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THE EFFECT OF VITAMIN D AND VITAMIN D DEFICIENCY ON NON-PREGNANT RATS

1. The materials and the method of experiment

Wistar-strain female rats were chosen as the experimental animal, and they were divided into "the mature group" and "the immature group." The criteria for choosing the mature group were: they weighed about 120g when born and had normal sexual cycles. The immature group's criteria were, 40 to 50g at birth and had vaginas that were not yet opened. In the deficiency experiment, the mature rats were divided into 30, 60, 90, 150, 200-day units with control groups for each of them. The immature rats were divided into units of 30, 60, 90, 120 days each with a control group. Some units for a recovery experiment were added to the latter five groups. In the experiment with those receiving Vitamin D_2; the rats whether they were to be sterilized or not, were divided into 4 groups--Placebo, 10, 100, 1000 I.U.--and were injected daily for a period of 20 days. Each group was composed of 5 to 8 rats.

For food, the composite feed listed in Table-1 was used. To the feed of the control groups in the Vitamin D deficiency experiment, 30 I.U. of Vitamin D_2 was added per 100g of feed. All the feeds used in the experiment of administration of Vitamin D contained no Vitamin D. D-solvent was used in the doses for oral administration. An injection was composed of D-solution[1], Tween 80, and physiological saline; and was arranged in such a way that the necessary amounts of components could exist in 0.1ml of the injection. A mixture of Tween 80 and physiological saline was used for Placebo. Feed and water

[1]Esai, K. K. product, Chocola D

were provided as much as the rats wanted. The rat's room was prepared in such a way that the temperature was from 20°C to 25°C, and the light was on from 6 a.m. to 6 p.m. The windows of the room had double glass; the outer sheet was 9mm thick and opaque and had a wire net in it, the inner was common window glass. A blue curtain shut out direct sunlight.

TABLE 1

Vitamin D Free Feed

granule sugar	66.9
vitaminless casein	20.0
DL methionine	0.5
OLEIC ACID	3.0
LINOLEIC ACID	0.15
VITAMIN ADDED CELLULOSE	5.0
MIXTED SALT	4.0

ADDED VITAMIN (mg%)		Mameesh No. 446	mixed salt 混合塩
A	0.8	NaCl	243.2
E	1.2	$K_3C_6H_5O_7 \cdot H_2O$	533
K_1	1.0	K_2HPO_4	174
B_1	1.0	$CaHPO_4 \cdot 2H_2O$	800
B_2	1.0	$CaCO_3$	368
Ca-PaA	5.0	$MgCO_3$	92
PIN-HCl	0.5	$FeC_6H_5O_7 \cdot 5H_2O$	36
NiA	2.0	$CuSO_4 \cdot 5H_2O$	0.4
FA	0.1	$MnSO_4$	2.8
B_2	0.1	$K_2Al_2(SO_4)_4 \cdot 2H_2O$	0.2
VIOTINE	0.1	KI	0.1
Base coline	100.0	$CoCl_2 \cdot 6H_2O$	0.2
		$ZnCO_3$	0.1
		NaF	0.002

2. The ordinary state and sexual cycle of rats

1. In the case of Vitamin D-deficiency

According to the length of the D-deficiency period, the fur of the rats became rough, lost its luster. Some of the rats got boils on their tails, and some became inactive. In the mature group, there was not as big a difference in the weight increase curve as compared with the control group.

But in the immature group, the rate of weight increase is less than the control group, and after 40 days all weight increase stopped. Seven rats which strongly suffered from sexual cycle disorder were chosen for the recovery experiment. These rats were orally given 50 I.U. of Vitamin D every day after the 67th day of Vitamin D deficiency. As a result, all of them recovered their weight loss and ordinal sexual cycle. (Fig.1, Tab.2)

TABLE 2

The growing curve of white rats under Vitamin D deficiency

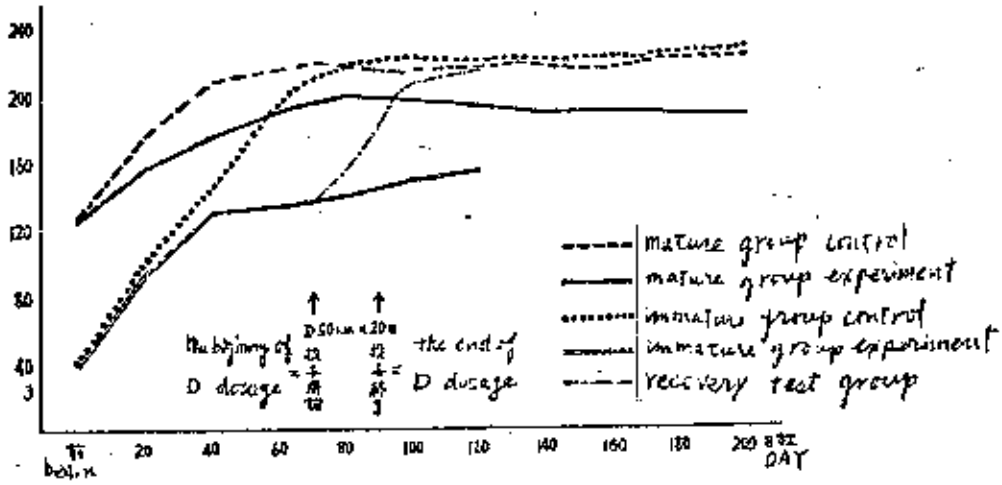


Table 2.

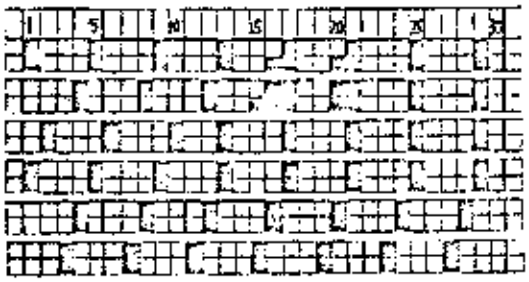
EXPERIMENT GROUPS		#	MEAN (g)	THE STANDARD DEVIATION (±σ)	RELIABILITY (95%)	TEST	STATISTICAL SIGNIFICANCY
A	control group	5	47.6	26.8	37.2	A & B	$P<0.05$
B.	recovery test g.	7	51.1	21.9	21.9	A & C	$0.3<P<0.4$
C	DEFICIENCY G.	12	31.5	13.6	9.1	B & C	$P<0.01$

Sexual cycles were observed at about 10 a.m. everyday using a vagina-smear. In the mature group, symptoms of estrus were found frequently around the 50th day from the beginning of the deficiency. Near the 90th day, some rats showed symptoms of estrus constantly, but after this time, they gradually began to show insufficient keratinizations.

After around 150th day of deficiency, they entered the period of Dioestrus. (Fig. 2, 3). In the immature group, the rats' vaginas opened at about the same time. Though all the rats of the control group had favorable sexual cycles about 25 days after the beginning of the experiment, more than half of the rats of the deficiency group suffered disorder. After the 40th day, the deficiency groups' rats showed symptoms of Dioestrum extension and insufficient keratinization, and from around the 60th day, these rats entered a period of continuous Dioestrum. (Fig. 4). But all of them were restored to normal sexual cycles after going through the recovery experiment. (Fig. 5). Among the deficiency groups, some rats didn't suffer from any kind of abnormality, therefore, there were individual differences.

Figure 2

The Effect of D-Deficiency on the Sexual Cycle of the Mature Rats (1)



30日

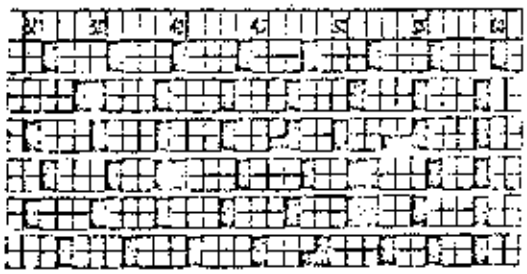


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Figure 3

The Effect of D-Deficiency on the Sexual Cycle of the Mature Rats (2)

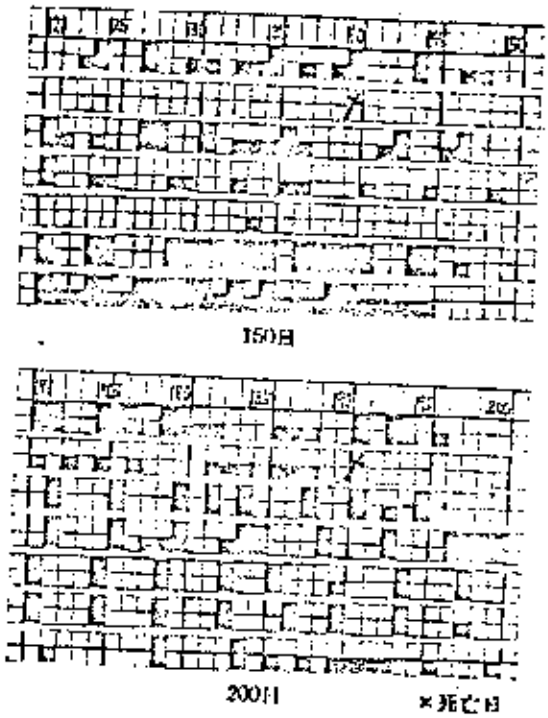


Figure 4

The Effect of D-Deficiency on the Sexual Cycle of the Mature Rats (3)

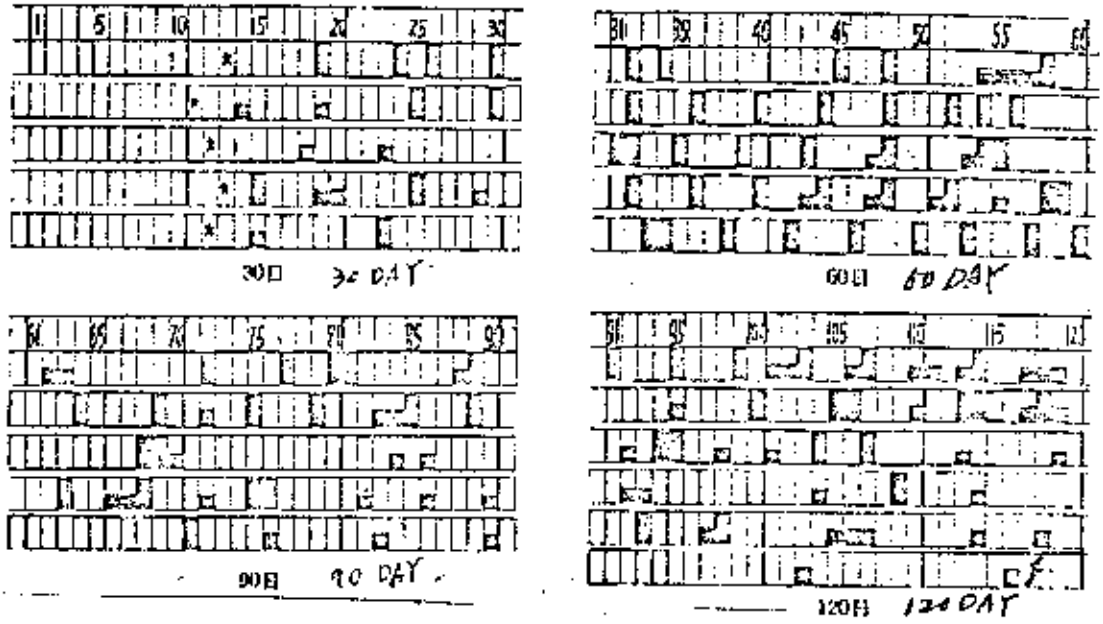
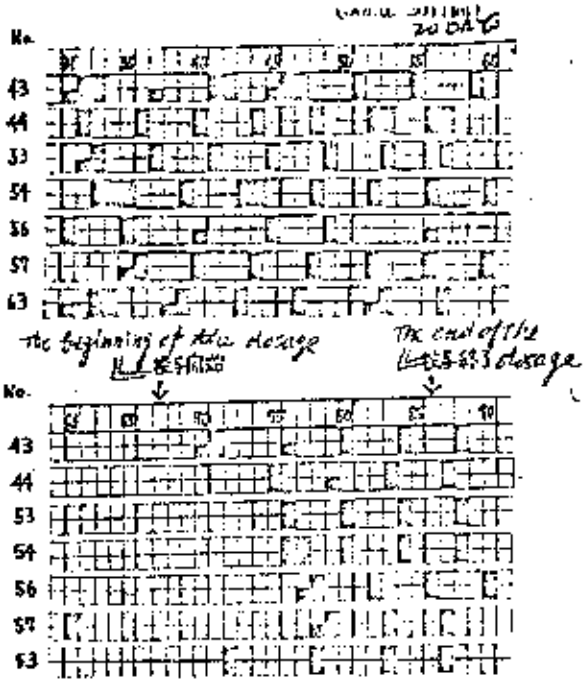


Figure 5

The Recovery Test of the D-Deficient Rats



2. In the case of Vitamin D administration

The ratios of rats' weight increase in each group were rather similar. Sexual cycles were quite normal except for one case. In this case, the insufficient keratinization was observed with 1000 I.U. Vitamin D administration in the non-sterile mature group. (Fig. 6). In the immature group, the rats' vagina opened at almost the same time, and their sexual cycles were similar to each other. (Fig. 7). All the sterile groups showed symptoms of continuous Dioestrus. So these rats were injected with 0.01 μg Estradiol[2] and Vitamin D_2-10,100, or 1000 I.U.-; and the respective control groups were also injected with Estradiol-0.05,0.02, or 0.01 μg and the mixed liquid

[2]OVAHOLMONE (TEIKOKU ZOKI Co. Ltd)

Figure 6

The Sexual Cycle of the White Rats
in Non-Sterilized Mature Group Under Vitamin D Dosage

