Vitamin D Balance and Distribution in Rats
Given a Massive Dose of Ergocalciferol

PART I. EXPERIMENTAL RESULTS

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Since no entirely satisfactory chemical or physical method is yet available for the quantitative estimation of vitamin D in animal tissues, resort must be had to laborious biological assays. Consequently there is little information about the amount and site of storage of the ingested vitamin. Nor is there much experimental evidence to indicate what proportion of an ingested dose can be recovered from tissues and excreta. Windorfer (1938) reported that in rachitic infants receiving 10–15 mg. of ergocalciferol, less than 10 % was excreted within 10 days after dosing, and in one instance in which an autopsy was performed less than 10 % was found in the tissues. Vollmer (1939) and Houet (1946) found that the tissues of children who had received a massive dose of ergocalciferol contained only a small proportion of the ingested vitamin. Morgan & Shimotori (1943) studied the distribution in the tissues of young dogs 3 days after a massive dose. The vitamin did not seem to be stored in any one organ. The greatest concentrations were found in heart, kidney and intestine, but all tissues contained small amounts, from 1 to 5 i.u./g.

The present paper reports experiments designed to ascertain, in rats given a large dose of ergocalciferol, (a) the distribution of the vitamin in tissues and excreta at various time intervals, and (b) the percentage of the dose recovered. A preliminary communication has already been presented (Cruickshank & Kodicek, 1952*a*).

EXPERIMENTAL

The studies consisted of one main and two subsidiary experiments. In the former, assays were carried out on freeze-dried tissues and faeces, in the latter on fresh. It was thus possible to assess the effect of freeze-drying on the vitamin D content.

Material

In all experiments the test substances were derived from piebald rats depleted of vitamin D, i.e. rats maintained from weaning on the rachitogenic diet 2965 of Steenbock for about 3 weeks, by which time florid rickets had developed. They were then given ergocalciferol *per os*, the same diet being continued. Material from deficient rats which did not receive the vitamin was also assayed.

In the main experiment (Exp. 1) the rats were divided into five groups, each comprising five rats. Each rat in four of the groups received a single dose of 1 mg. ergocalciferol (40000 i.u.) in arachis oil. The rats in the remaining group were given arachis oil only, and served as controls. The design of the experiment is shown in Table 1.

Crystalline ergocalciferol (Glaxo Laboratories) with an $E_{1\text{cm}}^{1\%}$ of 467 at 265 m μ ., was dissolved in 98 % ethanol. The appropriate amount of solution was transferred to a palette, the ethanol evaporated off at about 40° and the ergocalciferol taken up in arachis oil and given to the rats.

On each of the 4 succeeding days a group was killed and the pooled livers, intestines and the remainder of the carcasses from each group were taken for assay. The faeces from each group were collected daily. A pooled sample of urine was also collected from a group of rats and assayed as

Table 1. *Design of Exp. 1*

(*F* = faeces; *L* = liver; *C* = 'carcass'; *I* = intestines.)

Group	No. of rats	Sex	Dose of ergocalciferol (mg.)	Day of collection of faeces				Day of killing	Tissue samples
				1st	2nd	3rd	4th		
<i>a</i>	5	4 M. 1 F.	1	<i>F</i> _{a1}	—	—	—	1st	<i>C</i> _a , <i>L</i> _a , <i>I</i> _a
<i>b</i>	5	2 M. 3 F.	1	<i>F</i> _{b1}	<i>F</i> _{b2}	—	—	2nd	<i>C</i> _b , <i>L</i> _b , <i>I</i> _b
<i>c</i>	5	3 M. 2 F.	1	<i>F</i> _{c1}	<i>F</i> _{c2}	<i>F</i> _{c3}	—	3rd	<i>C</i> _c , <i>L</i> _c , <i>I</i> _c
<i>d</i>	5	3 M. 2 F.	1	<i>F</i> _{d1}	<i>F</i> _{d2}	<i>F</i> _{d3}	<i>F</i> _{d4}	4th	<i>C</i> _d , <i>L</i> _d , <i>I</i> _d
<i>e</i>	5	2 M. 3 F.	None	<i>F</i> _{e1}	<i>F</i> _{e2}	<i>F</i> _{e3}	<i>F</i> _{e4}	4th	<i>C</i> _e , <i>L</i> _e , <i>I</i> _e

such, but since no vitamin D was found 24 hr. after dosing no further assays were made.

