The Intracellular Distribution of [$1\text{-}^3\text{H}$]Cholecalciferol in the Intestine of Vitamin D-Deficient and -Supplemented Rats

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(Received 26 September 1966)

1. [$1\text{-}^3\text{H}$]Cholecalciferol was administered orally at two dosages to vitamin D-deficient and -supplemented rats, and the intracellular distribution of the vitamin in the intestinal mucosa studied. 2. The concentration of cholecalciferol was highest in a fraction consisting of brush borders and nuclei. The microsomal fraction contained a higher concentration of the vitamin than the mitochondrial fraction in deficient rats, irrespective of the dose, whereas in the vitamin D-supplemented rats the concentration was the same in the two fractions. 3. Appreciable metabolism of the cholecalciferol occurred only in the supplemented rats and the metabolites were found predominantly in the mitochondrial fraction. 4. The cholecalciferol is more tightly bound to the microsomal fraction than to the mitochondrial fraction. 5. Experiments conducted *in vitro* have shown that all the intracellular particles combine with the vitamin either when dissolved in ethanol or bound to albumin. However, such an uptake does not account for the high concentration of radioactivity found *in vivo* in the fraction containing nuclei and brush border, nor for the tightly bound vitamin in the microsomal fraction.

Recently [$1\text{-}^3\text{H}$]cholecalciferol has been synthesized (Callow, Kodicek & Thompson, 1966) with a specific activity sufficient to allow physiological doses of the vitamin to be administered to rats, permitting investigations to be made into the tissue distribution of the vitamin and into the identification of the metabolites of the vitamin (Fraser & Kodicek, 1965). After an oral dose of $10\mu\text{g.}$ of [$1\text{-}^3\text{H}$]cholecalciferol the proportion of the vitamin found 24 hr. later in the various tissues was: liver, 4.7%; bones, 3.9%; kidney, 2.8%; intestine, 2.5%. Most of the radioactivity (36.7%) was present in the faeces and gut contents, but of this only 64% was due to the unchanged vitamin. The intracellular distribution of vitamin D has been studied in the liver (Kodicek, 1963) and in the intestine and kidney of vitamin D-deficient rats (Norman & DeLuca, 1964). In this present work, we have extended the observations on the intracellular distribution of cholecalciferol in the intestine of vitamin D-supplemented and -deficient rats to obtain information on the nature of the radioactivity and of the binding of the vitamin within the various cellular fractions.

EXPERIMENTAL

[$1\text{-}^3\text{H}$]Cholecalciferol. The [$1\text{-}^3\text{H}$]cholecalciferol 3,5-dinitrobenzoate was synthesized according to Callow *et al.* (1966). The dinitrobenzoate was dissolved in dioxan, an equal volume of 2*N*-KOH in methanol added and the

mixture kept at room temperature for 1 hr. Then 5 vol. of water was added and the cholecalciferol extracted with several volumes of diethyl ether. Then labelled vitamin was further purified by thin-layer chromatography. It was applied as a streak to silica-gel G plates and two markers of the non-labelled vitamin were added, one at each side of the streak. The plate was developed with chloroform until the solvent front had reached the top of the plate. The non-labelled markers were visualized with SbCl_3 in CHCl_3 (antimony trichloride solution for vitamin A test; British Drug Houses Ltd., Poole, Dorset). The narrow band of silica gel between the markers was scraped off the plate into a small sintered-glass funnel and the [$1\text{-}^3\text{H}$]cholecalciferol eluted with several volumes of ethanol and then diethyl ether. To achieve maximum recoveries of the vitamin from the silica gel it was essential not to dry the plate before elution. The specific activities of two batches of the [$1\text{-}^3\text{H}$]cholecalciferol were $16.8\mu\text{C/mg.}$ and $18.7\mu\text{C/mg.}$

Animals. Male and female piebald weanling rats were raised on either the synthetic diet described by DeLuca, Guroff, Steenbock, Reiser & Mannatt (1961), or that described by Numerof, Sassaman, Rodgers & Schaefer (1955), which is a modification of the diet used by Steenbock & Black (1925); some of the rats were supplemented from weaning with $12.5\mu\text{g.}$ of vitamin D_3 in arachis oil per week. The rats were maintained on these diets 3–4 weeks before the experiment. The dose of [$1\text{-}^3\text{H}$]cholecalciferol was administered to each rat in 0.2 ml. of arachis oil by stomach tube. The rats were kept without food for 24 hr. before killing by cervical dislocation. The small intestine was quickly removed, washed through with ice-cold 0.9% NaCl, opened longitudinally, rinsed with cold 0.25*M*-sucrose and the mucosa scraped off with a glass slide.