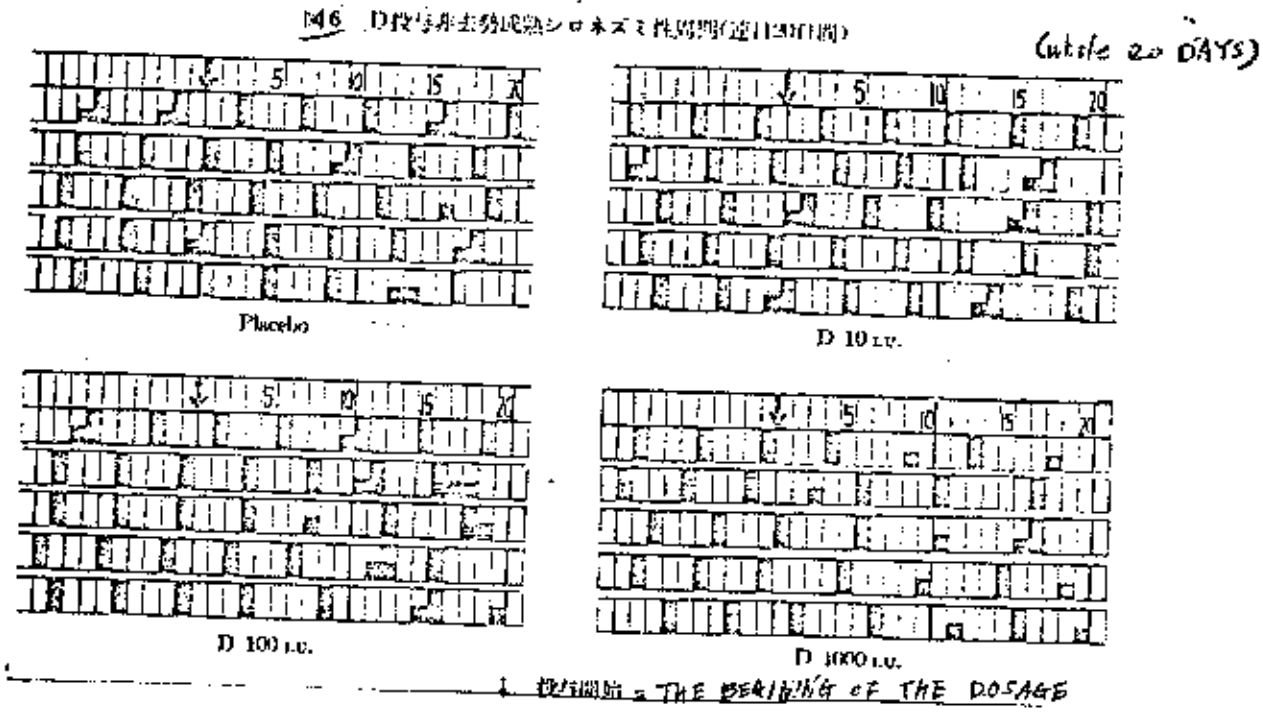
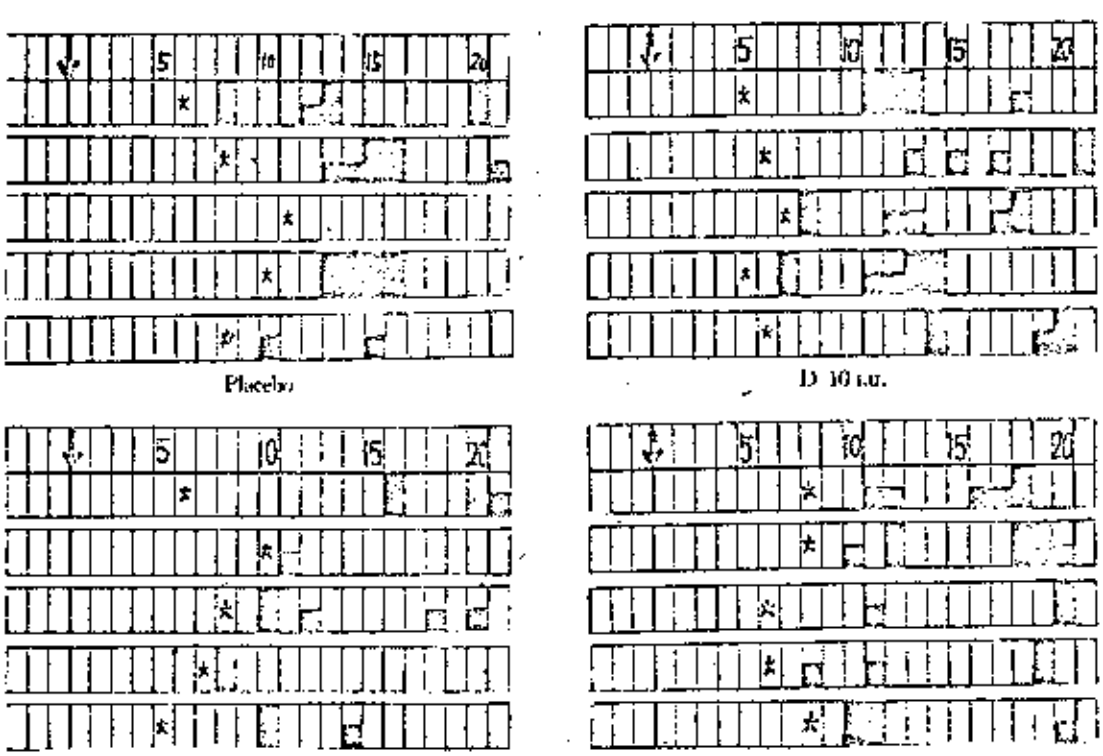


Figure 7

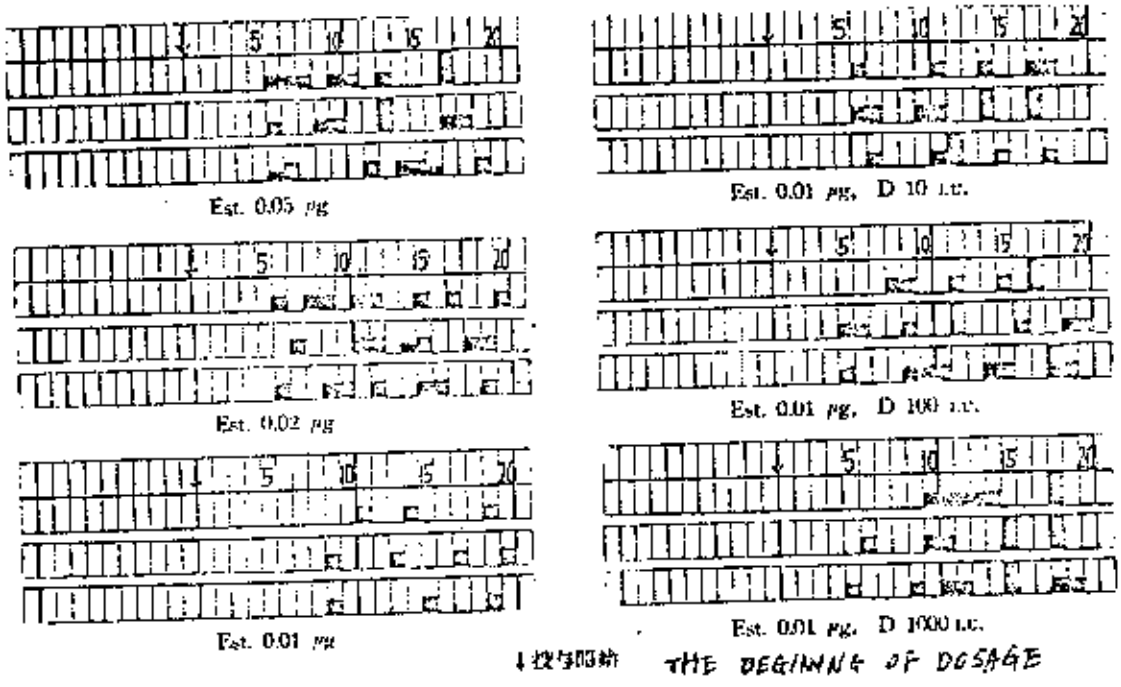
The Sexual Cycle of the White Rats in Non-Sterilized
Immature Group Under Vitamin D Dosage



of Tween 80 and physiological saline. The result is shown (Fig. 8). According to this, sufficient keratinization was hardly seen in the control group, but in the case where Vitamin D was added into the injection, the rate of sufficient keratinization had grown as the amount of the dose increased.

Figure 8

The Effect of Vitamin D and a Little Amount of Estrogen on the Sterilized Rats



3. A short summary

Up to now, Steenbock-Black No. 2965 has been used as the feed for Vitamin D experiments, but nowadays, from the dietetic point of view, this feed is said to be unsuitable for the Vitamin D deficiency experiment. Therefore, a composite feed in which various vitamins have been added was chosen for this experiment. 14). Because a dark room affects the sexual cycle of the rats, it was not used. 15). And since all effective wave lengths of ultraviolet light which activate pro-D are absorbed by common window glass, it was used.

Vitamin D stimulates the TCA cycle promptly 16) and causes the promotion of appetite 17) and the secretion of gastric juice 18); these are considered the main reasons for "the growth promoting effect of Vitamin D." The ratio of Calcium/Phosphorus in the feed, affects the growth--if this ratio is small, then growth is promoted, and vice versa 19) Mr. Miyasaki 20) reported that in the case of a large ratio of Ca/P, the growth rate of rats in the Vitamin D-deficiency group exceeded the other groups' growth rate, though they suffered from rickets. But if this ratio was near 1, rickets was not found in any rats. In my experiment, the Ca/P ratio of the feed was 1.5/1.0, and none of the rats in the deficiency group suffered from rickets. In the mature group, the deficiency group suffered from a slow growth rate, compared with the control group but these rats recovered quickly when administered Vitamin D and also resumed their normal sexual cycles. Therefore, it is possible to say that the slow growth rate was caused by Vitamin D-deficiency.

The Vitamin D-deficiency effect on sexual cycle was weak, and was found after the 50th day. In the mature group, the course of this effect was recognized as follows: first, the frequent occurrence of estrus symptoms; second, the continuous symptoms of estrus; the third, insufficient keratinization and the dioestrum extension. But in the immature group, this process was different. Most of them seldom showed the symptoms of estrus, and afterwards, all of them entered a period of continuous dioestrus. These effects were caused by Vitamin D deficiency as ascertained by the recovery experiment. Though there were still some differences between the individuals, these were considered to have occured because of the difference in the inherent accumulated amount of Vitamin D in their bodies or by sexual immaturity. Some species of rats (including Wistar strain rats) naturally show their

sexual desire continuously even late in their life 21). Continuous lighting and an administration of sexual hormone just after their birth, raised their sexual desire. Mr. Takewaki explained these phenomenon as the underdevelopment of the sexual center or senility. In my experiment, there were no abnormal sexual cycles in the control group; it was estimated that Vitamin D-deficiency causes the irregularity of the ovaries or the central sexual function, and disturbed their sexual cycle.

The necessary amount of Vitamin D for white rats in a day is said to be about 1 I.U. (D_2 0.025 μg), and when the administration of Vitamin D exceeds more than 20 to 1000 times the necessary amount, it causes toxic symptoms. In my experiment, though, the rats were given 10 to 1000 I.U. for 20 days; there was no effect on their sexual cycles in any of the groups, sterilized or non-sterilized. But in another experiment, a rat was injected with a little amount of Estrogen--the amount itself has no effect at all--along with Vitamin D, and sufficient karatinization was observed. Therefore, Dr. Oniwa's report, that Vitamin D reinforces the estrogen effect is in aggreement.

THE EFFECT OF VITAMIN D ON THE PITUITARY GLAND, THE ADRENALS, AND THE OVARIES

1. The material and the method of the experiment

The same kind of Wistar strain female rats which were observed for their ordinary states and sexual cycles in the above-mentioned experiments were used. The rats of each group were beheaded, and immediately, the pituitary glands, the adrenals, the ovaries, and the uteri were extracted and weighed on a Torsion balance, and were fixed by a fixation liquid for the purpose of histological research. The weight of the viscera was expressed by a percentage as compared with the total weight. In making up each histological specimen, the pituitary gland was fixed by chromium-alum, and then, some pieces of the central-forehead part of this gland were cut off and were made into paraffin-specimens. These specimens were dyed by the "Elfmann's[3] 30) 31)--Schiff dyeing" and the "Trichrome dyeing" methods. The number of cells in the mature grouping was counted using Dr. Inoue's" 32) Three-vision method." The adrenals, the ovaries, and the uteri were fixed by a formalin liquid, and the adrenals, were dyed by H.E. dyeing, Vines-dyeing and Sudan IV dyeing; the ovaries and the uteri were dyed by H.E. dyeing. The classifications of the follicles and the lutein body were based on Dr. Koga's method.

2. In the case of Vitamin D deficiency

1. The change of Viscera weights

In the mature group, the pituitary gland began to enlarge significantly in the 60th day of deficiency, and the ovaries became atrophied significantly after the 90th day; the uteri started to shrink in the beginning of the deficiency experiment, but no change was found in the adrenals. (Table 3).

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[3]AF = Aldehyde Fuchsin.

Table 3

The Weight of Organs Per Rats-Weight
of D-Deficiency Exp. (Mature G.)

器官 1	实验日数 2	例数 3	平均重量 (mg%) 4	标准偏差 (±σ) 5	信赖度 (95%) 6	检定 7
8 下垂体	对照 CONTROL 0	8	5.0	0.25	0.37	—
	30	6	5.1	0.25	0.34	0.8< P <0.9
	60	6	4.9	0.23	0.36	0.6< P <0.7
	90	5	6.6	0.13	0.21	P <0.01
	150	6	5.5	0.11	0.23	0.3< P <0.4
	200	6	4.9	0.11	0.18	0.6< P <0.7
9 副肾	对照 CONTROL 0	8	21	2.1	2.7	—
	30	6	24	2.8	3.6	0.3< P <0.4
	60	6	21	2.0	2.7	0.6< P <0.7
	90	5	23	2.7	3.2	0.6< P <0.7
	150	6	21	1.5	1.8	—
	200	6	20	0.7	1.2	—
10 卵巢	对照 CONTROL 0	8	29	1.4	2.2	—
	30	6	30	1.0	2.0	0.6< P <0.7
	60	6	35	1.3	1.7	0.05< P <0.1
	90	5	18	2.8	3.1	P <0.001
	150	6	16	1.7	2.1	P <0.001
	200	6	13	2.3	2.6	P <0.001
11 子宫	对照 CONTROL 0	8	330	24	2.9	—
	30	6	195	30	3.7	P <0.001
	60	6	220	13	1.6	P <0.01
	90	5	262	20	2.2	P <0.05
	150	6	242	22	3.0	P <0.05
	200	6	172	24	3.3	P <0.01

1-Organs
2-Day Units
3-Instances
4-Average Weight
5-Standard Deviation
6-Reliability
7-Test
8-Pituitary Gland
9-Adrenals
10-Ovary
11-Uterus

In the immature group, the ovaries and the uteri had begun to shrivel distinctly at the same time as the mature group, but the pituitary gland and the adrenals did not show any distinct changes. (Table 4).

