Biologically active metabolite of vitamin D_3 from bone, liver, and blood serum

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ABSTRACT Radioactive metabolites present in bone, blood, liver, and feces of rats given ^{3}H vitamin D_3 have been isolated. Of these the aqueous soluble metabolite(s) from tissue and all those isolated from feces did not cure rickets in rats, while all the others were at least partially active in this regard. One of the metabolites proved to be as active as the parent vitamin in curing rickets and was found in large amounts in liver, blood, and bone.

As much as 50–80% of the radioactivity in bone was found in this metabolite after a 500 IU oral dose of ^{3}H vitamin D_3. With 10 IU doses of 1,2-^{3}H vitamin D_3, most of the radioactivity of the organs examined was found in this metabolite fraction. This metabolite appears to be more polar than vitamin D and is not an esterified form of the vitamin nor a complex of the vitamin with tissue lipids. Its possible role as the metabolically active form of the vitamin is discussed.

KEY WORDS vitamin D_3 · metabolite(s) · biologically active · bone · liver · serum · low doses · rickets · rat · man

UNTIL RECENTLY, it had been accepted that of the metabolites of vitamin D found in tissue, none possessed ability to cure rickets (vitamin D activity). Thus, in previous work, it had been repeatedly emphasized that all of the metabolic products detected could be considered "breakdown products," presumably because of lack of vitamin D activity (1, 2). It was primarily for this reason that the possibility of a metabolically active form of vitamin D other than the unaltered vitamin seemed remote. However, with the preparation of radioactive vitamin D of moderately high specific activity (3), the development of mild extraction procedures (4), and new chromatographic methods (5), this possibility could be examined anew (5). In that investigation, at least four metabolites of vitamin D were detected in intestine and kidney, of which three possessed at least partial ability to cure rickets (6).

This finding has now been confirmed and extended. In this manuscript it will be demonstrated that one of these metabolites is found in high concentration in bone, blood, and liver, and that its relative proportion is increased as vitamin D dosage is decreased. This metabolite is chromatographically distinct from vitamin D, does not yield vitamin D on saponification, and is as biologically active as the parent vitamin. Its possible function as the metabolically active form of vitamin D is suggested.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (Holtzman Company, Madison, Wis.) were maintained individually in hanging wire cages and given free access to food and water. They were obtained as weanlings and fed a purified diet (containing 0.47% calcium and 0.3% phosphorus) that does not induce rickets. This diet was similar to that described by DeLuca and coworkers (6, 7). The rats were maintained on this vitamin D-free diet for 3–4 weeks up to the beginning of the experiments, at which time the animals were vitamin D-deficient as judged by the criteria of Steenbock and Herting (8).

The ^{3}H vitamin D_3 prepared in this laboratory (3) was generally used, although similar results were obtained with 1,2-^{3}H vitamin D_3 (9) and 4-^{14}C vitamin D_3 as well as other labeled vitamin D preparations. Radiochemical purity of the radioactive vitamin preparations was demonstrated in at least three chromatographic systems, as previously described (3, 9). The ^{3}H vitamin D_3 was administered either orally (usually 500 IU) in 0.2 ml of cottonseed oil (Wesson), intraperitoneally, or intracardially in 0.2 ml of 0.10% Tween 20 (polyoxyethylene sorbitan monolaurate; Atlas Chemical Industries, Inc., Wilmington, Del.)–0.9% NaCl in water. The aqueous

solution was prepared by layering the ^3H vitamin D_3, dissolved in a few drops of ether, over the necessary amount of Tween solution and bubbling nitrogen through the solution at 40°C until the ether was removed. In the most recent experiments the intrajugular doses of ^3H vitamin D_3 were given in 0.05 ml of 95% ethanol.

The rats were decapitated at specified times (usually 24 hr) after ^3H vitamin D_3 administration. Blood was collected in centrifuge tubes and centrifuged after 1–2 hr. The serum was decanted and frozen for later analysis. Livers were removed immediately and placed in ice-cold isotonic sucrose solution. They were either extracted immediately or frozen for later extraction.