In the two subsidiary experiments, groups of five rats were also used. In Exp. 2 a group of rachitic rats and a group which received 1 mg. ergocalciferol/rat were killed after 48 hr. and the livers only were assayed. In Exp. 3 two groups of rats were dosed and killed 24 hr. later and the livers from each group, and the faeces from one, were assayed.

Preparation of material

After most of the fur had been shaved off, the rats were skinned and the liver and intestines removed. The latter were split open and the contents removed by rinsing in warm saline. The term 'intestines', therefore, refers to the intestinal tissue only. The contents were discarded to obviate estimating them twice, namely as gut contents and later as faeces. The remainder of the carcasses, including the skin (referred to below as 'carcass') were minced, the individual tissues from each group were pooled and, in Exp. 1, were freeze-dried and kept at -20° until required for assay. In the other two experiments the fresh substances were tested immediately. In all experiments the assays were made on the tissues and faeces as such and not on the unsaponifiable matter.

Method of assay

Vitamin D was estimated biologically on rats by the method of Bourdillon, Bruce, Fischmann & Webster (1931) in which the degree of healing produced is assessed by means of X-ray pictures of the tibiae. In each assay three levels of the International Standard were used and in most instances three levels of the test preparation, six rats being assigned to each dose level. In all about 700 rats were used.

In Exp. 1 five series of assays were carried out between 7 July 1950 and 8 March 1951. The amount of the test preparations administered depended on their expected potency, which had been ascertained approximately in pilot experiments. The following range of dose levels, in mg., of dried material was used: 'carcass', 25-400; liver, 5-100; intestinal tissue, 2.5-200; faeces, 0.5-100; urine, 0.1 ml. The dried preparations from the undosed rats were administered at levels of 500-1000 mg. In assays on fresh

material (Exps. 2 and 3) the doses given were approximately equivalent, allowing for water content, to those used for the dried substances.

In assaying the test preparations which had been freeze-dried, appropriate amounts, finely ground, were given to the rats on palettes as a single dose on the first day of the curative period, as also was the standard. When the required dose was less than 40 mg. the samples were diluted with lactose before being weighed out. In every instance these were mixed with a few drops of arachis oil before being administered, as this made the materials more palatable and also prevented loss by scattering. Lactose and oil were also given to all rats which received undiluted doses and to the undosed controls. In Exps. 2 and 3, on fresh material, the liver was weighed out on rice paper and then given to the rats on palettes as a single dose.

RESULTS

The effect of the administration of the massive dose on the development of calcification in the rachitic metaphysis was observed by X-ray examination of the tibiae at daily intervals. No sign of healing was noted on the first day; on the second day a slight trace was visible and by the fourth, healing was appreciable.

Table 2. *Vitamin D assays of tissues and faeces of deficient rats*

Material	Wt. of material		Vitamin D	
	Fresh (g.)	Dried (g.)	(i.u./g. dry wt.)	Total (i.u.)
'Carcass', C_6	51.1	15.2	0.7	10
Liver, L_6	4.30	1.17	1.2	1
Intestines, I_6	4.94	0.77	1.5	1
Faeces, F_{61}	1.12	0.98	5.0	5
Liver, Exp. 2	3.07	—	0	0

The assays of tissues and faeces of deficient rats (Exps. 1 and 2) showed that virtually no vitamin was present (Table 2). It was therefore assumed

Table 3. *Average weight per rat of tissues and faeces of rats given 1 mg. ergocalciferol (40000 i.u.). Exp. 1*

	TISSUES							
	1st day (group a)		2nd day (group b)		3rd day (group c)		4th day (group d)	
	Fresh (g.)	Dried (g.)	Fresh (g.)	Dried (g.)	Fresh (g.)	Dried (g.)	Fresh (g.)	Dried (g.)
'Carcass'	51.2	15.6	47.2	14.1	55.0	16.8	54.8	16.2
Liver	3.74	1.03	4.16	1.15	4.42	1.22	4.40	1.19
Intestines	4.66	0.69	4.70	0.66	5.30	0.80	5.20	0.76