Table 4

The Weight of Organs Per Rats-Weight of D-Deficiency Exp. (Immature Group)

器官 1	实验群欠乏日数 2	例数 3	平均重量 (吨%) 4	标准偏差 (±σ) 5	信赖限界 (95%) 6	检定 7
TARY 8	对照 control G.	5	4.9	0.15	0.28	—
	30	5	5.2	0.12	0.23	0.7<P<0.8
下垂体	60	5	5.7	0.27	0.47	0.5<P<0.6
AND.	90	5	4.1	0.21	0.36	0.3<P<0.4
	120	5	4.1	0.20	0.26	0.3<P<0.4
ENALS 9	对照 control G.	5	21	1.4	1.9	—
	30	5	23	1.6	2.0	0.6<P<0.7
副腎	60	5	28	1.7	2.8	0.4<P<0.5
	90	5	20	1.4	1.9	0.6<P<0.7
	120	5	18	1.6	2.0	0.5<P<0.6
RIES 10	对照 control G.	5	29	2.4	3.4	—
	30	5	28	2.0	2.9	0.1<P<0.2
卵巢	60	5	26	2.3	3.1	0.1<P<0.2
	90	5	18	2.6	3.4	P<0.001
	120	5	14	2.8	4.1	P<0.001
ERUS 11	对照 control G.	5	320	15	25	—
	30	5	221	18	32	P<0.02
子宫	60	5	143	11	20	P<0.001
	90	5	148	18	23	P<0.001
	120	5	135	20	31	P<0.001

1-Organs
2-Day Units
3-Instances
4-Average Weight
5-Standard Deviation
6-Reliability
7-Test
8-Pituitary Gland
9-Adrenals
10-Ovaries
11-Uterus

2. The histological observation

Anterior pituitary gland

In the control group, the acidphilic cells (alpha-cells) were favorable to Acid Fuchsin or Organ G. These cells were found especially in the central and back part of the anterior pituitary, and accounted for an average 32.7%. Melanophobic cells (gamma-cells) were 62.3% of the total and this amount was the highest percentage. The basophilic cells were the smallest percentage, 3.4%, and these were divided into pink Gonadofroph (delta-cells) and dark violet

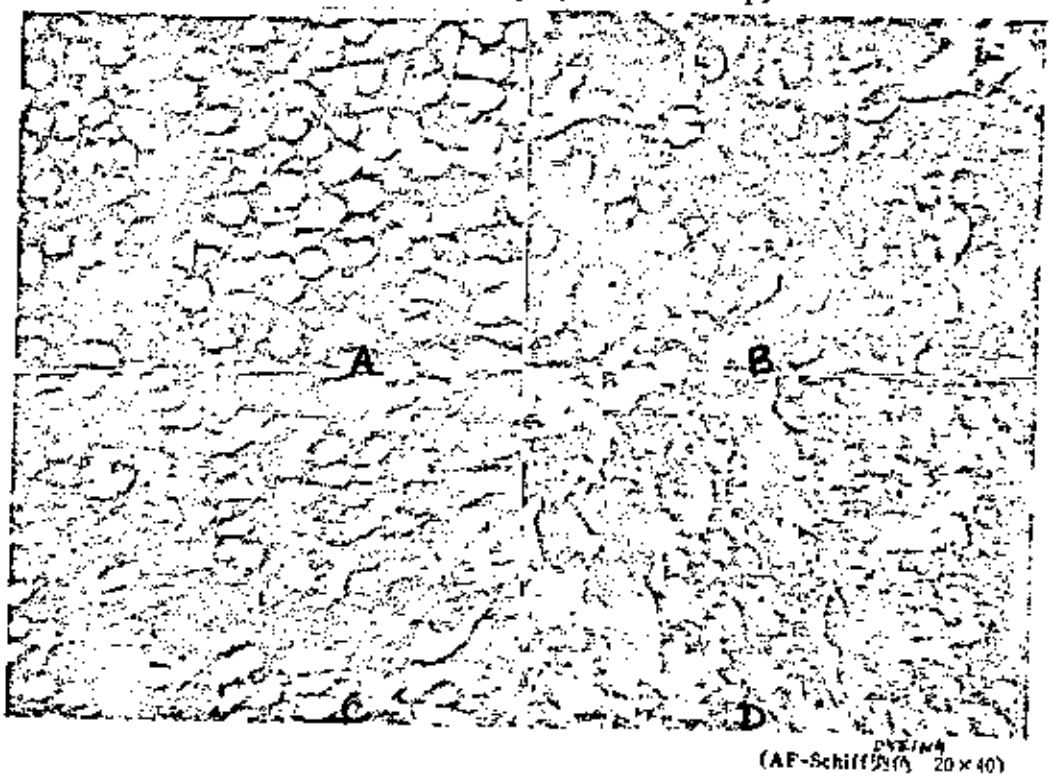
Thyrotroph (β-cells), using the AF-Schiff dyeing 34) 35). Most of the basophilic cells were β-cells (80.2%), and these were mainly scattered in the central part of the anterior pituitary. There were few delta-cells, particularly in the mature female rats (19.8%). The change in the number of these cells because of the sexual cycle was not so remarkable: on the average, α-cells 3.7%, γ-cells 3.2%, and β-, δ-cells 0.8%.

In the mature Vitamin D-deficiency groups, the number of α-cells had become small after the 60th day of the experiment, and the follicles were also found to become atrophied. Also, around this day, the secretory granules from the α-cells were found to decrease in the mature group, but in the immature group, the secretory granules decreased from the beginning of the experiment.

The basophilic cells in the mature group remarkably increased 9.0% in number by the 30th day of deficiency, and until the 60th day, the cells increased slowly, but after that, they decreased. Both β-and δ-cells increased by the 30th day: the β-cells had a 45.2% increase, and the δ-cells had a 54.8% and showed more remarkable growth than the β-cells. At the 60th day the β-cells recovered their normality. But after that, though they gradually decreased in size and number, their secretory granules didn't decrease but became more concentrated. The δ-cells increased slightly by the 150th day, but the follicles shriveled, and the secretory granules decreased. In the immature group, the β-cells had decreased from the beginning of the experiment; δ-cells also decreased when the deficiency continued a rather long period. (Fig. 9, 10, Table 5, 6).

Figure 9

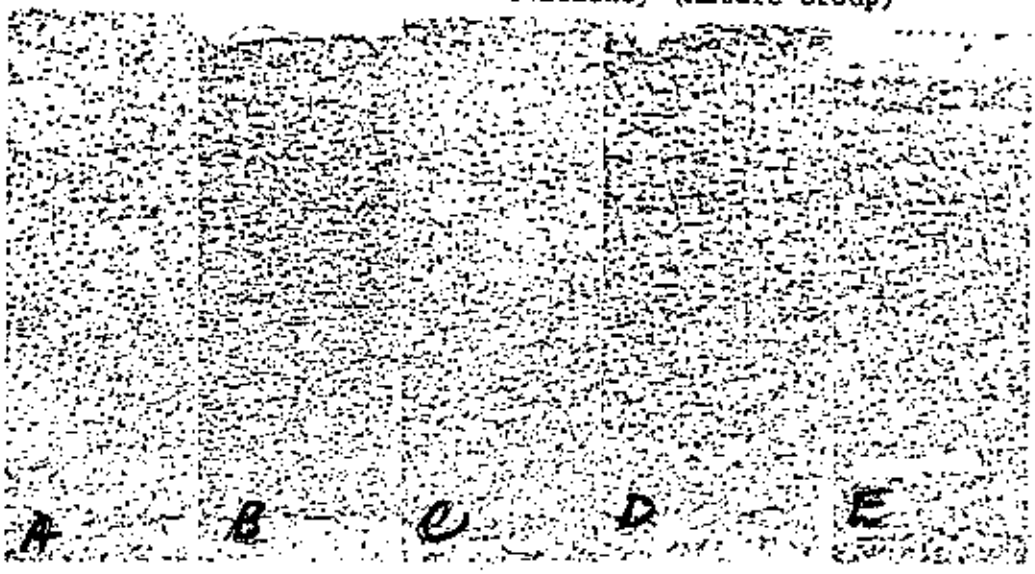
The Change of the Anterier Pituitary Cells
Under D-Deficiency (Mature Group)



A-Control Group
B-30 Day Deficiency β-δ-Cell Increases
C-90 Day Deficiency. No change in β-cell, decline in δ-cell
D-200 Day Deficiency βδ-cell decreases

Figure 10

The Adrenal Cortex Under D-Deficiency (Mature Group)



(H-E DYEING)

A-Control Group
B-30 Day Deficiency
C-60 Day Deficiency
D-150 Day Deficiency
E-200 Day Deficiency

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Table 5

The Change of the Anterior Pituitary Cells Under D-Deficiency

実験群 (欠乏日数)	好酸性細胞(%)	好塩基性細胞(%)	色素嫌性細胞(%)	計 total
対照	32.8(1999)	4.9(298)	62.3(3803)	(6100)
30	30.4(1284)	9.0(380)	60.6(2562)	(4226)
60	26.3(1314)	5.3(266)	68.4(3425)	(5005)
90	23.1(1487)	3.2(205)	73.7(4752)	(6444)
150	22.9(1630)	4.3(304)	72.8(5185)	(7119)
200	25.4(1099)	3.2(138)	71.4(3091)	(4328)

( )内は実測細胞数
(#) is survey number

1-Day Units of Deficiency
2-Acidphilic Cell
3-Basophilic Cell
4-Melanophodic Cell
5-Total
6-Control G.

Table 6

The Change of the Basophilic Cell of the Anterior Pituitary Under D-Deficiency

表6 D欠乏と下垂体前葉好塩基性細胞の変動

実験群 (欠乏日数)	β-細胞(%)	δ-細胞(%)	計 total
対照	80.2(239)	19.8(59)	(298)
30	45.2(225)	54.8(155)	(380)
60	66.9(178)	33.1(88)	(266)
90	68.2(140)	31.8(65)	(205)
150	64.8(197)	35.2(107)	(304)
200	74.6(103)	25.4(35)	(138)

( )内は実測細胞数
(#) is survey number

1-Day Units of Deficiency
2-β-Cell (%)
3-δ-Cell (%)
4-Total
5-Control Group

The Adrenal Cortex

The Observation by H.E. dyeing

As for the zona glomevularis, the shortening of the zona laminalis, the declining of the cytoplasm solidity, and the concentration of the nucleus were observed. As for the zona fascicularis, the shortening of the zona laminalis, the disorder of cellular fascicle, the declining of the cytoplasm solidity, the concentration of the nucleus, and nucleolysis were observed in the mature group after the 90th day. On the other hand, in the immature group, the acidphilicity of the cell of the zona fascicularis was slightly raised around the 30th day of deficiency, but after the 60th day, the same kind of retrogressions which were observed in the mature group were also noticed in this group. As for the zona reticularis, the follicle had swollen slightly and the acidphilicity of the cytoplasm was raised by the 60th day, and after the 90th day, the zona reticularis had become atrophied, and after that, a high degree of atrophy was observed in the mature group. In the immature group, this atrophy was earlier than the mature group; it began at the 60th day. As for the paramedular cortex, the atrophy was found in an earlier period of the experiment, and after the 90th day, this tissue almost disappeared. (Fig. 10).

The Observation by Vines-dyeing

In each zona laminalis of both groups, the diminishing in number of the granules was noticed from an early period in the experiment. (Table 7).

3327

Table 7

The Change of Vines-Positive Granule of the Adrenal Cortex Under D-Deficiency

DAY UNITS OF DEFICIENCY	1	2	3	4	5	6
7 対照 control 30	−	+	+	+	+	+~++
60	(±)	+	+	+	+	+~++
90	(±)	+~±	+~±	±~+	+~±	+~±
150	−	±~++	±~+	+	±	±~+
200	−	±	±	±	±	±~(+)
8 対照 control 30	±	+	+	+	+~++	++
60	±	+~±	+~±	+~±	+~±	+~±
90	±	±~+	±	±	±	±
120	(±)	±	±	±	±	±
	(±)	±	±	±	±~++	+~±

1-Day Units of Deficiency
2- Zona (Glomevularis)
3-Zona (Fascicularis Exterior)
4-Zona Fascicularis Interior)
Zona Fascicularis
5-Zona Reticularis
6-Paramedular Cortex
7-Mature Non-sterilized control
8-Immature Non-sterilized control

The Observation by Sudan IV dyeing

In both groups, the number of fat granules in the zona fascicularis interior and the zona reticularis decreased in the early part of the experiment but after the 90th day, conversely, they began to increase and gradually became coarse particles. (Table 8).

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Table 8

Lacking of Mature Follicle Multifollicular Degeneration

	DAY UNITS of D-DEFICIENCY 1	ZONA GLOMEVULARIS 2	INTERMEDIATE ZONA 3	ZONA FASCICULARIS (EXTERIOR) 4	ZONA FASCICULARIS (INTERIOR) 5	ZONA RETICULARIS 6	PARAMEDULAR CORTEX 7
MATURE	control	++	−	++	++	++	±~(+)
	30	++~++	−	++~++	++~++	±~(+)	±
成熟	60	++	−	++~++	++~++	+~(±)	±~+
非去勢	90	++	−	++	++	++~(±)	±~+
NON-STERILIZED	150	++	±	++~++	++	+~(±)	±~+
	200	++	±	++~++	++	+	+
IMMATURE	control	++~++~(±)	−	++	++	±~(+)	±
	30	++~(±)	++~(−)	++~++	++~++	±~(+)	±
幼若	60	++~(±)	++~(−)	++~++	++~++	±~(+)	±~(+)
去勢	90	(++)~(±)	−~(+)	++	++~++	±~+	±~(+)
NON-STERILIZED	120	(++)~(±)	−~(+)	++	++~++	±~(+)	±~(+)

1-Day Units of D-Deficiency
2-Zona Glomevularis
3-Intermediate Zona
4-Zona Fascicularis (Exterior)
5-Zona Fascicularis (Interior)
6-Zona Reticularis
7-Paramedular Cortex

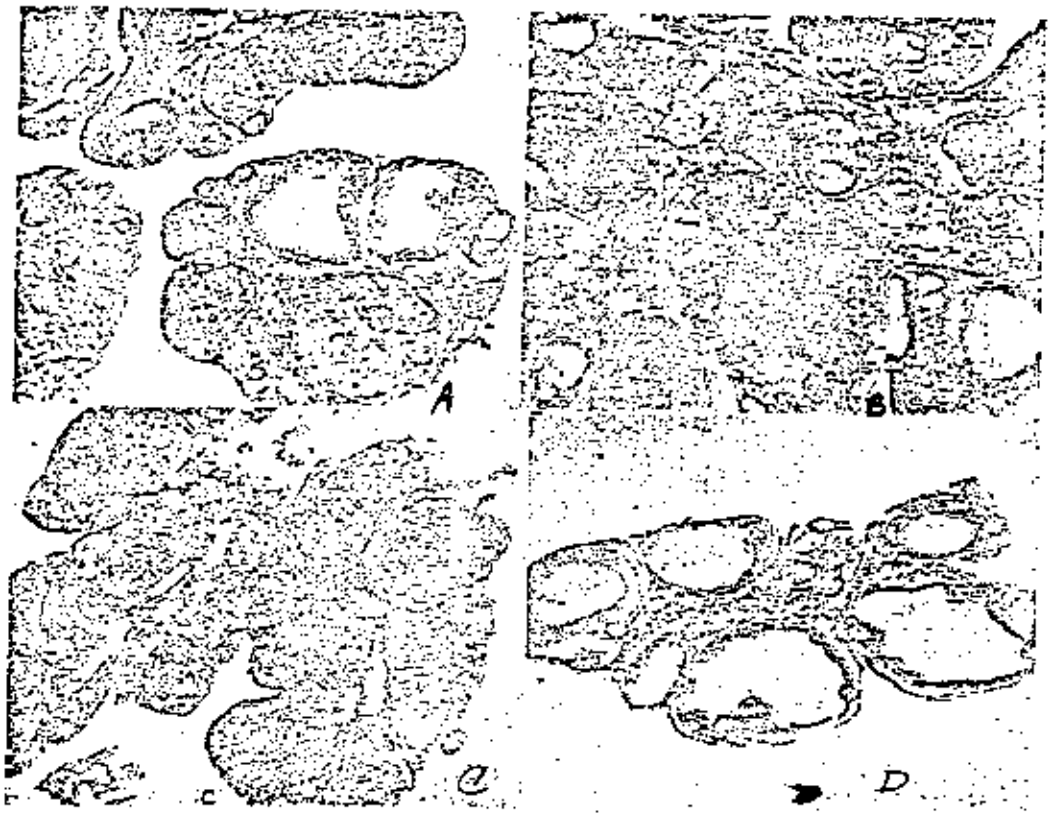
The Ovaries

There were two kinds of change in the ovaries. One was that the lutein bodies became remarkable lacking and the cell became gigantic. The other was that the contents of the cell consisted only of minor follicles and small lutein body remnants. The former pattern was found in the mature group, though the major follicles had decreased, there was no difference in the lutein body remnants. The former pattern was found in the mature group, though the major follicles had decreased, there was no difference in the lutein bodies between the deficiency group and the control group, until the 60th day of the experiment. But after the 90th day, the gigantic cell size which was more than 1000μ in the largest diameter, appeared; large, middle and small follicles occupied most of the ovaries; and the lutein bodies were few, chronic, and small. These symptoms show the so-called multifollicular degeneration.

