be biologically active in the USP line test. Therefore, we conclude that the radioactivity of peak III represented unaltered vitamin D<sub>3</sub>. Peak IV had the same mobility as peak IV found by DeLuca using the same chromatographic system, and presumably consisted of a mixture of 25-HCC and other metabolically active and inactive polar metabolites of vitamin D<sub>3</sub> (18, 27).

The relative proportion of radioactivity in fat and plasma associated with each peak, 1 and 44 days after vitamin D<sub>3</sub> supplements had been withdrawn, is shown in Fig. 4. In subcutaneous fat, approximately 50% of the

radioactivity was associated with peak III (unaltered vitamin); this proportion did not change in these two samples bracketing a 34 day time span. However, there was a 40% decrease in the total amount of unaltered vitamin D<sub>3</sub> (from 3.5  $\mu\text{g/g}$  fat). During the same period, the proportion of total radioactivity in peak IV declined in fat from 21% to 12%, representing a 50% decline in total polar metabolites (from 1.3 to 0.48  $\mu\text{g/g}$ ). In contrast, there was an increase in the proportion of radioactivity in peak I, representing doubling of content of total vitamin D<sub>3</sub> esters (from 0.45 to 0.84  $\mu\text{g/g}$ ). Plasma radioactivity was located primarily in peak IV, the unaltered vitamin (peak III) being found present only during and immediately after the period of supplementation. We did not detect any plasma radioactivity in peak I, in agreement with previous reports (26).

Subcutaneous and epididymal fat, liver, kidney, and skin in depleted animals before the period of treatment were all found by bioassay to be free of antirachitic activity, but all these tissues were shown to contain activity throughout the entire period of observation after treatment.

TABLE II  
<sup>14</sup>C Content of Various Body Fat Depots, Group B\*

Day	Body wt. ‡	Specific gravity carcass §	Total body fat	Concentration <sup>14</sup> C in fat depots				
				Subcutaneous	Epididymal	Perirenal	Mesenteric	Brown ¶
g		g		dpm/g				
Supplementation								
1	117.3	1.0248	37.4	800	977	2870	1342	—
2	131.8	1.0244	39.0	1653	1415	—	—	—
4	162.0	1.0312	38.5	2675	1847	—	—	—
6	148.3	1.0377	39.8	3877	3698	3528	3771	—
8	165.9	1.0183	55.4	3285	3049	3440	4102	—
10	189.4	1.0141	60.9	3996	3300	—	—	—
12	176.2	1.0210	53.5	5696	5165	5148	6560	8391
Deprivation								
14	204.5	1.0428	55.2	4909	4832	4182	6059	—
18	211.0	1.0412	59.6	4245	4056	3107	4882	—
21	245.1	1.0506	63.0	4520	3948	—	—	—
27	236.1	1.0488	63.2	3841	3641	2447	3972	4319
35	288.9	1.0484	77.7	2567	1805	—	—	—
55	332.9	1.0533	87.1	2271	1446	—	—	—
71	310.8	1.0598	76.6	1835	1580	995	1330	1378
99	350.3	1.0440	100.0	1368	913	—	—	—

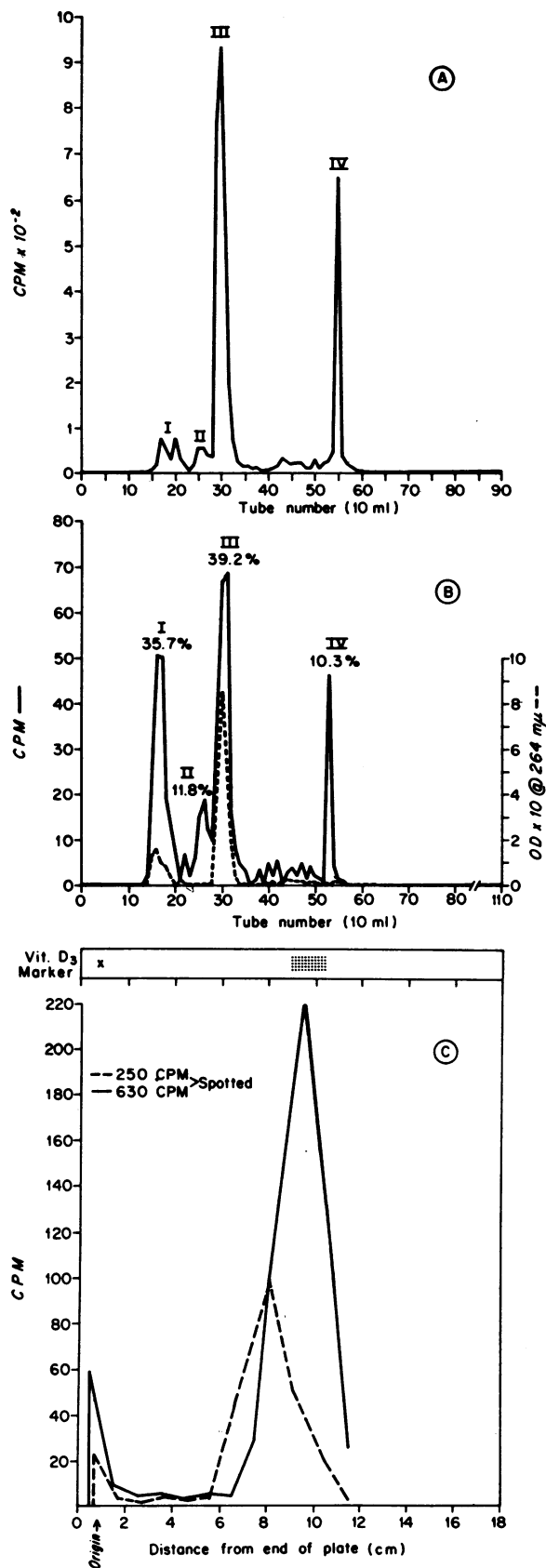
\* All values represent means of analyses on tissues from three animals.