Tissue fractionation. In Expts. 1–4, the weighed intestinal

mucosa was homogenized in 0.25 M-sucrose with an all-glass Potter-Elvehjem homogenizer to give a 10% homogenate. The homogenate was fractionated by a modified method of Schneider & Hogeboom (1950). The cell debris, mitochondrial and microsomal fractions were collected at 700g for 10 min., 10000g for 10 min. and 105000g for 60 min. respectively. The debris, mitochondrial and microsomal pellets were all resuspended in 0.25 M-sucrose, all fractions being made up to a known volume.

In Expt. 5, the intestinal mucosa was fractionated by a different method. The mucosa was washed with Krebs-Ringer phosphate buffer (Krebs & Henseleit, 1932) and centrifuged at 500g for 2 min. and the supernatant discarded. Epithelial-cell 'ghosts' were then prepared by the method of Clark & Porteous (1965) and their intracellular fractions III-X prepared as described by Porteous & Clark (1965). All fractions were resuspended and made up to known volumes. All tissue manipulations were carried out at 4°.

Extraction of intracellular fractions. Portions of the suspension of mitochondrial and microsomal fractions and also the cytoplasmic fluid from Expts. 1-4 were extracted three times with an equal volume of diethyl ether, and the ether extracts were combined and evaporated to dryness (ether-soluble extract). Ethanol was then added to the suspension to give a 30% (v/v) solution and the suspension left at room temperature for 10 min. The suspension was again extracted three times with an equal volume of diethyl ether, and the combined extract evaporated to dryness (ether-ethanol-soluble extract). The remaining suspension was then saponified by the addition of 3 vol. of ethanol and solid KOH (6g./10ml. of suspension) and refluxed for 10 min. It was then cooled and an equal volume of water added and the whole extracted three times with diethyl ether. The combined ether extracts were washed three times with water and evaporated to dryness (ether-soluble extract after saponification). A suspension of the cell debris was also saponified with ethanolic KOH without prior extraction and the unsaponifiable material extracted with diethyl ether. In Expts. 3 and 4, with high doses of cholecalciferol, a portion of the mitochondrial and microsomal suspension and of the cytoplasmic fluid was similarly saponified without prior extraction. In Expt. 5, samples from all fractions were directly saponified and the unsaponifiable material was counted.

Thin-layer chromatography. Thin-layer chromatography of lipids was carried out on silica-gel G thin-layer plates, with chloroform as the developing solvent. The silica gel was scraped off in 1 cm. bands from the origin to solvent front, transferred to polythene counting vials and the radioactivity assayed as described below. Non-labelled cholecalciferol was run as a marker in each chromatograph and detected with SbCl_3 in CHCl_3 .

Radioactive measurement. The radioactive assays were made in a Packard automatic Tri-Carb liquid-scintillation spectrometer with a counting solution consisting of 100 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 4.0g. of 2,5-diphenyloxazole per l. of toluene. Quenching was corrected for by the addition of $n[1,2\text{-}^3\text{H}^2]$ -hexadecane as an internal standard.

Protein estimation. The protein content of the intracellular fractions was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as the reference standard.

RESULTS

The distribution of the intestinal radioactivity within the intracellular fractions after the dose of 2.5 $\mu\text{g.}$ of $[1\text{-}^3\text{H}]$ cholecalciferol is shown in Tables 1 and 2. Irrespective of the dosage, the concentration of the total radioactivity in the fractions from the deficient rats was highest in the microsomal fraction followed by the mitochondrial fraction. In the vitamin D-supplemented rats, however, the position was reversed, with the mitochondrial fraction having the highest concentration of the total radioactivity. The cytoplasmic fraction from these homogenates contained at least 40% of the total radioactivity in the final three centrifugal fractions, although the specific activity was always the lowest.