The legs were removed from the animals immediately after the livers and packed in ice. A short time later, the muscle tissue was removed from the leg bones and the bones were split open in order that the marrow could be removed. Care was taken to keep the bones at 0°C whenever possible.

Extraction

The radioactivity present in the tissues was extracted by an adaptation of the procedure of Bligh and Dyer (4). A 30% homogenate of liver or feces was made in water by means of a Potter-Elvehjem homogenizer fitted with a Teflon pestle (Arthur H. Thomas Co., Philadelphia, Pa.). Homogenate (0.8 part) was mixed with two parts of methanol and one part of chloroform and the mixture was magnetically stirred overnight at 12°C. One part of chloroform was subsequently stirred in, followed by one part of water. The two-phase system thus formed was filtered with suction through Whatman No. 1 paper and then allowed to separate at 12°C for at least 2 hr. The lower chloroform phase was drawn off and the upper aqueous phase was routinely reextracted twice with chloroform.

The serum was extracted similarly except that it was necessary to change the solvent proportions slightly if an inseparable emulsion was not to result. Accordingly, only 0.1 part of water was added before filtration, rather than the previously described 1.0 part.

Special treatment was needed to extract the bone. The pieces of bone were frozen in a bath of acetone–dry ice. A large mortar and a pestle were placed in a similar bath and when they were cold, the bones were placed in the mortar with an equivalent amount of crushed dry ice. After the mixture had been crushed as fine as possible, the particles were triturated with 200 ml of methanol–chloroform 2:1. The mixture was transferred to a 1 liter Erlenmeyer flask. An additional 100 ml of the 2:1 solution was used to rinse the mortar and pestle. The mixture was magnetically stirred overnight at 12°C. Then 100 ml of chloroform was stirred in, followed by 180 ml of water.

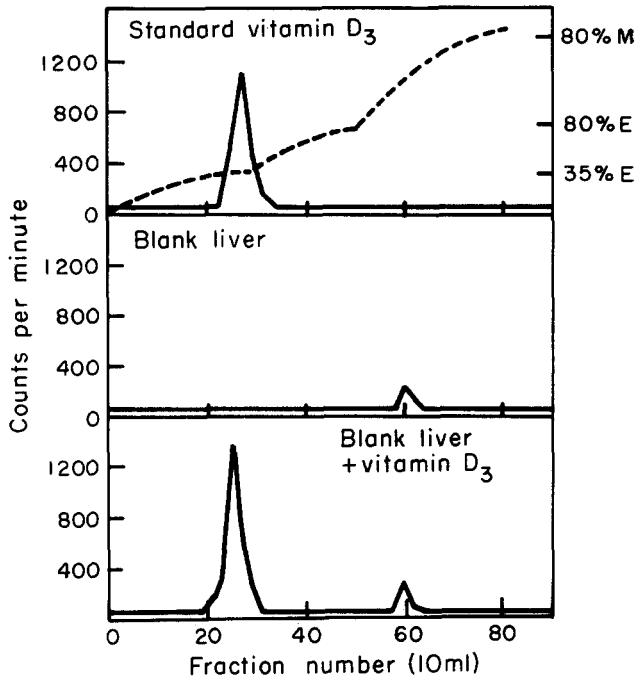

FIG. 1. Silicic acid column chromatography of standard vitamin D and extracts of control tissues.

Standard ^3H vitamin D_3 was dissolved in petroleum ether and chromatographed on a silicic acid column. The gradient used in elution is indicated by the dashed line: from 0 to 35% diethyl ether in petroleum ether (*35% E*), from 35% to 80% diethyl ether in petroleum ether (*80% E*), and from there to 80% methanol in diethyl ether and petroleum ether (*80% M*). The same gradient was used for all column chromatography described in this communication.

A 30% homogenate in water was prepared from the livers of two vitamin D-deficient rats. Half of the homogenate was extracted as described in the text and chromatographed. Twenty-five units of ^3H vitamin D_3 in 0.1 ml of ethanol was added to the other half which was then extracted and chromatographed.

Tritium was measured in aliquots of all column fractions.