Group	FAECES							
	1st day		2nd day		3rd day		4th day	
	Fresh (g.)	Dried (g.)	Fresh (g.)	Dried (g.)	Fresh (g.)	Dried (g.)	Fresh (g.)	Dried (g.)
a	1.24	0.98	—	—	—	—	—	—
b	1.12	0.96	1.08	0.89	—	—	—	—
c	1.06	0.92	0.90	0.81	1.26	1.04	—	—
d	1.08	0.89	0.80	0.70	1.50	1.15	1.62	1.18
Av.	1.13	0.94	0.93	0.80	1.33	1.10	1.62	1.18

Table 4.* *Vitamin D content per g. dry weight and per whole tissue of rats given 1 mg. ergocalciferol (40 000 i.u.). Exp. 1*

Tissue	TISSUES											
	1st day (group a)			2nd day (group b)			3rd day (group c)			4th day (group d)		
	(i.u./g.)	Total (i.u./rat)	95 % fiducial limits (%)	(i.u./g.)	Total (i.u./rat)	95 % fiducial limits (%)	(i.u./g.)	Total (i.u./rat)	95 % fiducial limits (%)	(i.u./g.)	Total (i.u./rat)	95 % fiducial limits (%)
'Carcass'	44.5	690	67-150	44.9†	630	75-133	45.7†	770	71-141	31.1	500	65-154
Liver	1402†	1440	69-144	44.3	510	67-150	337	410	57-176	339	400	66-151
Intestines	366	250	69-145	142	90	68-147	101	80	54-185	54.1	40	65-154
Total	—	2380	—	—	1230	—	—	1260	—	—	940	—
Group	FAECES											
	1st day			2nd day			3rd day			4th day		
	(i.u./g.)	Total (i.u./rat)	95 % fiducial limits (%)	(i.u./g.)	Total (i.u./rat)	95 % fiducial limits (%)	(i.u./g.)	Total (i.u./rat)	95 % fiducial limits (%)	(i.u./g.)	Total (i.u./rat)	95 % fiducial limits (%)
a	6587	6460	67-150	—	—	—	—	—	—	—	—	—
b	3195	3070	62-161	2249	2000	67-149	—	—	—	—	—	—
c	3987	3670	60-167	1390	1130	57-175	241	250	56-180	—	—	—
d	6992	6220	66-151	1462	1020	66-151	126	150	55-181	21.8	30	56-179
Av.	5190	4855	—	1700	1383	—	184	200	—	21.8	30	—

* In all tables the potency per g., when less than 100 i.u., was calculated to the first decimal, otherwise to the nearest whole number. The total content in i.u. was rounded off to the nearest 10.

† Weighted geometric mean from replicate assays (see Table 7).

that the amount found in the material obtained from the rats given ergocalciferol was derived from the dose.

Experiment 1

The average weight, per rat, of tissues and faeces before and after freeze-drying is shown in Table 3. Not all the moisture was removed; about 5% remained. The average weight of the rats when killed was about 70 g. Table 4 shows the average vitamin D content per g. of dried tissues and faeces, and also the total content, per rat, of individual tissues and the total faecal excretion, per rat, per day.

The results for the first day after dosing show that, of the 40 000 i.u. administered, a total of only about 7000 i.u. could be accounted for. Only one-third of this amount, namely 2380 i.u. was found in the body. A large proportion was present in the liver, the amount being about twice as much as in the remaining 'carcass' and over five times as much as in the intestinal tissue. The faeces from the different groups varied considerably in their vitamin D content, from 3000 to 6000 i.u. The average vitamin content of the faeces was about twice that of the body. No vitamin D was found in urine.

On the second day, the total amount of the vitamin in the tissues and particularly in the faeces had decreased considerably. The fall in the body vitamin was accounted for mainly by a significant decrease of the vitamin in the liver, to about one-third of its value on the first day. The vitamin D in the intestinal tissue had also decreased significantly from 250 to 90 i.u. The vitamin content of the 'carcass' showed practically no change. Though, as on the first day, considerable variation between groups was observed, there was a significant fall in faecal excretion from about 4900 i.u. on the first, to about 1400 i.u. on the second day, a figure approaching the total present in the body.