Besides these in the ovaries, the granule-cell-stratum became atrophied and the follicles were dark and small, and the nuclear concentration was found, but the outer cell layer was rather swollen up and the interstitium was rough. It was remarkable that the connective tissue invaded the lutein bodies, so that the cell border line became indistinct and vacuous parts were found in the cytoplasm. After the 150th day, two types of ovaries, a multifollicular degenerated one and a rough atrophied one were found. In the immature group, the atrophy of the ovaries was observed after the 60th day, and also the lack of mature follicles and the transformation of follicles were found. (Fig. 11, Table 9).

Figure 11

The Ovary Under D-Deficiency (Mature Group)



A-Control
B-60 Day Deficiency
C-150 Day Deficiency Lacking of Mature Follicle
D-150 Day Deficiency MultiFollicular Degeneration

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Table 9

The Uterus Under D-Deficiency (Mature Group)

	DAY 欠乏日数 UNITS OF D-DEFICIENCY	発育卵胞 growing-follicle 巨大 1 gigantic	大 2 major	中 3 medial	小 4 minor	ORIGIN 原始卵胞 follicle 5	黄体 LUTEIN BODY 巨大 6 gigantic	大 7 major	中 8 medial	closed 閉鎖卵胞 9 follicle
mature 成熟	対照 control	−	+	+	+	+	+	+	+	++
	30	−	±	+	±~+	++	+	+	+	++
	60	−	+	++	+~++	+	+	+~++	+	++
	90	+	+	+	+	+	−	∓	±	++
	150	+~++	+	+	±~+	+	−	−	−	++
		−	±	±	±	+~±	−	+~++	+	++
	200	+	+	+	±~+	±~+	−	−	−	++
		±	+	+	±~+	±	+	+	+	++
immature 幼若	対照 control	−	+	+~++	+~±	+	±	+	+	++
	30	−	+	+	±~+	±	±	+	+	++
	60	−	±	+~++	±~+	±~+	±	+	+~++	++
	90	−	±	+	+~++	+	±	+	+~++	++
	120	−	±	+	+~++	+~++	±	+	+	++

1-Growing-Follicle (Gigantic)
2-Growing-Follicle (Major)
3-Growing-Follicle (Medial)
4-Growing-Follicle (Minor)
5-Origin Follicle
6-Lutein Body (Gigantic)
7-Lutein Body (Major)
8-Lutein Body (Medial)
9-Closed Follicle

3. A short summary

Because of the Vitamin D deficiency, the weight of the pituitary gland increased during the 60th to the 90th days and after that it began to decrease, but the weight of the adrenal gland didn't show any remarkable change. But from the histological point of view, both the pituitary and the adrenal glands suffered serious effects. In the anterior pituitary, the deterioration of α-cell function and the fluctuation of the cell-ratio of aciphilic cells were observed.

In the beginning of the experiment, both β- and δ-cells increased; especially remarkable was the increase of δ-cells, but soon after, they began

to decrease in number, and showed cytoplasm atrophy and functional deterioration. In the adrenal cortex, at the beginning, the Vitamin D deficiency experiment, the zona fascicularis interior showed stronger acidphilic tendencies, the fat-particles became smaller, and the other slight functional accelerations were observed, but gradually the function slowed down. The weight of the ovaries began to decrease after the 90th day, and the ovaries became atrophied histologically, and were divided into two patterns--one was the lack of the mature follicles, and the other was the lack of lutein bodies. The atrophy of the uteri was observed earlier than any other impediments of the pituitary, the adrenals, and the ovaries.

3. In the case of Vitamin D administration

1. The change of Viscera weight

THE NON-STERILIZED GROUP

In the mature group, the viscera weight didn't change so remarkably. In the immature group, when the rats were administered Vitamin D_2 1000 I.U., the functional deterioration was significant, and when 100 I.U. and 1000 I.U. Vitamin D_2 were administered, there were some increases in the size of the uterus. (Table 10, 11).

THE STERILIZED GROUP

In the mature group, the administration of 10 I.U. and 100 I.U. of Vitamin D caused the increase in the size of the adrenals, and 100 I.U. and 1000 I.U. of Vitamin D caused a significant increase in the size of the uterus. Even in the immature group, the administration of 10 I.U. of Vitamin D caused the decrease in the size of the pituitary gland, and the uterus increased

Table 10

The Weight of Organs Per Rats-Weight of D-dosage Group (Mature Non-Sterilized Group)

臓器 1	実験群 D投与量(I.U.) 2	例数 3	平均重量 (mg%) 4	標準偏差 (±σ) 5	信頼限界 (95%) 6	検定 7
PITUITARY 下垂体 GLAND	Placebo	5	5.1	0.21	0.32	—
	10	5	4.8	0.45	0.51	0.5<P<0.6
	100	5	5.2	0.36	0.42	0.7<P<0.8
	1000	5	5.0	0.22	0.30	0.7<P<0.8
ADRENALS 副腎	Placebo	5	26	2.6	3.3	—
	10	5	23	2.0	3.0	0.4<P<0.5
	100	5	21	3.2	4.1	0.1<P<0.2
	1000	5	26	2.7	3.8	0.8<P<0.9
OVARIES 卵巣	Placebo	5	34	2.1	2.8	—
	10	5	38	3.4	4.1	0.2<P<0.3
	100	5	37	2.0	2.6	0.4<P<0.5
	1000	5	36	2.2	3.0	0.7<P<0.8
UTERUS 子宮	Placebo	5	216	23	3.2	—
	10	5	229	32	3.9	0.7<P<0.8
	100	5	253	27	3.8	0.2<P<0.3
	1000	5	281	11	4.9	0.1<P<0.2

1-Organs
2-Amount of D-Administration
3-Instances
4-Average Weight
5-Standard Deviation
6-Reliability
7-Test

in size by using 100 I.U., and 1000 I.U. of Vitamin D. Comparing the administration of only Estradiol with the Vitamin D and Estradiol dosage, in the latter case, the size increasing tendency of the adrenal and the uterus was found. (Tables 12, 13).

Table 11

The Weight of Organs Per Rats-Total Weight of D-Dosage Group

ORGANS 臓器 1	AMOUNT OF DOSAGE 実験群 D投与量(I.U.) 2	INSTANCES 例数 3	AVERAGE WEIGHT 平均値 (mg%) 4	STANDARD DEVIATION 標準偏差 (±σ) 5	信頼限界 (95%) 6	RELIABILITY 検定 = TEST 7
PITUITARY 下垂体 GLAND	Placebo	5	4.3	0.16	0.46	—
	10	5	3.6	0.12	0.22	0.1<P<0.2
	100	5	3.9	0.26	0.34	0.5<P<0.6
	1000	5	3.3	0.24	0.42	0.1<P<0.2
ADRENALS 副腎	Placebo	5	22	1.7	2.3	—
	10	5	21	2.9	4.5	0.6<P<0.7
	100	5	23	1.3	2.5	0.6<P<0.7
	1000	5	22	3.9	5.2	0.6<P<0.7
OVARIES 卵巣	Placebo	5	36	2.3	3.8	—
	10	5	36	1.5	2.5	—
	100	5	33	2.4	3.2	0.3<P<0.4
	1000	5	27	2.9	3.1	P<0.02
UTERUS 子宮	Placebo	5	124	24	38	—
	10	5	153	16	23	0.1<P<0.2
	100	5	192	26	39	P<0.02
	1000	5	196	25	36	P<0.01

1-Organs
2-Amount of Dosage
3-Instances
4-Average Weight
5-Standard Deviation
6-Reliability
7-Test

2. Histological Observations

ANTERIOR PITUITARY

THE NON-STERILIZED GROUP

The swelling up and the increase of the number of α-cells was observed in both the mature and immature groups, but the α-cells in the mature group became atrophied by the administration of 1000 I.U. of Vitamin D. The acid-philic cells decreased in number a little with the 10 I.U. dose, but it began to increase with the amount of the dose. This increase was found expecially in the β-cells, and there was not as remarkable a change in the δ-cells. On the contrary, when the amount of the dose was increased, the number of δ-cells decreased. (Fig. 13, Tables 14, 15).

Table 12

The Weight of Organs Per Rats-Weight of D-Dosage Group (Mature-Sterilized G.)

器官 1		D投与量(i.u.) 2	例数 3	平均値 (mg%) 4	標準偏差 (±σ) 5	信頼限界 (95%) 6	検定 7
MATURE 成熟去勢 STERILIZED	PITUITARY 下垂体	Placebo	5	3.5	0.21	0.28	—
		10	5	3.7	0.16	0.22	0.4 < P < 0.5
		100	5	3.4	0.14	0.24	0.6 < P < 0.7
		1000	5	4.3	0.20	0.28	0.05 < P < 0.1
	ADRENALS 副腎	Placebo	5	20	1.6	3.1	—
		10	5	24	1.5	2.6	0.02 < P < 0.05
		100	5	27	2.1	3.1	P < 0.02
		1000	5	26	2.4	3.5	0.1 < P < 0.2
	UTERUS 子宮	Placebo	5	26	1.4	1.8	—
		10	5	29	1.8	2.1	0.1 < P < 0.2
		100	5	38	2.0	1.6	P < 0.01
		1000	5	32	2.6	2.5	0.02 < P < 0.05
IMMATURE 幼若去勢 STERILIZED	PITUITARY 下垂体	Placebo	5	4.2	0.16	0.18	—
		10	5	3.3	0.16	0.22	P < 0.0
		100	5	3.5	0.26	0.24	20.05 < P < 0.1
		1000	5	4.7	0.24	0.31	0.2 > P < 0.3
	ADRENALS 副腎	Placebo	5	21	1.2	20	—
		10	5	22	1.0	15	0.8 < P < 0.9
		100	5	19	2.4	28	0.2 < P < 0.3
		1000	5	26	2.2	23	0.2 < P < 0.3
	UTERUS 子宮	Placebo	5	26	1.6	24	—
		10	5	28	1.2	18	0.7 < P < 0.8
		100	5	38	2.2	30	P < 0.01
		1000	5	35	2.1	26	0.02 < P < 0.05

1-Organs
2-Amount of Vitamin D Dosage
3-Instances
4-Average Weight
5-Standard Deviation
6-Reliability
7-Test

Figure 13.

The Change of the Anterior Pituitary Cell
by D-Dosage (Mature-Non-Sterilized Group)

(AF-Schiff's, 20×40)

A-Placebo
B-β-Cell Increases a Little
C-β-Cell Increases, δ-Cell Decreases a Little
D-β-Cell Increases, δ-Cell Decreases

STERILIZED GROUP

The α-cells decreased in number a little in the Placebo groups but the cytoplasm swelled up slightly, and the size of the secretory granules decreased, and became hard to dye. The following was a remarkable change:

Table 13

The Weight of Organs Per Sterilized Rats-Weight Under Estradiol with Vitamin D For 20 Day Dosage

AMOUNT OF 投与量 DOSAGE Estradiol (μg)	D (I.U.)	PITUITARY 下垂体 GLAND 2	前 腎 ADRENALS 3	UTERUS 子宮 4
0.05	—	4.5	26	35
0.02	—	3.6	14	21
0.01	—	4.6	17	28
0.01	10	3.6	16	45
0.01	100	4.6	19	36
0.01	1000	4.2	35	42

1-Amount of Dosage
2-Pituitary Gland
3-Adrenals
4-Uterus

Table 14

The Change of the Anterior Pituitary Cells by D-Dosage

D投与量(I.U.) AMOUNT OF D-DOSAGE 1		好酸性細胞 (%) 2	好塩基性細胞 (%) 3	色素嫌性細胞 (%) 4	5 計 TOTAL
NON-STERILIZED 非去勢	Placebo	31.5(1671)	3.6(193)	61.9(3441)	(5305)
	10	30.4(1554)	3.0(151)	66.6(3406)	(5111)
	100	37.0(2140)	3.5(20.6)	59.5(3436)	(5780)
	1000	33.9(2195)	5.4(347)	60.7(3933)	(6475)
STERILIZED 去勢	Placebo	20.2(582)	20.1(577)	59.7(1714)	(2873)
	10	41.9(2461)	11.0(649)	47.1(2770)	(5880)
	100	48.3(1449)	13.4(401)	38.3(1149)	(2999)
	1000	35.3(1493)	15.5(653)	49.2(2079)	(4225)

1-Amount of D-Dosage
2-Acidphilic Cell (%)
3-Basophilic Cell (%)
4-Melanophobic Cell (%)
5-Total

Table 15

The Change of the Basophilic Cell in the Anterior Pituitary by D-Dosage

D投与量(I.U.) 1 Amount of D-dosage		β-細胞% 2 β-cell %	δ-細胞% 3 δ-cell %	計 4 total
NON-STERILIZED 非去勢	Placebo	68.4(132)	31.6(61)	(193)
	10	74.8(113)	25.2(38)	(151)
	100	71.1(145)	28.9(59)	(204)
	1000	78.1(271)	21.9(76)	(347)
STERILIZED 去勢	Placebo	22.5(130)	77.5(447)	(577)
	10	35.6(231)	64.4(418)	(649)
	100	42.9(172)	57.1(229)	(401)
	1000	39.2(256)	60.8(397)	(653)