‡ Weight of the shaved animal.

§ (Weight in air)/(weight in air minus weight in water); see Methods.

|| As calculated from the relationship between specific gravity and chemical determinations for body lipid in the rat; see Methods.

¶ Interscapular.



*Effect of dose on tissue concentration of radioactivity.* The general pattern was remarkably consistent for all groups, considering that the difference between the dose of group A and group C animals was 250-fold (Fig. 2). The concentration of radioactivity expressed as a fraction of administered total dose never differed by more than a factor of 5 and usually by no more than a factor of 2. The striking exception to this pattern was the kidney, where there was a higher initial fraction of the dose retained and a much slower rate of loss in group A than from either of the groups B or C.

## DISCUSSION

Reversal of a state of severe vitamin D<sub>3</sub> deficiency by administering only radioactive vitamin D<sub>3</sub> in the absence of sunlight permits us to make the assumption that subsequent tissue radioactivity in our experimental animals reflected the location and movement of all bodily vitamin D.

Although all tissues examined contained some radioactivity, indicating the presence of vitamin D<sub>3</sub> or its derivatives, adipose tissue was found to have the characteristics of a depot for the storage and release of the vitamin. This conclusion is supported by (a) the appearance within 24 hr of a substantial concentration of radioactivity in all five adipose tissue sites examined, (b) the subsequent very slow decrease in their radioactivity, (c) the consequent marked increase in total radioactivity in the adipose tissue relative to all other body tissues, and (d) the finding that a constant proportion of radioactivity in adipose tissue was present as unaltered vitamin D<sub>3</sub>, a form which was virtually absent from plasma throughout most of this same period of depletion. Thus, within 6 wk after vitamin D<sub>3</sub> administration, 80% of all of the radioactivity measured in the body was present in adipose tissue, and more than half of this was found to have the chromatographic characteristics of unaltered

FIGURE 3 Silicic acid column chromatography and identification of peaks. (A) Actual (but representative) chromatogram of extract of subcutaneous fat, day 12. Extract of 1 g tissue (4500 cpm) applied to column at room temperature, hyperbolic gradient, hexane  $\rightarrow$  diethyl ether  $\rightarrow$  methanol. (B) Cochromatography of hydrolyzed peak I fractions (pooled from two columns, one of which is shown above) with 10,000 IU unlabeled vitamin D<sub>3</sub> (Mann Research Labs, Inc.), column loaded, and developed as in (A). Optical density at 264  $m\mu$  (maximum absorption of vitamin D) of each fraction (in 3 ml ethanol) was measured in a Beckman DU spectrophotometer. Percentages above each fraction represent fraction of total radioactivity applied to column. (C) Thin-layer chromatogram of pooled fractions of peak III as shown in (A). In separate test lanes, two aliquots and cold vitamin D<sub>3</sub> in hexane were applied to a 250  $\mu$  thick alumina plate in nitrogen atmosphere and developed for 1 hr in chloroform. Successive 1 cm sections of each test lane were scraped into dioxane fluor containing 17% water.