On the third and fourth days the total vitamin in the tissues did not change significantly from the level observed on the second day. The only significant decrease observed was in the vitamin in the intestine. The faecal excretion of vitamin D was also greatly reduced, to 200 and 30 i.u. respectively.

Considering next the concentrations of the vitamin, i.e. i.u. of vitamin D/g. of fresh weight, it will be noted (Table 5) that on the first day the faeces contained, per g., about 100 times as much as the body, and on the second day about 70 times as much. On the fourth day the concentrations were practically equal, though the total content of the body was 940 i.u. as compared with 30 i.u. in the faeces, in consequence of the greater weight of the former.

The concentration in the individual tissues followed a pattern similar to that of the total content (compare Figs. 1 and 2). Throughout the period, the

Table 5. *Vitamin D concentration in body and faeces of rats given 40 000 i.u. of ergocalciferol*

	(i.u./g. fresh weight.)			
	1st day	2nd day	3rd day	4th day
Body	39.9	21.9	19.5	14.6
Faeces*	4293	1536	149	18.5

* The concentration in faeces has been obtained for each individual group and the figure shown is the arithmetic mean.

Fig. 1. Vitamin D concentration of fresh tissues of rats given 40 000 i.u. of ergocalciferol. ○—○, liver; ●—●, 'carcass'; ◐—◐, intestines.

Fig. 2. Vitamin D content of tissues of rats given 40 000 i.u. ergocalciferol. ○—○, liver; ●—●, 'carcass'; ◐—◐, intestines.

concentration of the vitamin was much higher in the liver than in the intestines or the 'carcass', particularly on the first day when the liver contained almost 400 i.u./g. fresh weight. The concentration dropped to 120 i.u. on the second day and remained practically at that level. The concentration in the 'carcass' varied very little; it ranged from 9 to 14 i.u./g. of fresh weight. In the intestinal tissue the concentration decreased steadily from 50 i.u. on the first, to 7 i.u. on the fourth day. On the first day it was significantly higher than that of the 'carcass'.

Experiments on fresh material

In the subsidiary Exps. 2 and 3, the assays were carried out on fresh substances. The relevant data and the vitamin D content of the samples tested are shown in Table 6. The total vitamin contents of the fresh livers of rats killed one day after dosing were 1300 and 1680 i.u. respectively. These values are not significantly different from the value of 1440 i.u. found in the main experiment (Table 4) on dried material. Likewise the vitamin D content of the liver on the second day was similar to that of the liver on the corresponding day in Exp. 1. The value obtained for the fresh faeces on the first day fell within the range found in the dried faeces in Exp. 1. The data, therefore, afford no evidence that freeze-drying had materially affected the vitamin D content of either tissues or faeces.

Reproducibility of assays

Replicate assays were made on one specimen of dried liver and on two specimens of 'carcass', the time interval between the original and the replicate

assay in each case being 4 months. Reference to Table 7 shows that there is no significant difference between the two estimates of potency on the same sample. There is, therefore, no evidence that loss of vitamin D occurred in the freeze-dried samples during storage at -20° for 4 months.

Recovery of dose administered

Table 8 was compiled from the results of all experiments, that is, on both fresh and dried preparations, and shows the percentage of the dose recovered in the tissues and faeces together with the 95 % fiducial limits. It will be seen that, of the 40000 i.u. administered, a total of 20.4 % was accounted for in the body and faeces on the first day, two-thirds being present in the faeces. On the 3 subsequent days the proportions found in the faeces decreased steadily to 0.1 % on the fourth day. For assessing the total recovery, the excretion on the second day has been included. The excretion on the last 2 days contributed little to the total recovery. A further possibility is that it may not have been derived directly from the dose, but indirectly from re-excretion into the gut through the bile.