In Table 2 are also shown the specific activities of the various fractions based on the radioactivity in the unsaponifiable matter only. Thin-layer chromatography on the unsaponifiable matter of these and similar fractions revealed only one peak of radioactivity, which was located in the position of $[1\text{-}^3\text{H}]$ cholecalciferol, only a trace being detectable at the origin. On this basis therefore it was concluded that in the microsomal fraction almost all the radioactivity was due to cholecalciferol irrespective of the vitamin D status of the animal. Thus in the vitamin D-deficient rats the specific activity of the microsomal fraction based on total radioactivity was 18315 disintegrations/min./mg. of nitrogen, and based on unsaponifiable material radioactivity was 16970 disintegrations/min./mg. of nitrogen.

Table 1. *Specific activities of intestinal intracellular fractions from vitamin D-deficient and -supplemented rats dosed with 2.5 $\mu\text{g.}$ of $[1\text{-}^3\text{H}]$ cholecalciferol per rat*

In Expt. 1 five vitamin D-deficient rats (av. wt. 111 g.) and in Expt. 2 six vitamin D-supplemented rats (av. wt. 167 g.) were each given an oral dose of 2.5 $\mu\text{g.}$ of $[1\text{-}^3\text{H}]$ cholecalciferol. All the rats were raised on the diet described by DeLuca *et al.* (1961). The homogenates of the intestinal mucosa from individual rats were pooled for the preparation of the intracellular fractions. The results are expressed as disintegrations/min. and disintegrations/min./mg. of N for the total radioactivity and specific activity measurements respectively.

Fraction	Deficient		Supplemented	
	Total extracted radio-activity	Specific activity	Total extracted radio-activity	Specific activity
Mitochondrial	139	13.5	224	54.5
Microsomal	406	20.0	516	37.5
Cytoplasmic	968	14.0	548	10.0
Debris	700	8.0	3780	19.5

Table 2. *Specific activities of intestinal fractions from vitamin D-deficient and -supplemented rats dosed with 500 μ g. of [1- 3 H]cholecalciferol per rat*

In Expt. 3 six vitamin D-deficient rats (av. wt. 106 g.) and in Expt. 4 three vitamin D-supplemented rats (av. wt. 114 g.) were given an oral dose of 500 μ g. of [1- 3 H]cholecalciferol. The supplemented rats were raised on the diet described by DeLuca *et al.* (1961) and the deficient on the diet described by Numerof *et al.* (1955). The homogenates of the intestinal mucosa from individual rats were pooled for the preparation of the intracellular fractions. The results are expressed as disintegrations/min. and disintegrations/min./mg. of N for the total radioactivity and specific activity measurements respectively.

Fraction	Total extracted radioactivity				Radioactivity in unsaponifiable matter			
	Deficient		Supplemented		Deficient		Supplemented	
	Total radio-activity	Specific activity	Total radio-activity	Specific activity	Total radio-activity	Specific activity	Total radio-activity	Specific activity
Mitochondrial	35470	13135	70320	14175	29600	10965	23700	4780
Microsomal	199660	18315	89615	5335	184975	16970	84750	5045
Cytoplasmic	188100	5300	122000	2635	138300	3895	101600	2190
Debris	1007175	8605	272800	2820	1007175	8605	272800	2820

Similarly in the vitamin D-supplemented rats the specific activities of the microsomal fraction were 5335 disintegrations/min./mg. of nitrogen for total radioactivity and 5045 disintegrations/min./mg. of nitrogen for the unsaponifiable material.

In the mitochondrial fraction of the deficient rats there was a slight fall in specific activity from 13135 disintegrations/min./mg. of nitrogen for total radioactivity to 10965 disintegrations/min./mg. of nitrogen for the unsaponifiable material. However, in the mitochondrial fraction of the vitamin D-supplemented rats there was a much greater fall in specific activities, from 14175 to 4780 disintegrations/min./mg. of nitrogen for the two equivalent fractions. In the cytoplasmic fraction there was only a slight fall in specific activities in both deficient and supplemented rats.