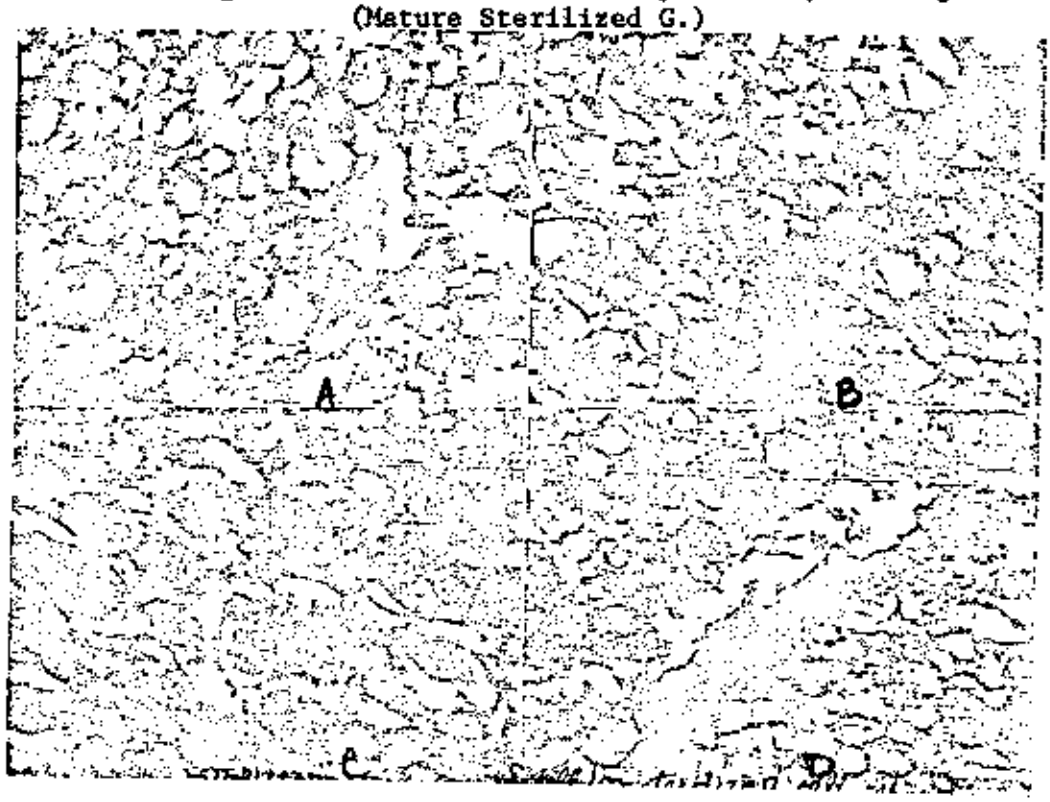
( )内は実測細胞数
(注) no a survey

1-Amount of D-Dosage
2-β-Cell (%)
3-δ-Cell (%)
4-Total

the significant increase of the number of acidphilic cells and the swelling up of the cytoplasm. The nucleus was unevenly distributed, colloid substances remained in the cytoplasm. So-called "sterilized cells" were observed. These cells could be dyed pink by the AF-Schiff dyeing method and it was possible to distinguish then from the β-cells which were AF-positive and became a dark violet color through this same method. The changes in these cells through Vitamin D administration are listed in Table 15. According to this table, α-cells increased in number and their affinity for the dye was enhanced. Besides these, the number of sterilized-cells became less, and the improvement of the cell vacuoles was observed. The rate of appearance of the sterilized-cells was not so largely different among the groups by a single dose of Estradiol 0.05 to 0.01μg, but in the case of Vitamin D being added to the Estradiol, when the amount of Vitamin D was 10 I.U., the appearance rate of sterilized-cells was lower than in the case of administering only Estradiol (0.05μg). And corresponding to the increase in the Vitamin D dose, the sterility became progressively normal. (Fig. 14, 15).

Figure 14

The Change of the Anterior Pituitary Cells by D-Dosage
(Mature Sterilized G.)



A-Placebo Sterilized Ring Cell (δ Cell)
B-10 I.U. Sterilized Cell--Shows no Difference from Placebo
C-100 I.U. Sterilized Cell Decreased
D-1000 I.U. Sterilized Cell Decreased

The Observation of the Adrenal Cortex by H.E. dyeing

In the non-sterilized group and the sterilized group, the shortening of the zona laminalis and the miniaturizing of the cells were found in the zona glomevularis. The acidophilic tendencies of the cells in the zona fascicularis interior was increased and the cytoplasm swelled a little, as well as there being an increase in the number of big nuclear and the bi-nuclear

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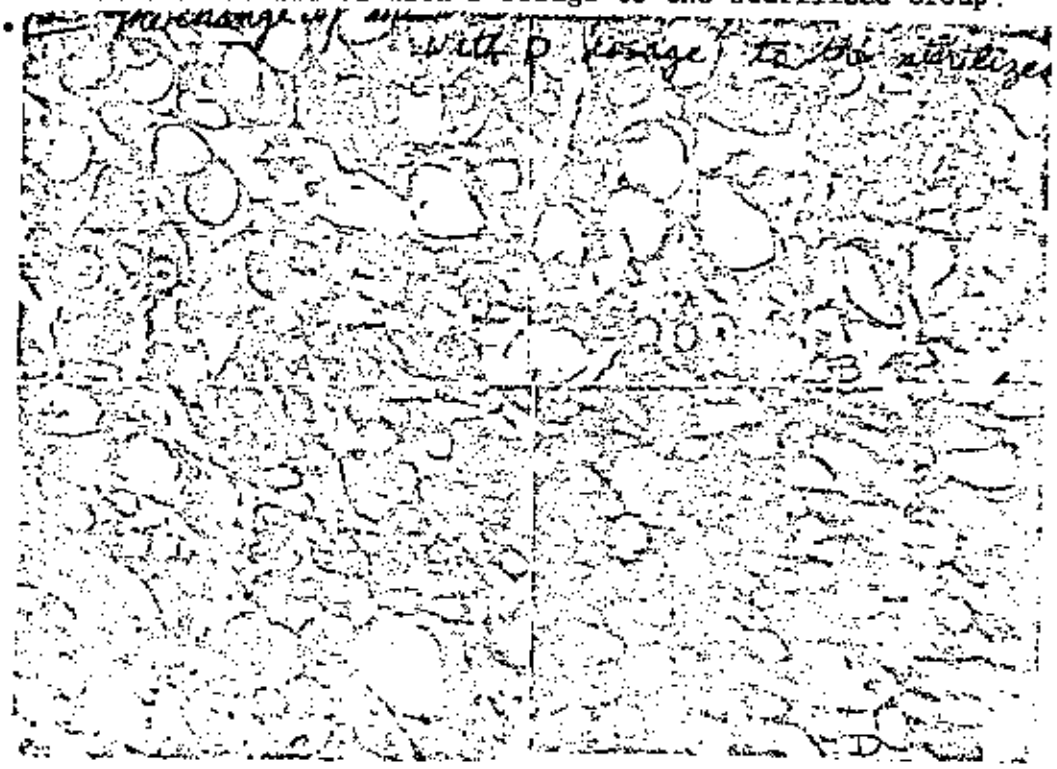
Figure 15

The Change of the Anterior Pituitary Cells in the Case of Estradiol with D-Dosage to the Sterilized Group.

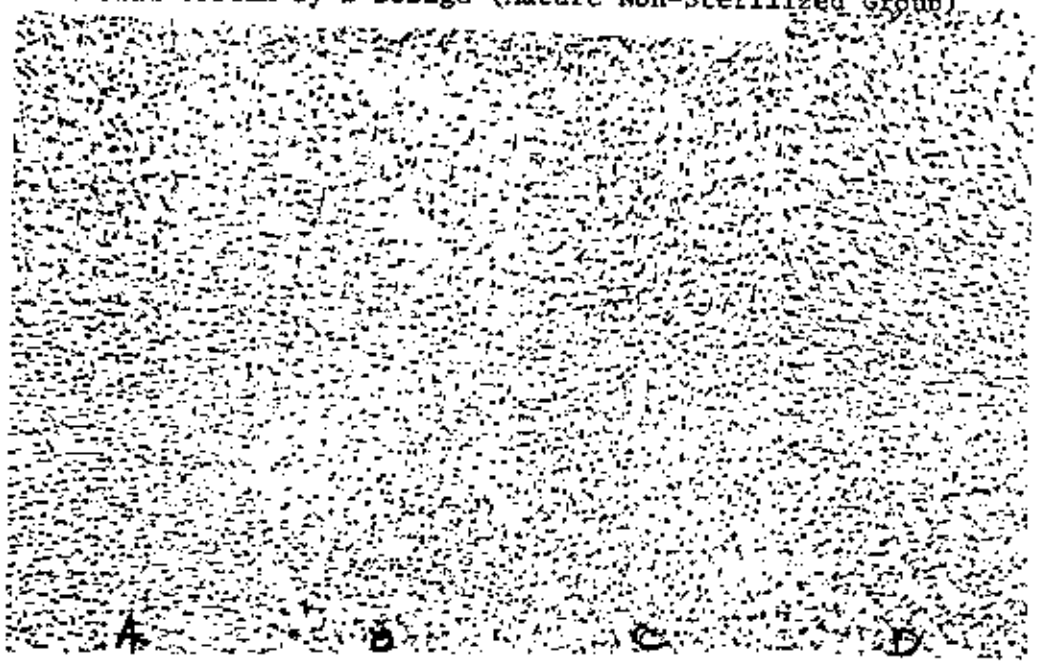


A-Estradiol 0.05μg Sterilized Ring Cell (δ) and Bird-Eye-Shaped Cell (β) increase
B-Estradiol 0.01μg + Placebo .
C-Estradiol 0.01μg + D 100 I.U. Though Sterilized Cell Increase, Significantly, Sterilized Ring Cell Decrease
D-Estradiol 0.01μg + D 1000 I.U. Sterilized Cell Is Rare

cells with the administration of Vitamin D. When 1000 I.U. Vitamin D was given, the rats showed the same symptoms as above, but, especially in the immature group, the disorder of the cellular fascicle was sometimes found. The function of the zona reticularis and the paramedular cortex was accelerated by the administration of 100 I.U. Vitamin D, but when 1000 I.U. of Vitamin D was given, the cytoplasm atrophied and became less dense. In the sterilized group, with a 100 I.U. Vitamin D dosage the recovery from sterile to normal was recognized, and with a 1000 I.U. Vitamin D dosage, the atrophy of the adrenal cortex was found though it was rare. (Fig. 16, 17).

Figure 16

Adrenal Cortex by D-Dosage (Mature Non-Sterilized Group)



A-Placebo
B-10 I.U.
C- 100 I.U.
D-1000 I.U.

Figure 17

Adrenal Cortex by D-Dosage (Mature Non-Sterilized Group)

A-Placebo
B-10 I.U.
C- 100 I.U.
D-1000 I.U.

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The Observation of the Adrenal Cortex by Vines dyeing

In both non-sterilized and sterilized groups, an increase of granules was found with a 10 I.U. Vitamin D administration. With a more than 100 I.U. Vitamin D dose, the granules became less. (Tables 16, 17).

Table 16

The Change of Vines-Positive Granule of Adrenal Cortex by D-Dosage

Amount of D-D投与量 1 (I.U.) dosage		2 球状带	束状带 3 外束状带 4 EXTERIOR	束状带 3 内束状带 5 INTERIOR	6	髓状带 7
mature	Placebo	(±)	∓	∓	+	+~∓
成熟	10	(±)	+	+~∓	+~∓	∓~+
非去势	100	(±)	+	+	+	+~∓
non-sterilized	1000	±	±~+	±~+	±~+	+
immature	Placebo	±	+	+	+~∓	+~∓
幼若	10	+	+	+	∓	∓
非去势	100	±	±	±~+	+~±	+
non-sterilized	1000	−	±~+	±~+	+	+

1-Amount of D-Dosage
2-Zona Glomevularis
3-Zona Fascicularis
4-Exterior
5-Interior
6-Zona Reticularis
7-Paramedular Cortex

Table 17

The Change of Vines-Positive Granule of Adrenal Cortex by D-Dosage

Amount of D投与量 1 (I.U.) DOSAGE		2 球状带	束状带 3 外束状带 4 EXTERIOR	束状带 3 内束状带 5 INTERIOR	6	髓状带 7
mature	Placebo	∓	∓	∓	∓	∓
成熟	10	−~(+)	±	±	±~(+)	±~+
去势	100	(±)	+~±	±~+	+	+~∓
sterilized	1000	−	±	±	±~+	±~+
immature	Placebo	(±)	±~+	±~+	±~+	+~∓
幼若	10	(±)	±~+	±~+	+~(∓)	+
去势	100	(±)	±~+	±~+	+~∓	±~∓
sterilized	1000	(±)	±	±	±~+	±~+

1-Amount of D-Dosage
2-Zona Glomevularis
3-Zona Fascicularis
4-Exterior
5-Interior
6-Zona Reticularis
7-Paramedular Cortex

The Observation of the Adrenal Cortex by Sudan IV dyeing

In the non-sterilized group, the granules were reduced with an administration of 100 I.U. Vitamin D, but an increase in the number of particles was observed with 1000 I.U. In the sterilized group, the number of particles were reduced by 100 I.U. (Tables 18, 19).

Table 18

The Change of Sudan IV Positive Granule of the Adrenal Cortex by D Administration

AMOUNT OF D投与量 (I.U.) D DOSAGE 1		2 球状带	束状带 3 外束状带 4 EXTERIOR	内束状带 5 INTERIOR	6 网状带	髓质皮质 7
成熟非去势	Placebo	++	++	++	±~(+)	±~(+)
	10	++~(±)	++~++	++~++	—~(±)	±
	100	++	++	++~++	±~(++)	±~(+)
	1000	++	++	++	±~(++)	±~(+)
幼若非去势	Placebo	++~++~±	++	++	±~(+)	±
	10	++~++	++~++	++	—~(+)	—
	100	++	++	++	±~(+)	±
	1000	++~++	++~++	++~++	±~(++)	±~(+)

1-Amount of D-Dosage
2-Zona Glomevularis
3-Zona Fascicularis
4-Exterior
5-Interior
6-Zona Reticularis
7-Paramedular Cortex

The Uteri

In the non-sterilized group, the proper layer of the mucous membrane was thickened and the uterine gland cells were atropied with the 100 I.U. administration, and as for the muscles of the uteri, the inner muscle became thick according to the increase of the dosage. In the sterilized Placebo group, there was a high degree of uteri atrophy, and especially remarkable was the atrophy in the proper layer of the mucous membrane and the inner muscle fibre stratum. When the Vitamin D administration was more than 100 I.U., these atrophies recovered somewhat, and in the case where both Estradiol and Vitamin D were given, the recoveries were more effective than with Vitamin D only. (Fig. 19).