DISCUSSION

The reasons for the low recovery of the dose, amounting to only 20–30 %, had to be considered. A loss could have occurred during the preparation of the calciferol for dosing, or through incomplete uptake of the dose by the rats. Losses might also have occurred on freeze-drying, and during storage of the materials. Since great care was taken in the preparation of the ergocalciferol solution and in

Table 6. *Vitamin D content of fresh liver and faeces of rats given 1 mg. ergocalciferol (40000 i.u.), Exps. 2 and 3*

Exp. no.	No. of rats	Material	Fresh wt. (g.)	Vitamin D content				95 % fiducial limits (%)
				1st day		2nd day		
				(i.u./g.)	Total (i.u.)	(i.u./g.)	Total (i.u.)	
2	5	Liver	3.20	—	—	141	450	75-134
3	5	Liver <i>a</i>	4.00	324	1300	—	—	71-141
		Liver <i>b</i>	4.10	411	1680	—	—	71-140
	5	Faeces	1.07	6135	6560	—	—	66-151

Table 7. *Comparison of replicate assays on dried material*

Date of assay	Material	Code no.	Vitamin D content (total i.u.)			95 % fiducial limits (%)
			1st day	2nd day	3rd day	
7. vii. 50	Liver	<i>L_a</i>	1900	—	—	56–179
8. xi. 50	Liver	<i>L_a</i>	1210	—	—	63–160
8. xi. 50	'Carcass'	<i>C_b</i>	—	620	—	67–149
7. iii. 51	'Carcass'	<i>C_b</i>	—	650	—	67–149
10. xi. 50	'Carcass'	<i>C_c</i>	—	—	710	53–190
7. iii. 51	'Carcass'	<i>C_c</i>	—	—	790	67–149

Table 8. *Percentage of dose (1 mg. ergocalciferol) recovered in tissues and faeces (Exps. 1-3)*

Tissue	1st day	2nd day	3rd day	4th day
	Recovery of dose			
	(%)	(%)	(%)	(%)
'Carcass'	1.7	1.6	1.9	1.3
Liver	3.7	1.2	1.0	1.0
Intestines	0.6	0.2	0.2	0.1
Total	6.1	3.0	3.1	2.4
95% fiducial limits	5.1-7.2	2.5-3.6	2.4-4.2	1.8-3.2
Faeces*	14.3	2.7	0.5	0.1
95% fiducial limits	10.6-19.3	2.1-3.4	0.33-0.75	0.05-0.18

* Estimated mean percentage recovery and fiducial limits from the regression line.

ensuring that it was completely consumed by the rats, it is unlikely that a loss of 70-80% could have occurred during the dosing procedure. Had a loss occurred at this stage, it is likely that erratic results would have been obtained in the different experiments, which was not the case. Losses during freeze-drying and storage could be ruled out as the result of experiments already referred to.

In addition to these control experiments, comparisons were made between the potency of fresh livers assayed as such and as the unsaponifiable matter obtained therefrom. Since concordant results were obtained, the simpler method of assaying the material was adopted.

From the present experiments it appears that the liver, both as regards content and concentration, is one of the main sites of absorbed vitamin, at least during the short period investigated and in the special conditions of our tests. This is in contrast with the results of Morgan & Shimotori (1943), who found that the liver of dogs is capable of only moderate storage, some of the other tissues having a higher concentration. On the other hand, Heymann (1937) states that in rabbits the liver can hold vitamin D more tenaciously than can the other organs.

In our experiments the high initial content of the liver was not maintained; a significant drop occurred on the second day. It is difficult to trace the loss of this 1000 i.u., but it does not appear to have been transferred to the other parts of the body. The possibility exists that it may have been changed into inactive products; alternatively, it may have been re-excreted into the gut via the bile. This, however, seems unlikely, since the bulk of the faecal vitamin D on the second day would then be derived from this re-excretion.

In view of the laborious nature of the assays, it was not possible in this investigation to examine the distribution of the vitamin in the different organs and tissues of which the 'carcass' is comprised. It should be noted, however, that on the first day, one-third of the absorbed vitamin was already present in

the 'carcass'. Little change in the content took place throughout the experimental period. The concentration was low, but this does not rule out the possibility that some organs, though small in size, may have had a rather high concentration of the vitamin. Later work (Cruickshank & Kodicek, 1952b) has indeed shown that, on the second day after dosing, kidney, adrenals, spleen and lungs contained concentrations of the vitamin similar to that of the liver.