In Table 3 the proportion of the radioactivity in the three extracts of mitochondrial, microsomal and cytoplasmic fractions is given both in terms of the amount recovered from within an intracellular fraction and from the fractions combined. About 50–70% of the radioactivity in the mitochondrial fraction could be extracted by diethyl ether–ethanol, whereas in the microsomal fraction the largest portion (50–70%) of the radioactivity could only be extracted into diethyl ether after saponification of the particles. The ether-soluble extract of the microsomal fraction contained very little radioactivity. With the cytoplasmic fraction, however, 80–90% of the radioactivity was extractable by ether or ether–ethanol. Comparison of the four groups shows that on increasing the dose of the vitamin 200-fold the increase in radioactivity in the intestine is spread throughout the various fractions but is particularly marked in the mitochondrial

ether–ethanol extract and in the cytoplasmic ether extract obtained after saponification.

The nine extracts from the vitamin D-supplemented rats dosed with the 500 μ g. of the [1- 3 H]-cholecalciferol were fractionated by thin-layer chromatography. Cholecalciferol was present in all the extracts and accounted for the largest proportion of the radioactivity in all fractions with the exception of the ether extract (a) of the cytoplasmic fraction (Table 4). In the latter, about 60% of the radioactivity was due to polar material that remained at the origin. Though some radioactivity was observed at the origin of all the chromatograms the only other extract to contain appreciable amounts of this substance was the ether–ethanol extract (b) of the mitochondrial fraction from the vitamin D-supplemented rats. In this case the total radioactivity was distributed almost equally between the polar material and the cholecalciferol. It was also observed that on saponification of extracts containing the polar material most of the radioactivity at the origin of the chromatogram disappeared, which almost certainly accounts for the decreased content of radioactivity in the unsaponifiable matter of the mitochondrial fraction compared with the total lipid extract (compare Table 3). In contrast, in the ether–ethanol extract of the mitochondrial fraction from the deficient rats only 8% of the radioactivity was due to this polar material, 85% being due to cholecalciferol (Table 5).

Recently the long-chain fatty acid esters of calciferol have been reported in rat liver and kidney after an oral dose of [1- 3 H]cholecalciferol (Fraser & Kodicek, 1965). Occasionally radioactivity was detected in the calciferol ester region of a thin-layer chromatogram (Tables 4 and 5, chromatographic

Table 3. *Proportions of radioactivity in the extracts from intestinal intracellular fractions*

The intracellular fractions from vitamin D-deficient and -supplemented rats (see the legends to Tables 1 and 2) were extracted as described in the text. A, Percentage of recovered radioactivity in the individual fraction; B, percentage of total recovered radioactivity from the mitochondrial, microsomal and cytoplasmic fractions.

Fraction	[1- ³ H]Cholecalciferol (2.5 µg.)				[1- ³ H]Cholecalciferol (500 µg.)			
	Deficient		Supplemented		Deficient		Supplemented	
	A	B	A	B	A	B	A	B
Mitochondrial								
Ether-soluble	24.1	6.7	19.2	10.3	11.2	4.0	7.3	4.6
Ether-ethanol-soluble	49.3	14.0	65.0	34.7	60.9	21.7	73.3	46.8
Ether-soluble after saponification	26.6	7.6	15.8	8.4	27.9	10.0	19.4	12.4
Total %		28.3		53.4		35.7		63.8
Microsomal								
Ether-soluble	3.9	1.6	8.8	3.3	5.0	2.4	14.6	3.5
Ether-ethanol-soluble	45.6	19.2	33.8	12.5	40.6	20.3	17.7	4.3
Ether-soluble after saponification	50.5	21.5	57.4	21.1	54.4	27.1	67.7	16.4
Total %		42.3		36.9		49.8		24.2
Cytoplasmic								
Ether-soluble	46.8	13.8	28.8	2.9	9.9	1.5	37.2	4.5
Ether-ethanol-soluble	50.0	14.6	62.1	6.0	79.1	11.4	39.9	4.8
Ether-soluble after saponification	3.2	1.0	9.1	0.8	11.0	1.6	22.9	2.7
Total %		29.4		9.7		14.5		12.0

Table 4. *Thin-layer chromatography of the extracts of intestinal intracellular fractions from vitamin D-supplemented rats*

The intracellular fractions from the vitamin D-supplemented rats (see the legend to Table 2) were extracted, chromatographed and the fractions assayed for radioactivity as described in the text. Values are expressed as percentages of the total recovered radioactivity. The vitamin D standard appeared in chromatographic fractions 5, 6 and 7. a, Ether extract; b, ether-ethanol extract; c, ether-soluble extract after saponification; d, mitochondrial ether-ethanol extract was saponified and rechromatographed.