The remainder of the procedure was as that described for liver, beginning with the filtration step.

Chloroform extracts were concentrated either in a stream of N_2 or in a flash evaporator and dissolved in petroleum ether for chromatography.

Measurement of Radioactivity

Radioactivity was measured by liquid scintillation in a Packard Tri-Carb model 3000 automatic counter as previously described (6). The toluene counting solution of Herberg (10) was used in all cases except for aqueous samples which were counted in Bray's (11) solution.

Chromatography

Silicic acid column chromatography of the tissue extracts was carried out essentially as described previously (6). The gradient is shown in Fig. 1. About 5 psi of air pressure was used to pack and run the column; this gave a flow rate of about 1.5 ml/min.

TABLE 1 DISTRIBUTION OF RADIOACTIVITY BETWEEN THE AQUEOUS PHASE AND THE CHLOROFORM PHASE IN VARIOUS TISSUE EXTRACTS OF RATS GIVEN ^{3}H VITAMIN D_3

Tissue	Aqueous	Chloroform
	%	%
Liver	11	89
Serum	20	80
Bone	19	81
Feces	36	64

Vitamin D-deficient rats were given 500 IU of ^{3}H vitamin D_3 (1000 dpm/IU) intraperitoneally in 0.1% Tween 20–0.9% NaCl solution; 24 hr later they were killed and the excised tissues and collected feces were extracted by the procedures described in the text.

Biological Assays

Various fractions from the column chromatography of lipid extracts of liver, serum, bone, or feces were biologically assayed in the rat for vitamin D activity at the Wisconsin Alumni Research Foundation by Mr. Lloyd Hein according to official USP (12) procedures, which are highly sensitive. The samples were prepared for assay as described previously (6).

Saponification

The sample was placed in a 100 ml round bottom flask with a side arm and 15 ml of 10% KOH in methanol was added. Nitrogen was constantly bubbled in through the side arm while the solution was refluxed for 30 min. Then 20 ml of water was added and the solution was extracted with three 20-ml portions of diethyl ether. The combined ether solutions were washed once with 3.6% HCl and twice with water and then dried over anhydrous $CaSO_4$. The filtered ether solution was evaporated and the residue dissolved in petroleum ether for chromatography.

RESULTS

It was demonstrated earlier that 24 hr after ^{3}H vitamin D_3 administration, a considerable quantity (49%) of the radioactivity of intestine appeared as water-soluble metabolites while only 17% of that in kidney appeared in this form (6). The present results show that in bone, liver, and blood, 20% or less of the radioactivity in these appeared in the aqueous phase (Table 1). In feces, however, considerably more radioactivity is found in the water-soluble fraction, which suggests that this fraction might represent degradation products.

Before trustworthy chromatography of the chloroform extracts of the tissues and feces could be carried out, artifacts had to be excluded. In Fig. 1, the chromatographic profile of the original ^{3}H vitamin D_3 on the silicic acid columns after gradient elution is demonstrated. Of interest are the tissue and silicic acid "blank" column profiles (the latter not shown in Fig. 1). Occasional batches of silicic acid tested on receipt from the supplier gave methanolic eluates containing rather large amounts of "apparent" radioactivity in the tritium "window" of the scintillation counter. In these cases it was found necessary to wash the silicic acid extensively with methanol, vacuum-dry it, and reactivate it at 140°C overnight. Occasionally, batches of silicic acid were discarded when it was impractical to remove all the impurities. Some "apparent" radioactivity was also found in the tissues of vitamin D-deficient rats that had not received ^{3}H vitamin D_3 (Fig. 1). These "blank" values were checked routinely and taken into account in the experiments reported here. Finally it was possible to show that the ^{3}H vitamin D_3 chromatographed as expected in this system even when it was added to the tissue before extraction. In experiments not shown, all of the radioactivity of the tissues a few minutes after an intrajugular injection of ^{3}H vitamin D_3 in ethanol was found in the vitamin D fraction (peak III, Fig. 2) on silicic acid chromatography. These experiments demonstrate that this system is free from artifacts that might result from complexing of the small amounts of vitamin D with the tissue lipids.