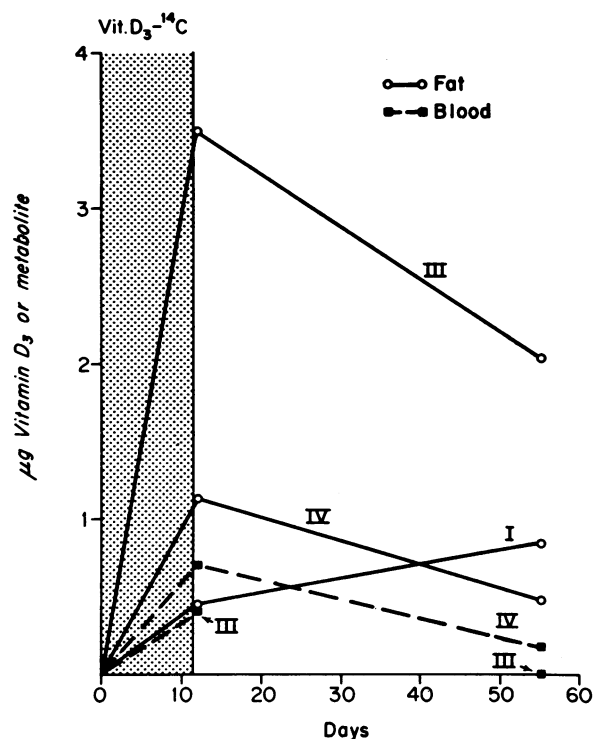


FIGURE 4 Total content of some chromatographic fractions in fat and blood of group B animals. The concentrations of vitamin  $D_3$  metabolites shown were calculated by means of an assumption that all metabolites had the same specific activity as the administered vitamin  $D_3$  ( $\mu\text{g} = \text{original specific activity} \times \text{dpm/g tissue extracted} \times \text{weight of tissue (g)}$ ). The total dose of vitamin  $D_3$  given to these animals was  $60 \mu\text{g}$ .

vitamin  $D_3$ . The large quantities of vitamin D-associated radioactivity in fat during and after supplementation resulted from the combination of substantial tissue concentration and a slow disappearance rate from this large organ mass.

Chromatographic analysis of the adipose tissue extracts disclosed that the peaks of radioactivity corresponded to those of multiple forms of vitamin  $D_3$  reported by DeLuca and coworkers for other tissues (3, 18). Of these, unaltered vitamin  $D_3$  (peak III) was present in the largest quantity throughout the entire experiment. Polar metabolites of vitamin  $D_3$  (peak IV) made up the next largest fraction of the total radioactivity in fat. The rates of decrease of these two fractions were similar, a finding consistent with their serving as sources of vitamin D during deprivation and consistent with the possibility that unaltered vitamin D in fat is the source from which the polar metabolites in fat and blood are replenished during deprivation. However, with respect to peak IV materials, the interpretation of this observation is uncertain since peak IV material includes metabolites which lack biological activity (1, 2) in addition to those which

are biologically active, such as 25-HCC. Furthermore, we do not know whether these polar metabolites of adipose tissue arose from liver, tissues other than liver, or were produced within the fat cells themselves.

After ceasing supplementation, a progressive increase in the total quantity of vitamin  $D_3$  esters (peak I) in fat was observed, whereas the content of all other forms of vitamin  $D_3$  was decreasing. Thus, by 6 wk after treatment was stopped, 20% of all radioactivity in fat was in the form of esters. It is known that a small quantity of these is formed during intestinal absorption (28, 29), and they have been detected in liver and kidney (26, 30). However, the amount present in fat by the end of the experiment was very large compared with the quantity of vitamin  $D_3$  esters outside adipose tissue; this suggests that vitamin  $D_3$  esters are formed from vitamin  $D_3$  within fat. Their continued accumulation, even while all other forms of vitamin  $D_3$  were diminishing, suggests a very prolonged storage in fat. They might serve as still another bodily source of vitamin D during starvation, when fat is mobilized and after other forms of vitamin D have been exhausted.

Our studies show that adipose tissue will accumulate a substantial proportion of a dose promptly (i.e. 12% in 24 hr), that about this same fraction is accumulated irrespective of dose over the very large range examined (from what is considered a maintenance dose for rats of this age (31, 32) to a high pharmacological dose), and that release is very slow and approximately proportional to the concentration of vitamin D in adipose tissue over this same large dose range. Thus, adipose tissue serves as a depot of large capacity which accumulates vitamin D proportionate to its concentration in plasma and releases it at a much slower rate that is proportionate to its concentration in fat. These features of accumulation and release would protect against toxicity during short periods of vitamin overdosage, would tend to maintain a relatively stable concentration in blood under natural conditions where absorption and production of the vitamin could vary significantly, and would provide continued maintenance of vitamin D in plasma for hepatic conversion to 25-HCC during a relatively prolonged period of low intake. The fact that the biological activity of plasma of humans falls exponentially after a pharmacological dose of vitamin D (8, 33) at a rate similar to that which we have observed in rats (i.e.  $t_{1/2} \sim 3-4$  months cf.  $t_{1/2} \sim 80$  days in rats) lends support to the concept that these same features may characterize the storage and release of vitamin D in humans.

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