The vitamin D content of the intestinal tissue reflected the amount passing through the alimentary tract, but it was low in proportion to the amount offered. On the first day the intestinal tissue contained about 6 µg. while 1000 µg. had been ingested.

The faeces contained a considerable amount of vitamin D, which decreased steadily throughout the experimental period. Nevertheless, the value on the fourth day showed that excretion had not ceased, since this amount was significantly higher than that found in the faeces of the undosed rats. The vitamin D occurring in the faeces on the last 2 days of the experiment might possibly have been derived from absorbed vitamin D which had been re-excreted into the gut, e.g. in the bile. This view is supported by the experiments of Heymann (1937-8) who stated that ingested vitamin D may appear later in the bile. On the other hand, Taylor, Weld & Sykes (1935) and Morgan & Shimotori (1943) found no evidence of re-excretion in dogs following administration of a massive dose. To ascertain the rate of movement of the ingesta, Sudan III was administered to a group of rachitic rats on the Steenbock diet. It was observed that the dye was still present in the faeces after 2 days. Possibly, therefore, traces of the vitamin derived from the dose were present in the excreta on the third day, though the passage of Sudan III may not necessarily correspond with that of the vitamin.

As previously mentioned, no vitamin D was detected in urine by biological assay. The possibility existed that it had been excreted in a conjugated

form. However, since the ether extract of urine, hydrolysed according to the method of Bachman & Pettit (1941) for conjugates of steroid hormones, showed no biological activity, it is considered unlikely that any appreciable amount of the vitamin had been excreted in this form.

From the data obtained, it appears that the total recovery in body and excreta amounted to only 23 %, or taking the extreme fiducial limits, to 30 %, so that about 70–80 % of the dose could not be accounted for. The fate of this large deficit is at present unknown, but it was presumably converted into inactive products not detectable by biological assay. This destruction or conversion might occur in the alimentary tract, in some body tissue, or in the faeces after excretion. It has been reported that cholesterol can be destroyed by certain micro-organisms (Turfitt, 1944; Wainfan, Henkin, Rice & Marx, 1952; Stadtman & Cherkes, 1952), but it is not known whether vitamin D can be so attacked. If it should be so, the destruction might occur in the caecum or in some other part of the digestive tract or, alternatively, in the faeces, which, in our experiments, after being voided, were exposed to a temperature of 21° in the experimental rat room until collected at 24 hr. intervals.

The results obtained in the particular conditions of the experiment are of special interest in view of the use of massive doses of vitamin D for the prevention and cure of rickets in infants.

SUMMARY

1. A massive dose of ergocalciferol (40 000 i.u.) was given *per os* to groups of weanling rachitic rats and on each of the 4 succeeding days the pooled

livers, intestines (minus contents), the remainder of the carcasses, the faeces and the urine were assayed biologically for vitamin D. Forty rats were used in the preparation of the material to be tested, and over 600 for the assays.

2. The assays were performed on freeze-dried, and in some instances, on fresh material. There was no evidence that loss of the vitamin occurred on freeze-drying or storage.

3. Little or no vitamin D was detected in rachitic undosed animals. The estimates of the vitamin D content of the body of rats receiving calciferol were 2380, 1230, 1260 and 940 i.u. on the 4 consecutive days, respectively. The decrease on the second day after dosing was mainly due to a fall in the vitamin in the liver, from a value of 1440 to 510 i.u., a level subsequently maintained. The intestinal tissue showed a steady decrease from 250 on the first, to 40 i.u. on the fourth day. The remainder of the carcass maintained a level of about 600 i.u. throughout the experimental period.

4. It appeared that the liver was the main site of deposition. No significant transfer of the vitamin from liver to other tissues of the body was observed within the period studied.

5. The estimated faecal excretion of vitamin D which was 4860 i.u. on the first day decreased to 1380, 200 and 30 i.u. on the subsequent days. No vitamin D was found in urine, assayed as such or as the ether extract of urine hydrolysed with concentrated hydrochloric acid.

6. About 23 % of the dose was accounted for. The fate of the remainder of the ingested vitamin is not known. It is assumed that it is converted into products having no antirachitic activity.

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