When the chloroform fractions of the tissue extracts were chromatographed (Fig. 2), the radioactivity was separated into four distinct components. Peak I, which was present in virtually all tissue extracts, represents an ester of vitamin D (Lund, Horsting, and DeLuca, data in preparation), while peak III migrates identically with crystalline vitamin D_3. Peaks II and IV are as yet unidentified.

The extract of feces gave a chromatogram somewhat different from that of the tissue extracts. For bone, peak IV represented the majority of the radioactivity present while vitamin D itself was present in smaller amounts. Rather large amounts of peak IV were also found in liver and blood, while in a previous investigation (6) only 5% of the radioactivity of kidney and intestine was found in this region.

The various column fractions obtained were bioassayed by the line test method (Table 2). As might be expected, the peak III material obtained from tissues gave full biological activity. The material from feces which migrated in the same general region as vitamin D (peak III) was virtually without biological activity and is therefore *not* vitamin D. This finding should serve as a warning against identifying a radioactive material simply on the basis of its behavior in one chromatographic system.

The important finding that peak IV gave biological activity equal to that of vitamin D is illustrated in Table 2. This was true whether the fraction was isolated from bone, liver, or blood. Previously it had been found that a similar preparation from kidney or intestine was only

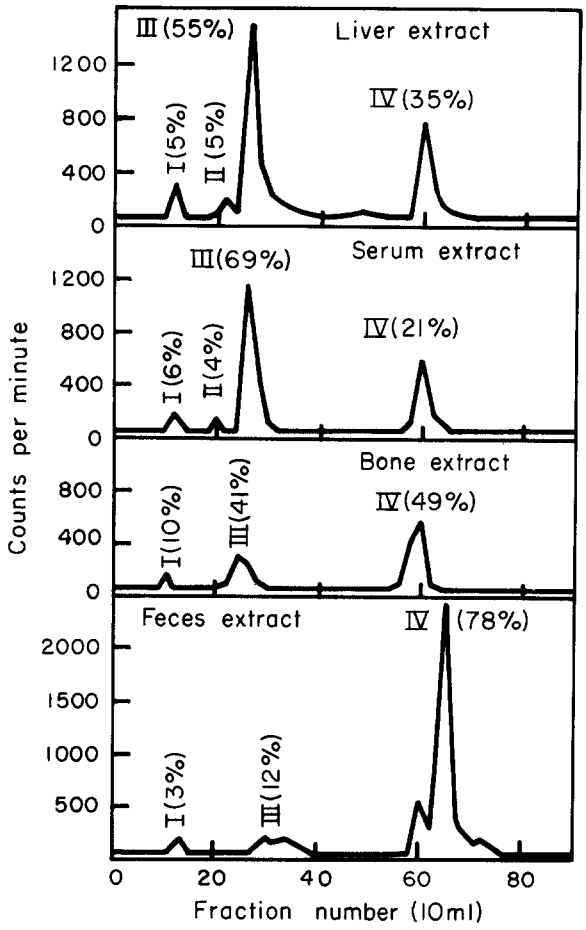

FIG. 2. Silicic acid column chromatography of lipid extracts from various tissues.

Rats were fed a vitamin D-deficient diet for 3 weeks and then given 500 IU each of ^{3}H vitamin D_3 either intraperitoneally in 0.2 ml of 0.1% Tween 20–0.9% NaCl solution or orally in 0.2 ml of cottonseed oil (Wesson). After 24 hr the animals were sacrified, tissues excised, and feces collected. Extracts were made and chromatographed as described in the text. Aliquots of the column fractions were used to measure tritium and then tubes comprising each peak were pooled for the bioassay. Thirteen rats were used for the feces extract and twenty for the other extracts.

partially biologically active. This may have been due to some oxidative destruction of the metabolite before bioassay was carried out; this question is under reinvestigation. Peaks I and II were also partially biologically active. However, in no case have we detected more than small amounts of these two fractions. Essentially no biological activity could be detected in any of the fractions from feces or in any of the water-soluble metabolites, which suggests that they probably represent degradation products.