Table 19

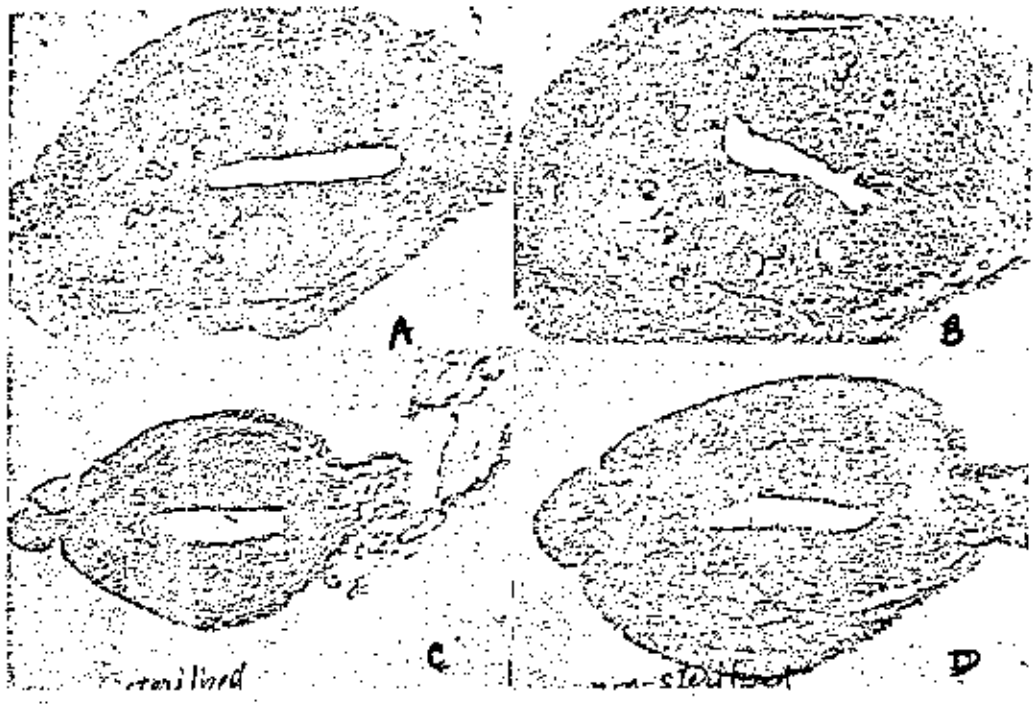
The Change of Sudan IV-Positive of Adrenal Cortex by D-Dosage

	AMOUNT OF D-DOSAGE (I.U.) 1	ZONA GLOMERULARIS 2	INTERMEDIATE ZONA 3	ZONA FASCICULARIS 4		ZONA RETICULARIS 7	PARAMEDULAR CORTEX 8
				EXTERIOR 5	INTERIOR 6		
成熟去勢	Placebo	++~+++	±~(+)	++	++	±~(+)	+
	10	++~+++	±	++	++	±~(++)	±~(+)
	100	++~(±)	—	++~(++)	++	±~(+)	±~(+)
	1000	++~+++	—	++~(++)	++~(++)	±~(+)	±~(+)
幼若去勢	Placebo	++	±~+	++~+++	++~+++	±~+	±~+
	10	++~+++	±~+	++~++	++~++	±~(++)	±~(+)
	100	++~(±)	—	++	++~±	±~(+)	±~(+)
	1000	++~(±)	—	++~(++)	++~+++	±~+	±~+

1-Amount of D-Dosage
2-Zona Glomevularis
3-Intermediate Zona
4-Zona Fascicularis
5-Exterior
6-Interior
7-Zona Reticularis
8-Paramedular Cortex

Figure 19

The Uterus By D-Dosage (Mature Group)



A-Non-Sterilized Placebo
B-Sterilized Placebo
C-Non-Sterilized Placebo
D-Sterilized Placebo

A Short Summary

The relation between the Vitamin D dosage and each viscus is expressed as follows: with 100 I.U. of Vitamin D administration, a functional acceleration of each viscus was found, but with 1000 I.U. of Vitamin D administration, the restraint of each viscus function was found, and the uteri was hypertropied according to the increasing amount of Vitamin D administer. The Vitamin D administration restricted the changes in the pituitary gland, the adrenals, and the uterus, to some extent, but even 1000 I.U. of Vitamin D was not enough.

THE EFFECTS OF VITAMIN D DEFICIENCY ON PREGNANCY

It is known that Vitamin D deficiency easily causes abortion and premature birth, but there are few reports on the observations of the placenta in such causes.

1. The Method of Experiment

Wistar-strain female rats who had normal sexual cycles were chosen as 'control groups'. For the Vitamin D deficiency group, the rats were divided as follows: (1) a group which was fed a Vitamin D-free diet from the first day of pregnancy, (2) a group which conceived after 30 days on a Vitamin D free diet, (3) a group which conceived after 40 days on a Vitamin D-free diet. Including the control group, the rats were divided into 5 groups. Rats from each group were beheaded after the 14th and 20th day of their pregnancy, and were observed for the existence of an embryo, the weight of the embryo, the weight of the placenta. Histological references of the placenta were done. Besides this, especially in the group which conceived after 30 days on a

Vitamin D-free diet, a histological study of the placenta was done on the 10th, 12th, 14th, 16th, 18th, and 20th days of their pregnancy. To establish pregnancy, the female rat lived with the male during the early part of the Praeoestrus. The next morning, if keratinized cells and spermatozoan were observed in the vaginal smear, that day was recognized as the first day of pregnancy.

2. The Results of the Experiments

1. The ratio of pregnancy, the ratio of embryo existence

These two ratios deteriorated in proportion to the Vitamin D deficiency period, especially after the 30th day. The difference between the 14th day and the 20th day's ratio of embryo existence became less remarkable in the last stage of pregnancy, especially after the 30th day of Vitamin D deficiency. (Fig. 20, 21).

Figure 20

Pregnant After 30 Days D-Deficiency (14th Day)

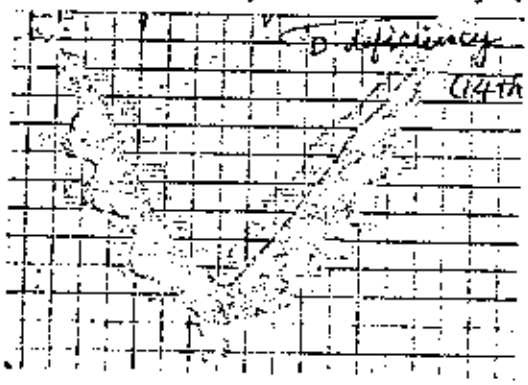
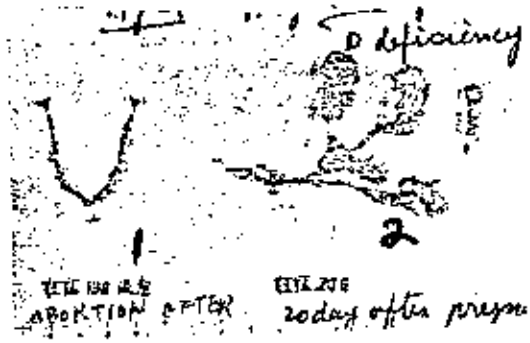


Figure 21

Pregnant After 30 Days D-Deficiency (20th Day)



1-Abortion After 13th Day of Pregnancy
2-Abortion After 20th Day of Pregnancy

2. The weight of the placenta, the weight of the embryo

The weight of the placenta and the weight of the embryo of each group are shown in Tables 21 and 22, and through this, one can see that there were not as large differences between each group and the control group, but inspection on the 14th day, shows the embryo weights decreased in the rats who belonged to the longer than 30 days Vitamin D-deficiency units.

3. The Histological Observations

In order to study the histological references, specimens from the central cross cutting part of the embedding part of the placenta, which was already fixed by formalin, were made into paraffin pieces, and were dyed by H.E. dyeing, Van Gieson dyeing, and PAS dyeing.

The placenta of the white rats was divided into two parts. One was the Yolk sack placenta, which comes out on the 12th day of pregnancy, and the other was the Labyrinthine placenta, which comes out the end of the 10th day of pregnancy. After the 12th day of pregnancy, Syncytial trophoblast which had the blood vessel of the urinary sac integrated reticularly into Cytotrophoblast that connected with the blood of the mother's womb, performed Labyrinth until

Table 21

The Effect of D-Deficiency Breeding on the White Rats' Pregnancy (14th Day of Pregnancy)

Experimental group 1	number of animal 2	The number of bed 3	生存胎仔数 4	平均生存率 (%) 5	生存胎仔の胎盤重量(mg) 6	
					7 湿重量 wet weight	8 乾燥重量 dry-weight
9	206	12	12			
	207	14	14			
対照	208	13	13	98.3	143.8±2.4	14.7±2.9
control group	209	12	11			
	210	10	10			
10 D-deficiency breeding	216	11	11			
妊娠前後	217	14	13			
	218	11	9	87.5	135.3±3.6	14.8±3.4
欠乏飼育	219	10	9			
just after pregnancy	220	10	7			
11 pregnant rats group after	226	11	8			
欠乏飼育	227	10	7			
	228	17	13	70.9	110.7±3.4	13.0±2.2
30日後妊娠	229	10	6			
30 days D-deficiency	230	—	—			
pregnant rats-group	236	11	5			
欠乏飼育	237	—	—		126.2	12.1
12	238	—	—	45.5	92.4	7.3
40日後妊娠	239	—	—		98.6	8.2
after 40 days D-deficiency	240	—	—			

1-Experimental Group
2-Number of Animal
3-The Number of Bed
4-The Number of Survival Fetus
5-Average Survival Rats (%)
6-The Weight of Placenta of Survival Fetus (mg)
7-Wet Weight
8-Dry Weight
9-Control Group
10-D-Deficiency Breeding Just After Pregnancy
11-Pregnant Rats Group After 30 Days D-Deficiency
12-Pregnant Rats-Group After 40 Days D-Deficiency

the 14th day of pregnancy. Including other parts' formation, the essential construction of the white rat's placenta is accomplished in the 14th day of pregnancy. All the placentas under the Vitamin D deficiency experiment suffered from abortion during the whole period, and this is shown in Fig. 22 and 23. At the 10th day of pregnancy, the abortion and the hyperemia of the Exto-

Table 22

The Effect of D-Deficiency Breeding on the White Rats Pregnancy (20th Day of Pregnancy)

Experimental 実験群 group 1	number 動物番号 of animal 2	生存者床数 of bed 3	生存胎仔数 4	平均生存率 5 (%)	平均生存胎仔体重 6 (g)	生存胎仔の胎盤重量(mg) 7 8 湿重量 wet-weight	9 乾燥重量 dry-weig
10 対照 control group	201	13	13	98.2	2.58±0.61	326.8±91.6	50.1±3.5
	202	11	11				
	203	12	11				
	204	11	11				
	205	10	10				
11 p-deficiency breeding 妊娠直後 欠乏飼育 just after pregnancy	211	14	10	86.2	2.54±0.31	292.3±27.8	48.2±2.4
	212	11	11				
	213	11	10				
	214	12	10				
	215	10	9				
12 pregnant group 欠乏飼育 30日後妊娠 after 30 days D-deficiency	221	7	3	48.3	2.64±0.40	295.2±19.6	49.0±3.1
	222	8	4				
	223	9	7				
	224	(10)	(0)				
	225	—	—				
13 pregnant group 欠乏飼育 40日後妊娠 after 40 days D-deficiency	231	12	4	30.4	2.50±0.48	307.1±11.9	48.1±2.6
	232	11	3				
	233	—	—				
	234	—	—				
	235	—	—				
14 pregnant group 欠乏飼育 50日後妊娠 after 50 days D-deficiency	241	7	0	0	—	—	—
	242	—	—				
	243	—	—				
	244	—	—				
	245	—	—				

( )内は妊娠13日流産

(#) is abortion

1-Experimental Group
2-Number of Animal
3-Number of Bed
4-The Number of Survival Fetus
5-Average Survival Rate (%)
6-The Weight Average of Survival Fetus (g)
7-The Weight of Placenta of Survival Fetus (mg)
8-Wet Weight
9-Dry Weight
10-Control Group
11-D-Deficiency Breeding Just After Pregnancy
12-Pregnant Group After 30 Days D-Deficiency
13-Pregnant Group After 40 Days D-Deficiency
14-Pregnant Group After 50 Days D-Deficiency
(#) Is Abortion the 13th Day of Pregnancy

placental cone (this is the Cytrophoblast which comes from the extradermal epidermis, and invades the decidua in a cone-shape, and also, the decidual vessels open in this place) could be observed, and partial bleedings were also found in the side of the dermal plate. At the 14th day, the whole Labyrinth stratum was thin, and the Syncytial trophoblast underwent atrophy; the concentration of the nucleus was also found in some parts. The corpuscles in the blood vessel of the urinary sac were normal, but the intravillous space (maternal blood space) was narrow and anemic, and sometimes it was found there were no blood corpuscles. In these Labyrinth, the necrosis of the Syncytial trophoblast and bleedings because of the breaking of the Allantoic villi were observed, and these caused bleeding-necrosis over a wide area. In some cases, at the beginning of Labyrinth formation, the Syncytial trophoblast had already died. The observation at the 20th day of the pregnancy, was rather the same as the observation at the 14th day; that is, incomplete formation of the Labyrinth, the death of the fetus by the bleeding-necrosis, and so on.

3. A Short Summary

Long-term Vitamin D deficiency aborts the fetus, the pregnancy rate and the fetus survival rate decreased, and the placenta weight declined at the 14th day of the pregnancy. But at the 20th day of pregnancy, the only difference between the experimental group and the control group was that the survival rate of the fetus decreased in the D-deficiency group. The surviving fetus' weight and the fetus' placenta weight weren't any different in either group. The abortion of the Labyrinth was noticed when the histological observation of the placenta was done, and this is thought to be the result of the incomplete formation of allantoic villi, that is the retrograde metamorphosis of the syncytial trophoblast which appeared in the early period of the experiment.

THE ANALYSIS OF THE EXPERIMENT

Dr. Schulze and others recognized the decline in the thyrotropic potency of the pituitary gland during the Vitamin D deficiency period. Also, Dr. Neuweiler and others reported the atrophy of the genitals, the ovary-follicular metamorphosis, the transformation of the cells of the pituitary gland. In my experiment, α-cells in the anterior pituitary began to decrease from the early stage of the D-deficiency experiment, and also other functional retrogradations such as the atrophy of the follicles were observed. Concerning the basophilic cells, the number of δ-cells of the gondotrophs increased remarkably, therefore, there is a conjecture that Vitamin D deficiency probably has an effect on this system: the mesencephalon-the pituitary gland-the ovary.

In the case when the deficiency ran for a long term, both β-and δ-cells began to atrophy and decline in number, and symptoms of functional retrogradation were noticed. Concerning the adrenal, some functional acceleration was found in the zona fascicularis interior and the paramedular cortex, at the early stage of the D-deficiency. The atrophy of the ovary and the uterus was observed in the early period of the Vitamin D-deficiency experiment, therefore, the degeneration in the pituitary gland can be judged as the secondary effect which was caused by the functional decline of the ovary. It cannot be denied, however, that there was also the direct effect of D-deficiency, judging from the symptoms of the atrophy in the late period of the experiment. Dr. Agduhr observed the weight increase of the pituitary gland, the significant increase of the number of acidphilic cells, and the slight increase of the basophilic cells which were caused by the dose of the Ergosterore. Dr. Ito reported the decrease in the number and the atrophy of the α-cells,

nuclear concentration in the α-cells, and the swelling in size and number increasing of the α-cells. In my experiments, the following were observed: until the 100 I.U. dose of Vitamin D_2, the α-cells in the non-sterilized group were swollen and large in number through out the Vitamin D administration, but the basophilic cells declined in number temporarily with the 10 I.U. administration, though they increased again until 1000 I.U. was administer. The number of β-cells changed significantly, but the δ-cells were constant or sometimes declined slightly. In the sterilized group, the rate of the appearance of the sterilized-cell decreased according to the increase of the amount of Vitamin D administration. Therefore, the direct effect of Vitamin D on the anterior pituitary or the hypothalamic sexual center can be conjectured.

Cholesterol in the adrenal cortex is the precursor of the steroid hormone. The Sudan-fat and the Ascorbic acid are affected by the function of the adrenal. According to Dr. Zimsser, the materials which are Vines-positive, can be chosen as the index of the cortex function, because they show inverse ratio with the cholesterol, the ascorbic acid, and the Sudan-fat 39). Thus, the adrenal cortex in the D-deficiency period, showed slight functional accelerations in the early period of the experiment. As the period of deficiency passed, all the strata of the cortex, expecially the strata under the zona fascicularis, began to atrophy significantly, and these changes were rather similar to the symptoms produced when they are sterilized. These changes are properly said to be the compensatory reaction against the functional deterioration of the sex gland, judging from the observations of the pituitary gland and the ovary. To say more: these are the stress reactions to the D-deficiency.