Chromatographic fraction	Mitochondrial fraction				Microsomal fraction			Cytoplasmic fraction		
	a	b	c	d	a	b	c	a	b	c
1	10	28.2	15.4	9.0	13.5	22.2	13.8	60	12.9	16.7
2	10	9.1	9.3	5.7	6.8	8.1	6.0	<1.0	9.7	2.2
3	10	9.1	4.0	4.6	4.0	3.0	1.7	<1.0	<1.0	4.9
4	<1.0	5.3	4.0	3.8	<1.0	<1.0	2.1	<1.0	<1.0	<1.0
5	<1.0	4.8	2.5	5.6	<1.0	11.1	4.3	<1.0	<1.0	11.9
6	43	31.4	49.5	54.8	64.9	44.4	62.5	40	48.4	47.6
7	17	3.7	9.3	4.6	<1.0	11.1	8.8	<1.0	<1.0	7.1
8	<1.0	2.1	3.0	2.9	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
9	<1.0	2.7	<1.0	4.9	<1.0	<1.0	<1.0	<1.0	16.1	<1.0
10	10	2.7	3.1	4.1	10.8	<1.0	<1.0	<1.0	12.9	9.5
11	<1.0	1.6	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
12	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0

fractions 9–11), but there was insufficient radioactivity to allow identification. This is in keeping with findings of D. R. Fraser & E. Kodicek (unpublished work), who found that only 1–2% of intestinal radioactivity was due to vitamin D esters.

At the completion of these studies Clark &

Porteous (1965) published a method for the preparation of epithelial-cell 'ghosts' and then used these cells for the preparation of intracellular fractions (Porteous & Clark, 1965). The advantage of this procedure is that it allows the division of the debris fraction of Schneider & Hogeboom (1950) into a number of fractions that have been partially

Table 5. *Thin-layer chromatography of the extracts of intestinal intracellular fractions from vitamin D-deficient rats*

The intracellular fractions from vitamin D-deficient rats (see the legend to Table 2) were extracted and the ether-ethanol extracts chromatographed as described in the text. Values are expressed as percentages of the total recovered radioactivity. Vitamin D appeared in chromatographic fractions 5, 6 and 7.

Chromatographic fraction	Mito-chondrial fraction	Microsomal fraction	Cytoplasmic fraction
1	5.8	4.9	3.2
2	2.4	1.5	1.3
3	1.8	1.6	1.2
4	1.6	1.1	1.0
5	11.3	1.3	4.0
6	70.4	84.6	82.4
7	3.3	2.3	4.1
8	<1.0	1.0	<1.0
9	1.2	<1.0	<1.0
10	1.2	<1.0	<1.0
11	<1.0	<1.0	1.7
12	<1.0	<1.0	<1.0

characterized. The distribution of radioactivity within these intracellular fractions after an oral dose of [1-³H]cholecalciferol to vitamin D-supplemented rats is shown in Table 6. The mitochondrial fraction VIII, 15 × 10⁴g-min., had a specific activity of the same order as that of the microsomal fraction, and similar to that found in unsaponifiable fractions of vitamin D-supplemented rats in Expt. 4. As expected from the large amount of radioactivity in the debris fraction in Expts. 1-4, fractions III-VII contained a high proportion of the radioactivity, but now by this new fractionation procedure a certain degree of localization was observed. Fraction IV, which consisted predominantly of brush borders with some contamination from nuclei, possessed the highest specific activity, followed by fraction VII, which, according to Porteous & Clark (1965), consisted of light mitochondria. Fraction VI, consisting of granules, had a high specific activity that is probably not of importance in understanding the function of vitamin D since its proportion of the total radioactivity was very low. Fraction III, which possessed by far the highest proportion of total radioactivity, consisted of whole 'ghosts' in addition to nuclei and brush border.

The uptake of the [1-³H]cholecalciferol *in vitro* by the intracellular fractions of the mucosa was also investigated (Table 7). As in the previous experiments the microsomal fraction contained the highest concentration followed by the mitochondrial fraction. However, in this case the debris contained

Table 6. *Expt. 5: specific activities of intestinal fractions from vitamin D-supplemented rats dosed with 400 µg. of [1-³H]cholecalciferol per rat*

Two vitamin D-supplemented rats, raised on the diet described by Numerof *et al.* (1955), were given an oral dose of 400 µg. of [1-³H]cholecalciferol. The two mucosae were pooled for the preparation of the epithelial-cell 'ghosts' and then the intracellular fractions. The results are expressed as disintegrations/min. and disintegrations/min./mg. of N for total radioactivity and specific activity respectively.