A control experiment was carried out in which the tissues of vitamin D-deficient rats were extracted, chromatographed, and bioassayed in exactly the same fashion as the tissues from rats treated with ^{3}H vitamin D_3. In no case was any biological activity found in the peak III and peak IV regions from the vitamin D-deficient rats. This experiment rules out the possibility of a false vitamin D test caused by a large amount of some tissue component, such as phospholipid, which would be located in the peak IV region.

The surprising discovery that a radioactive component of the tissue extracts from rats treated with ^{3}H vitamin D_3 was present in large amounts, especially in bone—a target tissue of vitamin D action—and that it is as biologically active as the parent vitamin stimulated our further interest in the substance. Could this component merely be a complex of vitamin D with some other tissue lipid? Rechromatography of peak IV material from liver or other sources demonstrated that it was not dissociating, with the release of vitamin D (Fig. 3). Cochromatography of "peak IV" with crystalline vitamin D_3 also demonstrated that it is distinct from the parent vitamin (Fig. 3). Finally it could be shown that most of this substance survives saponification and certainly does not yield vitamin D (Fig. 3). These experiments therefore establish that peak IV material is a distinct metabolite of vitamin D.

That the peak IV metabolite(s) is formed rapidly from vitamin D is demonstrated by the data shown in Table 3. Within 2 hr after an oral dose of the ^{3}H vitamin D_3 or 1 hr after an intravenous dose, significant amounts of the metabolite(s) appeared in blood. Four hours after an oral dose, a fairly constant amount of peak IV was found in blood for at least 48 hr.

TABLE 2 VITAMIN D ACTIVITY OF COLUMN FRACTIONS FROM VARIOUS SOURCES

Source	Aqueous Methanol*	Silicic Acid Column Fractions from Fig. 2 I	II	III	IV
		activity in IU/4000 dpm			
^{3}H vitamin D_3	—	—	—	4	—
Vitamin D-deficient blank†	0	—	—	0	0
Liver	0	1	0.5	4	4
Serum	0	—	2	4	4
Bone	0	—	—	4	4
Feces	0	0	—	0.8	0

The chloroform phase of the indicated extracts was chromatographed on silicic acid columns as shown in Fig. 2. Each fraction was dissolved in diethyl ether and that amount containing 4000 dpm was pipetted onto the diet of the test animals. The ether was allowed to evaporate and the food returned to the animals. The line test assay (rickets cure) was carried out as described in the U. S. Pharmacopoeia (1955).

* The aqueous methanol fraction of the extract was concentrated in a flash evaporator and the equivalent of 4000 dpm was given intraperitoneally to the test animals.

† An extract of an amount of tissue from vitamin D-deficient rats equal to that used from the ^{3}H vitamin D-treated rats was extracted and chromatographed as described in Fig. 2, and the fractions were assayed.

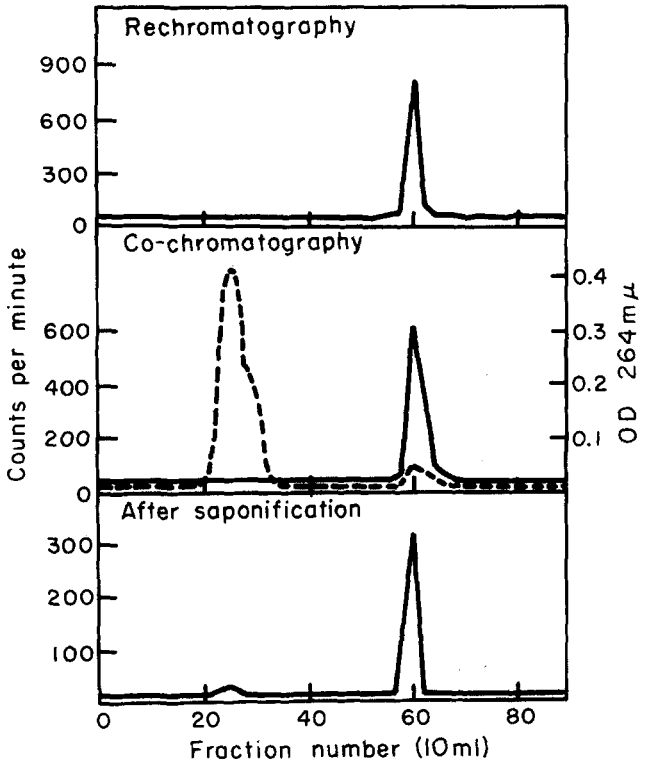

FIG. 3. Silicic acid column chromatography of peak IV after various treatments.