Dr. Ito 37) reported that mass administration of Vitamin D caused the atrophy of the cortex and the decrease of the fat. According to the results of my

experiments, in the non-sterilized group, the dose of 100 I.U. Vitamin D accelerates the cortex function, but conversely, 1000 I.U. dose of Vitamin D causes the functional retrogression of the cortex, and in the immature group, especially the cortex, atrophy was noticed in the histological research. In the sterilized group, a significant weight increase was observed in the mature group, and also the diminishing in size and number of the Sudan-fat granules was observed in the histological study. This is a symptom of recovery from sterility. As for the distribution of Vitamin D given; Dr. Cruickshank 42) stated that he observed it densely in the adrenal, but Dr. Kodecek 43) reported he found most of the D_2-C^{14} in the zona fascicularis; also Dr. Ryo 44) reported the same view in a histo-chemical study. In the experiment by Dr. Quarterman 45) which observed the weight of Vitamin D in the adrenal of white rats which had been given a Vitamin D dose, the adrenal showed a high density of a new substance after 30 minutes which was different from Vitamin D chemically because it had an antirachitic effect.

The ovary and the uterus suffered serious effects from the D deficiency experiment; the ovary became atrophied significantly after the 90th day of the experiment and the uterus already had begun to decline in weight even at the 30th day. The ovary showed two characteristics histologically; one was the lack of lutein bodies and the existence of major follicles only, the other was the existence of minor follicles and chronic lutein bodies. The former was found in the mature group and the latter was mainly observed in the immature group. According to the observations of Dr. Chinone 12) and Dr. Tsuzuki 3), the ovary showed the second characteristic, and by the 60th to 90th day of the deficiency, the vaginal smear showed Dioestrus. But conversely, in my

experiment, the symptoms of Oestrus were observed frequently. This occured because of the difference in the conditions for breeding the rats. Also in my case, the effect of Vitamin D deficiency seemed to appear slowly. In the observations of Dr. Goetigens 46) and Neuweiler 47), the multifollicular degeneration was observed in the ovaries of the rats who suffered with D-deficiency, and Dr. Parkes 48) reported that the vaginal smear showed continuous keratinization in this period. The reason for this multifollicular degeneration could partially be due to excessive amounts of Gonadotropin, judging from the remarkable increase of the number of the δ-cells in the early stage of the deficiency; but to my understanding, it is more reasonable to say that the main reason for this degeneration is the decline of the Feed-back for the sexual center which occured because of the Estrogen decrease in the ovaries from the early stage of the experiment. Dr. Parlow 49) reported that in sterilized rats any amount of Estradiol had a restrictive effect on the secretion of the pituitary LH[5], but in the case an infinitesimal amount of Estradiol (0.005μg), there was a stimulative effect on the secretion of the FSH[6]. The observation that there is multifollicular degeneration in ovaries which have a trace of recidual ovalium left by unsuccessful sterilization operations will be explained in these reports.

There was not so significant a change in the weight of the ovaries and the uterus in the non-sterilized group because of Vitamin D administration,

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[5]LH=Luteinzing Hormone

[6]FSH=Follicle Stimulating Hormone

but when 1000 I.U. of Vitamin D was given to the immature group, both the decline of the ovary-weight and the increase of the uterus weight were observed. When the dosage amount of Vitamin D exceeded 10 I.U., the weight of the uterus increased significantly in the sterilized group. Histologically in the ovaries, the number of growing-follicles and lutein bodies was increased by the 100 I.U. administration, but with the 1000 I.U., the growth of the follicles was hindered. In the uterus, Vitamin D administration promoted the growth of the muscular layer and thickened slightly the proper layer of the mucous membrane. According to Dr. Urist 50), the main reason for the ovary atrophy in D-deficient chickens is the hindrance of the synthesis of phosphorus albumin calicium or lipo-albumin. Dr. Aizawa reported estrogen affects the assimilation of inorganic phosphorous into the phospholipid in the uterine muscle of rats, and concluded this effect is very useful for uterine growth. Vitamin D also stimulates the metabolism of the phospholipid. Dr. DeLuca and others 52)-55), reported that the correct amount of Vitamin D is related to the parathyroid hormone and stimulates the release of calcium from the mitochondria, the metabolism of TPA, and cytoplasm respiration. But an excessive amount restricts these processes.

Vitamin D deficiency reduces the rate of pregnancy and the survival rats of the fetus. Comparing the 14th day and the 20th day of pregnancy, the hindrance of pregnancy was more serious at the 20th day. The surviving fetus' weights do not show any difference between the experimental group and the control group at the 20th day of pregnancy, but at the 14th day, the experimental group's rate is inferior to the control group's. Therefore, the reason for the fetus' death in the late stage of pregnancy is mainly the lack of the growth of the placenta. Pregnancy under Vitamin D deficiency causes the incomplete formation of the placenta. The most serious change is the abortion of the allantoic villi at the placental Labyrinth, and because of the retro-

grade metamorphosis of the syncytial trophoblast, the formation of Labyrinth is insufficient and the maternal blood space is narrow and suffers from anemia. After these symptoms, necrosis and bleeding were observed in the placental Labyrinth and, at last, the total necrosis of the placenta and the death of the fetus occurred.

The cause of the atrophy of the syncytial trophoblast is unknown, but for white rats, during any period of the pregnancy, the pregnancy is suspended by sterilization because of the atrophy. So if the pregnancy should continue, the ovary hormone is necessary. Therefore, besides the hormone environmental change caused by the D deficiency, the retrograde metamorphosis of all stratum of the placenta and the necrosis is caused immediately by the sterilization.

Dr. Abe 55) observed the Estradiol dose for the rats who suffered from Vitamin K deficiency forces the pregnancy's continuation. Dr. Kurokawa 57) has done detailed studies on the interior secretion glands under Vitamin A deficiency during conception and reported the changes caused by the pregnancy on the ovaries and the other interior secretion glands were slight, compared with the control group. But in these Vitamin A-, K-, deficiencies, the lesion of the placenta was found in the decidua and the gigantic cell stratum, in the early stage of the experiment, therefore, these differ from the case of Vitamin D deficiency. The organization of the chorion during the pregnancy needs a vigorous metabolism for itself and the growing fetus. Vitamin D deficiency is thought to hinder this metabolism, but it needs to be pursued more.

CONCLUSION

After the studies on the influence of Vitamin D upon the female sexual function under the experimental D deficiency administration, the ordinary

state of the rats, the change of the sexual cycle, the histological study of the endocrine glands and the sexual organs, and the D deficiency influence on pregnancy have been examined, and the following is my conclusion:

1. The effect on growth and the sexual cycle

D deficiency hindered growth of the immature group's rats. This was cured speedily by the administration of Vitamin D_2. The weight growth rate of the rats in the D administration group, is different from the control group. The sexual cycle got out of order around the 50th day of the deficiency. In the mature group, the symptoms of estrus were observed frequently at first; then many rats showed dioestrus. In the immature group, there were many rats who showed the symptoms of continuous dioestrus from the beginning of the experiment. In the non-sterilized group, estrus was found rarely with the 1000 I.U. Vitamin D, because of D administration. In the sterilized group, only the rats who were given Vitamin D with estrogen recovered their normal sexual cycle.

2. The effect on the endocrine organs and the sexual organs

In the case of D deficiency, the anterior pituitary suffers from atrophy and number decrease of the α-cells from the beginning of the experiment. The number of basophilic cells increased significantly at the beginning of the deficiency, but as the deficiency continued over a long period, atrophy and other functional deterioration were observed. In the adrenal cortex, a temporary functional acceleration was found at the beginning of the experiment, but later, the function deteriorates. In the ovaries, the atrophy was remarkable especially after the 90th day of deficiency. The deterioration pattern of the ovaries was divided in two; one was the multifollicalr degeneration and the other was the lack of the mature follicles. The uterus began to atrophy from the beginning of the deficiency. The experimental animals of the non-

sterilized group under the Vitamin D administration showed functional acceleration of the endocrine gland with the 100 I.U. dose, but with the 1000 I.U. dose, some restraint of the function was observed. The uterus grows in size according to the amount of administration. The Vitamin D administration in the sterilized group improved sterilization to some extent.

3. The effect on pregnancy

The Vitamin D deficiency caused the lowering of the pregnancy rate, the fetus-survival rate, and the abortion of the placenta. Histologically, under the deficiency, the incomplete formation of the placental Labyrinth, the retrograde metamorphosis of the syncytial trophoblast, and necrosis was observed. Necrosis and bleeding in the Labyrinth was the cause of the fetus' death.

Therefore, Vitamin D is found to have a similar kind of effect as estrogen on the female sexual function of white rats.

At the end of this thesis, I express my sincere gratitude to Professor Yoshimaro Jin for his guidance and revisions. I appreciate the advice and aid of Assistant Professor Ishihama, Instructor Sato, Instructor Iida, and especially Instructor Hamatsu for his long-term guidance. I also express my thanks to Esai Co. and Teikokuzoki Co. for the offer of the medicines used.

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ビタミンDの雌性性機能におよぼす影響にかんする実験的研究

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EXPERIMENTAL STUDIES ON THE INFLUENCE OF VITAMIN D UPON THE FEMALE SEXUAL FUNCTION

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To investigate the influences of vitamin D on female sexual function, Wistar strain rats were fed on vitamin D-free diet or were administered with vitamin D. (1) By feeding on vitamin D-free diet the sexual cycle of the rats were put out of order. In the adenohypophysis, α-cells decreased throughout the period of the experiment and δ-cells increased in just the early period. Atrophy of the adrenal cortex and the ovaries were seen after the middle stage of the period. Uterine atrophy began in early period. (2) In proportion of the dosis of vitamin D_2, both α- and β-cells were increased in the adenohypophysis, and functional acceleration of the adrenal cortex and ovaries were observed with uterine hypertrophy. Administration of vitamin D_2 was seemed to inhibit the castration changes of the adenohypophysis to some extent. (3) Vitamin D-deficiency caused marked interference to fertility and injury to fetal development owing to early atrophy of the syncytial trophoblast in the placental labyrinth in pregnant rats. After all, vitamin D deficiency brings on the sexual disorder to the female.

Dは化学構造上ステロイドホルモンに類似していることから性機能との関係を推測させ，そのEstrogen様作用[1)-7)]，D欠乏にもとづく性周期の停止[3)4)8)]，下垂体前葉細胞の変化[9)]，下垂体摘除後様症状の報告などがある[10)]。教室の茅根[11)12)]はD欠乏による性器萎縮と妊孕力の障害，D投与による性機能の調整を，大庭[13)]はDのEstrogen増強作用を報告した。

わたくしは実験的D欠乏およびD投与シロネズミにつき性周期および下垂体，副腎，卵巣，子宮の形態学的変化，ついで妊娠にたいする影響を検索したので報告する。

非妊娠シロネズミにたいするD欠乏およびD投与の影響

(1)　実験材料と実験方法

実験動物にはWistar系雌シロネズミを用い成熟群としては初体重120g前後で性周期の正確なもの，幼若群としては初体重40～50gで膣開口前の動物をえらんだ。欠乏実験は期間により成熟群は30，60，90，150，200日および対照群，幼若群は対照，30，60，90，120日の5群に回復試験群を加えた。D_2投与実験は非去勢・去勢ともにPlacebo，10，100，1000t.u.の4群に分け20日間連続注射した，1群は5～8匹ずつとした。

飼料には表1の合成飼料を用い，欠乏群の対照には飼料100g中にD_2 30t.u.あて添加した。投与実験にはすべて欠乏飼料をもちい，経口投与にはD液[*1)]を，注射にはD注射液[*1)]をTween 80と生理食塩水とで0.1

*1) エーザイKK製チョコラD

表1　ビタミンD欠乏飼料(%)

グラニュ糖	66.9
ビタミン除去カゼイン	20.0
DL-メチオニン	0.5
オレイン酸	3.0
リノール酸	0.15
ビタミン添加セルロース	5.0
混合塩	4.0

添加ビタミン(mg%)		Mameesh No. 446混合塩	
A	0.8	NaCl	243.2
E	1.2	$K_3C_6H_5O_7 \cdot H_2O$	533
K_1	1.0	K_2HPO_4	174
B_1	1.0	$CaHPO_4 \cdot 2H_2O$	800
B_2	1.0	$CaCO_3$	368
Ca-PaA	5.0	$MgCO_3$	92
PIN-HCl	0.5	$FeC_6H_5O_7 \cdot 5H_2O$	36
NiA	2.0	$CuSO_4 \cdot 5H_2O$	0.4
FA	0.1	$MnSO_4$	2.8
Be	0.1	$K_2Al_2(SO_4)_4 \cdot 2H_2O$	0.2
ビオチン	0.1	KI	0.1
塩化コリン	100.0	$CoCl_2 \cdot 6H_2O$	0.2
		$ZnCO_3$	0.1
		NaF	0.002

mlに希望量が含まれるよう作成し，PlaceboにはTween 80・生理食塩水混合液を用いた．飼料と水は自由摂取とした．飼育室は20～25℃に調節し，午前6時から午後6時まで点燈した．飼育室の窓は外側が9mmの金網入りすりガラス，内側は普通窓ガラスとし，その内側は青色カーテンによつて直射日光をさえぎつた．

(2)　一般状態および性周期

1)　D欠乏のばあい

欠乏期間が長びくにつれ被毛があれ光沢を失い一部では尾に瘢皮を形成し，運動も不活発となつた．成熟群の体重曲線は対照と大差がなかつたが，幼若群では体重増加が対照より劣り40日以降は停止した．これらのうち性周期障害の強い7例に欠乏67日から毎日50 i.u. を経口投与したところ全例に体重増加，性周期の正順化をみた(図1，表2)．

性周期は毎日午前10時前後に膣スミアにより観察した．成熟群では欠乏50日ごろから発情期像が頻発し90日ごろには一部で持続発情期像を呈したが，その後漸次角化不全をきたし150日前後から静止期の延長を示した(図2，3)．幼若群では膣開口の時期に差がなかつたが，対照群においては実験開始後25日で全例性周

図1　D欠乏飼育シロネズミ発育曲線

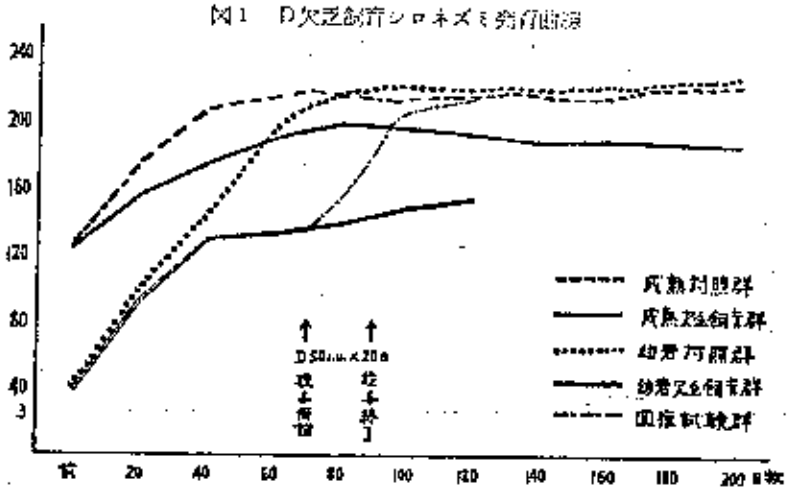
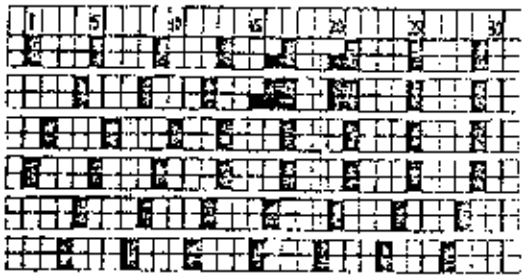