	Radioactivity in unsaponifiable fractions	
	Total radio-activity	Specific activity
Intestine less mucosa	252510	—
Residue from 'ghosts' prep.	86290	—
Supernatant from 'ghosts' prep.	22300	—
Epithelial-cell 'ghosts'	76500	6320
Fraction III (10 ³ g-min)	45945	5320
Fraction IV (4 × 10 ³ g-min.)	4685	11150
Fraction V (6 × 10 ³ g-min.)	415	2605
Fraction VI (10 × 10 ³ g-min.)	440	8760
Fraction VII (32 × 10 ³ g-min.)	660	9430
Fraction VIII (15 × 10 ⁴ g-min.)	1840	4845
Fraction IX (6 × 10 ⁶ g-min.)	2220	4190
Fraction X (final supernatant)	685	1290

the lowest proportion of the incorporated radioactivity. The results further show that the presence of metabolites in the intracellular fractions (see Tables 1 and 2) is not due to the known breakdown of the vitamin in aqueous solutions (Chen, Terepka, Kane & Marsh, 1965). When the microsomal, mitochondrial and cytoplasmic fractions were extracted with organic solvents the [1-³H]cholecalciferol was released into ether by treatment of the intracellular fraction with ethanol (Table 8). In the microsomal and cytoplasmic fractions this is in contrast with the experiments conducted *in vivo*.

To simulate more physiological conditions the vitamin was bound to albumin before adding to a suspension of the microsomal fraction in 0.28M-mannitol in 0.1M-tris-acid maleate-sodium hydroxide, pH 7.0. After incubation in air for 1 hr. at 4° about 25% of the radioactivity had become attached to the microsomal fraction. Again most of the radioactivity (73.7%) was recovered in the ether-ethanol extract. Further, the uptake of cholecalciferol by microsomal fractions on incubation at 37° for 30 min. in the mannitol-tris buffer in the presence of saturated urea, 10 µM-dinitrophenol, 1mM-sodium cyanide, µM-mercuric acetate or 0.1mM-EDTA was the same as in controls without inhibitors. Similar results were obtained with boiled or acetone-extracted microsomal fractions.

Table 7. *Specific activities of intestinal intracellular fractions from vitamin D-deficient rats treated with [1-³H]cholecalciferol in vitro*

[1-³H]Cholecalciferol (500000 disintegration/min.) in 0.1 ml. of ethanol was added to 3.55 g. of mucosa, from two vitamin D-deficient rats, suspended in 40 ml. of 0.25 M-sucrose at 0°. The tissue was homogenized and intracellular fractions were prepared by the method of Schneider & Hogeboom (1950). The four fractions were extracted and assayed for radioactivity. The results are expressed as disintegrations/min. and disintegrations/min./mg. of N for the total radioactivity and specific activity measurements respectively.

Fraction	Total extracted radioactivity		Radioactivity in unsaponifiable matter	
	Total radio-activity	Specific activity	Total radio-activity	Specific activity
Mitochondrial	46205	25815	42430	23705
Microsomal	113675	42260	107520	39970
Cytoplasmic	122710	7905	118975	7665
Debris	77635	1925	77635	1925

Table 8. *Proportions of radioactivity in the extracts from intestinal intracellular fractions from an experiment in vitro*

The intracellular fractions from vitamin D-deficient rats (see the legend to Table 7) were extracted as described in the text.

Extract	Distribution of radioactivity (%)		
	Mito-chondrial fraction	Micro-somal fraction	Cyto-plasmic fraction
Ether-soluble	5.6	3.0	22.1
Ether-ethanol-soluble	86.1	87.8	76.3
Ether-soluble after saponification	10.3	9.2	1.6

DISCUSSION

Until now it has been necessary in studies on the metabolism of labelled calciferol to administer a relatively large dose of the vitamin to the animal to detect it in the tissues. With the present preparation of [1-³H]cholecalciferol it was possible to investigate the nature of the radioactivity in the intestine after a near-physiological dose, i.e. 2.5 µg. (100 i.u.). The administration of the small dose gave results similar to those obtained with a dose 200 times as large. However, it would appear that the vitamin status of the animal is of importance. From the ratio of [1-³H]cholecalciferol in the mitochondrial and microsomal fractions in the vitamin

D-supplemented group it appears that the portion of the vitamin in excess of the animal's requirements is present in the mitochondria. Further, in this group there was a greater amount of the non-identified polar substances that were found in both the mitochondrial and cytoplasmic fractions.