Peak IV (^{3}H) was obtained from chromatography of liver extract from rats given ^{3}H vitamin D_3 24 hr before (Fig. 2). The appropriate fractions were dried under vacuum, dissolved in petroleum ether, and rechromatographed in the same system. Another sample of ^{3}H-peak IV was mixed with 3 mg of crystalline vitamin D_3 and cochromatographed in the same system. OD readings at 264 mμ indicated that the elution of the crystalline vitamin D and the UV spectrum of the eluent matched that known for the vitamin. The elution of peak IV was measured by radioactivity. ----, OD at 264 mμ; ———, radioactivity.

^{3}H-peak IV (12,000 dpm) was saponified, and the product was chromatographed in the same system. Thirty per cent of the radioactivity became water-soluble; nearly all of the remainder still chromatographed as peak IV.

Human serum also contains significant quantities of the peak IV material (Table 3). When normal adults were given 10,000 IU of ^{3}H vitamin D_3 intravenously, as much as 5.9% of the dose appeared as peak IV metabolite in the serum after 16 hr. The metabolite from human serum also proved to be as biologically active as the parent vitamin in the line test assay (Lund, Rosenbloom, DeLuca, and Lobeck, data in preparation).

We next determined the proportion of the peak IV metabolite as a function of vitamin D dosage. This investigation was made possible by the synthesis of 1,2-^{3}H vitamin D_3 with a specific activity of 26,000 dpm/IU (9). That the proportion of peak IV material is markedly increased as the vitamin D dosage is decreased to levels approaching nonstorage doses is shown in Fig. 4. Finally, the peak IV material is also effective in stimulating intestinal active transport of calcium, a response which will be reported in detail elsewhere (Lund and DeLuca, data in preparation).

TABLE 3 AMOUNT OF PEAK IV IN BLOOD AT VARIOUS TIMES AFTER DOSAGE WITH ^{3}H VITAMIN D_3

Source	Time after ^{3}H Vitamin D_3	Route of Dosage	% Dose as Peak IV
	hr		
Rat serum*	1	Intrajugular	1.4†
Rat blood (whole)*	2	Oral	0.4
	4	"	1.5
	8	"	1.0
	16	"	1.2
	24	"	1.1
	48	"	1.2
Human serum‡	16	Intravenous	5.9§
			3.3§

* 500 IU ^{3}H vitamin D_3 was given to each rat. There were at least 4 rats in each group.

† This assumes 6% of body weight is whole blood.

‡ 10,000 IU ^{3}H vitamin D_3 in 80% alcohol was administered to each patient. The values given are individual ones for 2 patients.

§ This assumes a blood volume of 5 liters/patient.

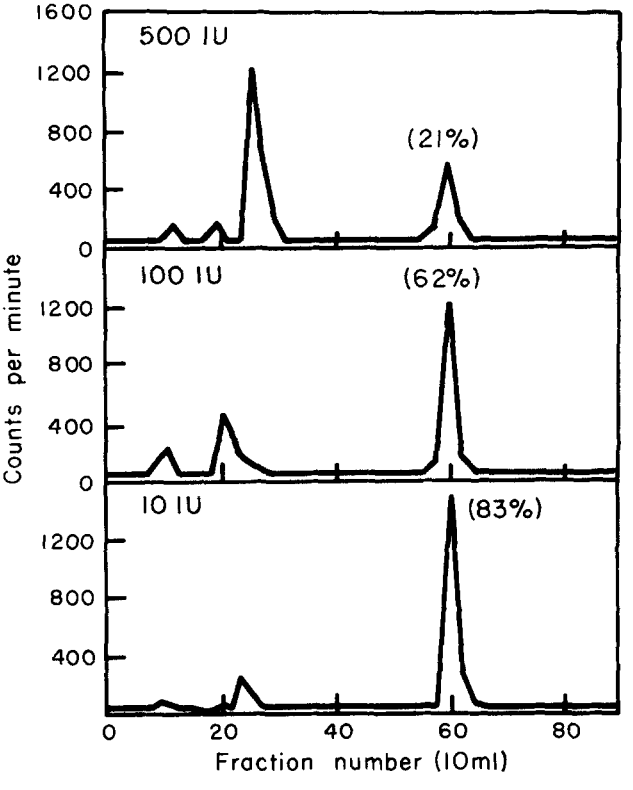

FIG. 4. Silicic acid column chromatography of serum lipids after various doses of ^{3}H vitamin D_3.

Rats were fed a vitamin D-deficient diet for 3 weeks and then given 500, 100, or 10 IU of 1,2-^{3}H vitamin D_3 orally in 0.1 ml of cottonseed oil. The animals were sacrified after 24 hr and the serum was obtained, extracted, and chromatographed.

DISCUSSION

Up to the present it has been assumed that the unaltered vitamin D molecule carries out the metabolic function of the vitamin. This is perhaps the result, at least in part, of experiments (1, 2) with radioactive vitamin D in which no biologically active metabolite of vitamin D could be detected. The specific activity of the preparations then used, however, necessitated experiments involving as much as a milligram of vitamin D per animal. These high doses, which greatly exceed the needs of the animal, might well have masked the appearance of a small amount of a functional vitamin D metabolite. In addition, Kodicek and coworkers did not employ silicic acid column chromatography, which was instrumental in locating the metabolites reported. These workers did report (1, 2) the presence of biologically inactive "breakdown" products of vitamin D, especially in liver and bone.

From our experiments it is clear that the possibility that the vitamin D is converted to a metabolically active form must once again be considered. As lower nonstorage doses of the vitamin are approached, the proportion of radioactivity in peak IV is greatly increased (Fig. 4). The peak IV metabolite(s) is formed within 1 hr after vitamin D administration and it is found in large amounts in human plasma (Table 3). That it is as biologically active as the parent vitamin in curing rickets in rats and that it is active in stimulating calcium transport in everted intestinal sacs add further support to the possibility that this material is a metabolically active form of the vitamin.

Much effort was expended to exclude artifacts which might account for the data reported here. The problem of false radioactive readings in effluents from columns made from certain batches of silicic acid must be recognized. Such readings may be caused by a long-lived phosphorescent material; in any case, it is essential that batches of silicic acid be checked for this behavior, and the impurity be removed by solvent washing. The apparent radioactivity that appears in tissue extracts from rats not treated with radioactive vitamin D must also be taken into account. Peak IV does not result from complexing of ^{3}H vitamin D_3 with tissue lipids, as tested in vitro (Fig. 1) and a few minutes after the intravenous administration of ^{3}H vitamin D_3.

That the peak IV material is not vitamin D was shown by cochromatography with crystalline vitamin D_3 (Fig. 3). Additional evidence that it is not merely vitamin D in some bound form is provided by the fact that saponification of peak IV does not yield vitamin D (Fig. 3). It does, however, yield water-soluble radioactivity in significant amounts (30% of peak IV becomes water-soluble) after this treatment, which suggests that it is more labile than vitamin D itself.

The chemical nature of peak IV material is not known at present. It is not a conjugated or esterified form of the vitamin D, since saponification of it does not yield the vitamin. Its behavior on silicic acid columns suggests that it is more polar than the parent vitamin. Work is now in progress to isolate large quantities of this metabolite for identification. Regardless of whether this metabolite of vitamin D proves to be the metabolically active form of the vitamin or not, its identification should provide information on the metabolism of vitamin D and indicate what modifications of the vitamin molecule are permissible for expression of vitamin D activity.

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