There is also a noteworthy difference in the nature of the solubility of the vitamin and its metabolites in the cell fractions. In the mitochondrial fraction most of the radioactivity can be extracted with diethyl ether after the addition of ethanol up to a final concentration of 30%. The remainder of the radioactivity, although soluble in aq. 50% ethanol, is insoluble in ether until saponified.

In contrast, in the microsomal fraction most of the radioactivity is more tightly bound to the particulate fraction, the binding only being broken by saponification. It is possible that the vitamin is bound to a different constituent in the two particles, but whether it indicates a different mechanism for its function it is not yet possible to say. These differences might also be explained by the occurrence of vitamin D conjugates.

Attempts to elucidate the parameters controlling the uptake of the vitamin by the intracellular particles have been undertaken, but without success. There are few reports in the literature on the uptake of lipids by intracellular particles *in vitro* (Reshef & Shapiro, 1965; De Venuto & Chader, 1966). In this present study it was found that irrespective of whether the vitamin was in ethanol or bound to albumin 30–35% was taken up by the microsomal fraction when incubated at 37°. Reducing the temperature to 0° only lowered the amount taken up by about 5%, and also the uptake was complete in less than 2 min. Decreasing the concentration of the vitamin increased the proportion bound to the microsomal fraction as occurred in the studies with cortisol (De Venuto & Chader, 1966), suggesting that there are a limited number of sites available to bind the vitamin. The affinity of the intracellular particle for that portion of the vitamin extractable with ether-ethanol appears to be a purely physical phenomenon, since it is not affected by the presence of metabolic inhibitors, or by the use of boiled or acetone-extracted microsomal fractions. However, it has not been possible to simulate *in vitro* the physiological conditions exactly so as to bind the vitamin as strongly as it was bound in the experiments *in vivo*, where most of it had to be saponified to remove it from the particle. These experiments *in vitro* show that results on the intracellular distribution of cholecalciferol should be interpreted with caution before any deductions as to its function are made. It appears, however, that the vitamin in the debris fraction and a portion, at least, of the vitamin in the microsomal fraction is of physiological significance.

In this connexion the high specific activity of fraction IV (Table 6) is noteworthy in that it consists almost entirely of brush border and nuclei. Eisenstein & Passavoy (1964) reported that actinomycin D had an inhibitory effect on vitamin D, thus implicating the vitamin as acting on protein synthesis at the nuclear level. This idea was supported by the experiments of Norman & DeLuca (1964), who reported the presence of vitamin D in hog-kidney nuclei. If the radioactivity in fraction IV were predominantly localized in the nuclei, the specific activity of pure nuclei would be higher than that of any other subcellular particle and would support the nuclear theory of vitamin D action.

Since 84–92% of the radioactivity in the intestinal mitochondrial and microsomal fractions of deficient rats is due to [^3H]cholecalciferol (see Table 5), this strongly suggests that cholecalciferol is the active form of the vitamin. Murray, Day & Kodicek (1965) have shown by gas-liquid chromatography that a dose of cholecalciferol can be recovered unchanged, at least in liver and kidney. In addition, Schachter, Kowarski & Finkelstein (1964) have found that vitamin D_3 has a direct effect on the intestine and is not activated by some other tissue. The time-lag, which occurs between the effect of the vitamin on the intestinal absorption of calcium and the administration of the vitamin (Kodicek, 1965), would thus appear to be due to an effect on the cellular constituents or metabolic processes rather than a conversion of the vitamin into some 'active' form.

We are grateful to Dr R. K. Callow and Dr G. A. Thompson for the supply of [^3H]cholecalciferol 3,5-dinitrobenzoate and to Mr D. R. Fraser for the supply of albumin-bound [^3H]cholecalciferol. We thank Mr S. G. Impey for his skilful assistance with animals.

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