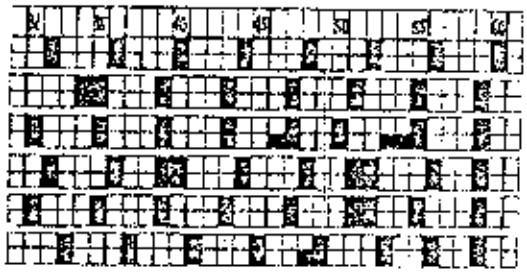
表2　回復試験期間における各群増加体重の比較(20日間)

実験群		例数	平均値 (g)	標準偏差 (±σ)	信頼限界 (95%)	検定	有意性
A	対照群	5	47.6	26.8	37.2	AとB	$P<0.05$
B	回復試験群	7	54.1	21.9	21.9	AとC	$0.3<P<0.4$
C	欠乏群	12	34.5	13.6	9.1	BとC	$P<0.01$

図2 D欠乏飼育が成熟シロネズミ性周期におよぼす影響(1)



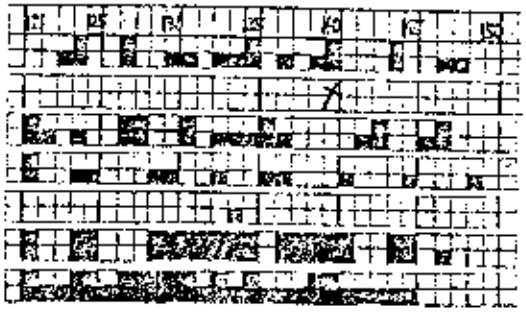
30日



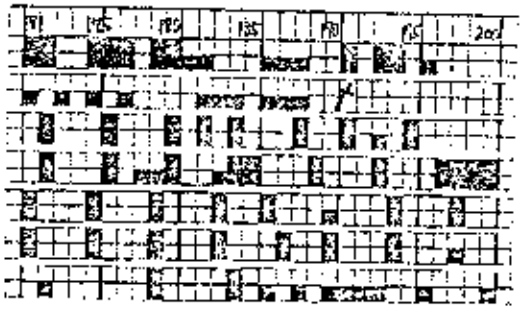
60日

90日

図3 D欠乏飼育が成熟シロネズミ性周期におよぼす影響(2)



150日



200日 ×死亡日

期が頻調となつたのに，欠乏群においてはなお半数以上が不整であつた．40日以降ではいずれも静止期像の延長，不全角化像について60日ごろから持続静止期像に移行した(図4)が回復試験の結果はすべて正常な性周期となつた(図5)．しかし欠乏群には全く性周期の変調をみないものもあり，個体差がみられた．

2) D投与のばあい

各群の体重増加率には投与量による差はみられなかつた．性周期も非去勢成熟群に1000i.u.のD投与で不全角化像をみたほかは変調がなく(図6)幼若群では腟開口期，性周期ともに各群間に差がなかつた(図7)．去勢群はすべて持続静止期像を示した．そこでこれらのシロネズミにEstradiol**) 0.01μgとD_2 10，100または1000i.u.を[illegible]Estradiol 0.05，0.02または0.01μgとTween 80・生理食塩水混合液とを注射した．その結果は図8のごとく役者ではほとんど完全角化像を認めなかつたが，D併用例では投与量に比例し完全角化像の発現率が増加した．

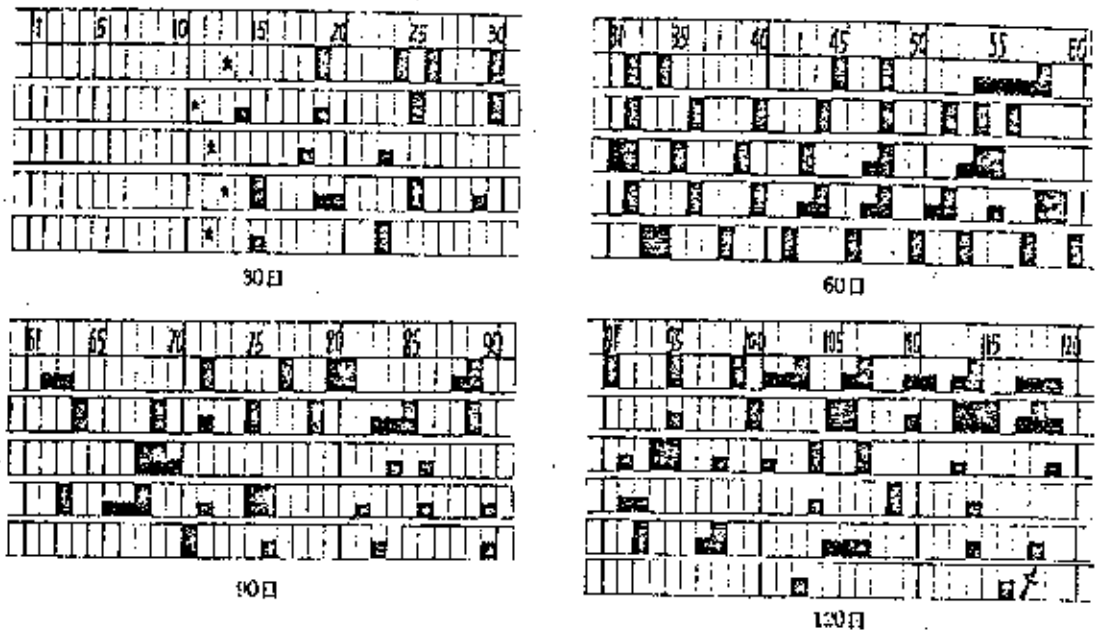
3) 小 括

従来Dの投与実験に用いられたSteenbock・Black No. 2965飼料は栄養上D欠乏飼料としては不適当とされ，現在では各種ビタミン添加の合成飼料が用いられている[14]．また暗室飼育は動物の性周期に影響を与えるし[15]，プロ-Dを活性化する紫外線の有効波長は普通窓ガラスではほとんど吸収されるので，この実験では暗室飼育は行なわなかつた．

Dの発育促進効果はDがTCAサイクルに促進的に作用し[16]，食欲増加[17]，胃液分泌増加[18]を招くことが主因と考えられている．また飼料中のCa/P比も関与しこれが小さいときは発育を促進し，大きいときは抑制するという[19]．宮崎ら[20]もCa/P比の大きいときの発育はむしろD欠乏群がよく，クル病の発現をみるが，比が1に近いときはクル病は起こらないという．本実験での飼料のCa/Pは1.5/1.0で欠乏各群ともクル

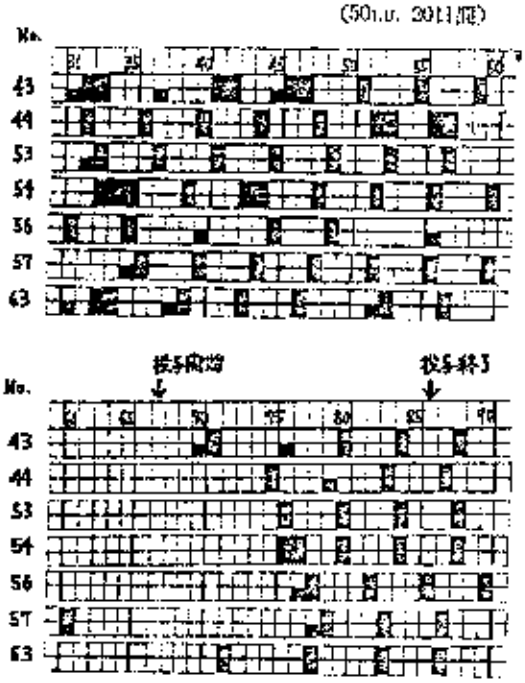
**) オバホルモン(帝国賓器KK)

図4　D欠乏飼育が幼若シロネズミ性周期におよぼす影響(3)



＊腟開口日　　×死亡日

図5　D欠乏飼育シロネズミの回復試験
(50i.u. 20日間)

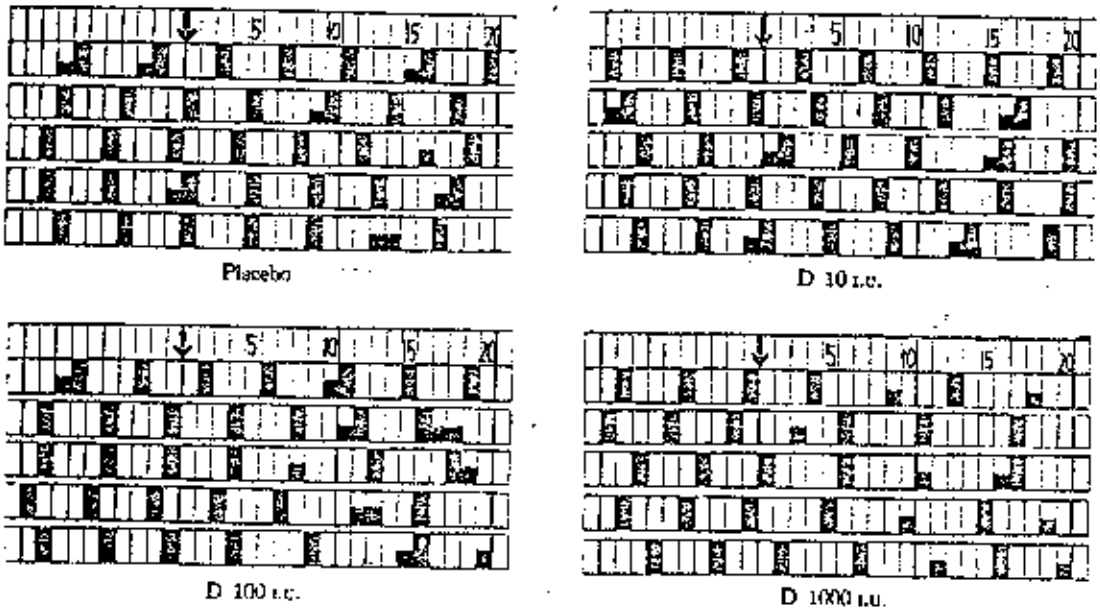


病所見はなかつた。成熟群では欠乏飼育例と対照との間に体重増加が抑制され，これはD投与で速やかに回復し性周期も正常に復したので，体重増加の抑制はD欠乏によるものとみてよいであろう。

性周期にたいするD欠乏の影響は緩慢で一般に50日以降にみられた。成熟群では発情期像の頻発，持続，ついで不全角化像から静止期像の延長を，幼若群では大部分希発発情期像から持続静止期像への移行をみとめた。回復試験によりこれらはD欠乏によることを確認できたが，個体差があるのは体内蓄積Dの多少や性機能未熟などによるものであろう。ある種のネズミ(Wistar系も含めて)は老齢で自然に連続発情像を示し[21]，連続照明，出生直後の性ホルモン投与[22,23]なども持続発情像を惹起させる。竹脇[25]はこれらの現象は性中枢の未分化または老化が原因であろうと述べている。本実験のばあいは対照群の性周期には異常がなかつたので，D欠乏は卵巣あるいは性中枢の変容を招き性周期を乱したものと推測できる。

シロネズミではDの1日必要量はほぼ1 i.u.(D_2 0.025 μg)とされ一般に必要量の20～1000倍量で中毒症状を起こすとされている。本実験の10～1000i.u. 20日間の投与では去勢・非去勢両群とも性周期に影響がなかつた。しかし単独では去勢シロネズミに完全角化を起こし得ない微量のEstrogenにDを併用すると完全角化像を起こし得る。このことからDがEstrogenの作用を増強させるという大庭[26]の報告が肯定される。

図6 D投与非去勢成熟シロネズミ性周期(連日20日間)



Placebo

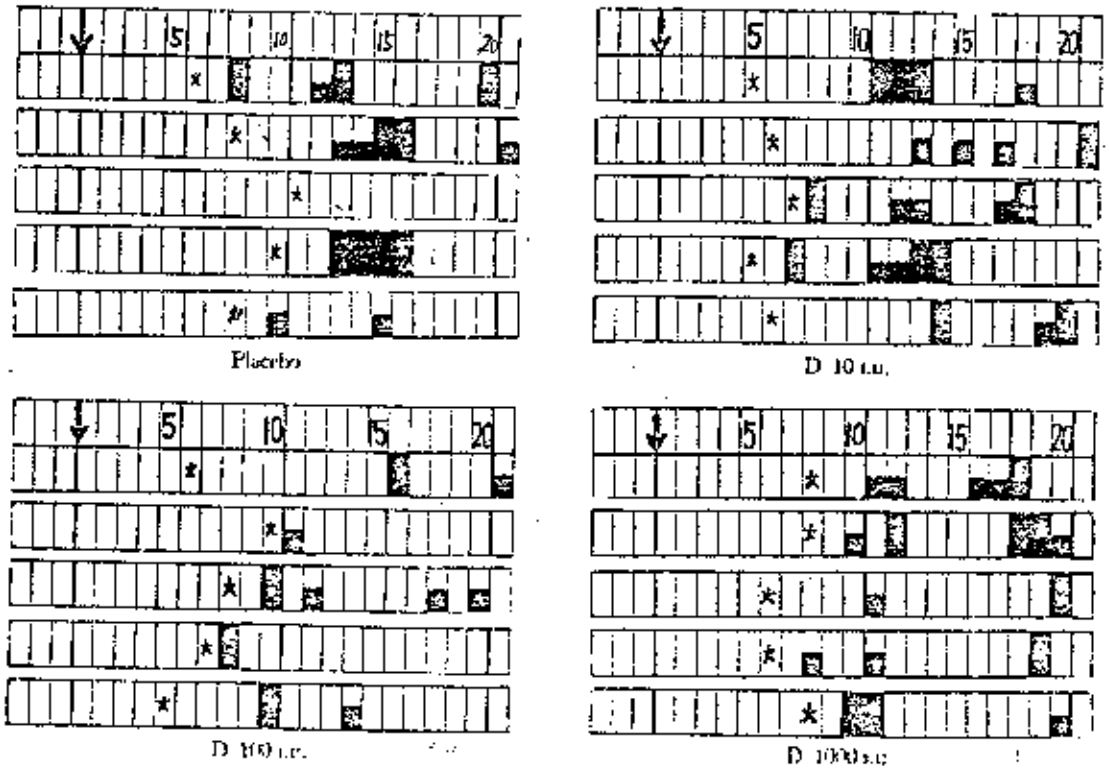
D 10 I.U.

D 100 I.U.

D 1000 I.U.

↓ 投与開始

図7 D投与非去勢幼若シロネズミ性周期(連日20日間)



Placebo

D 10 I.U.

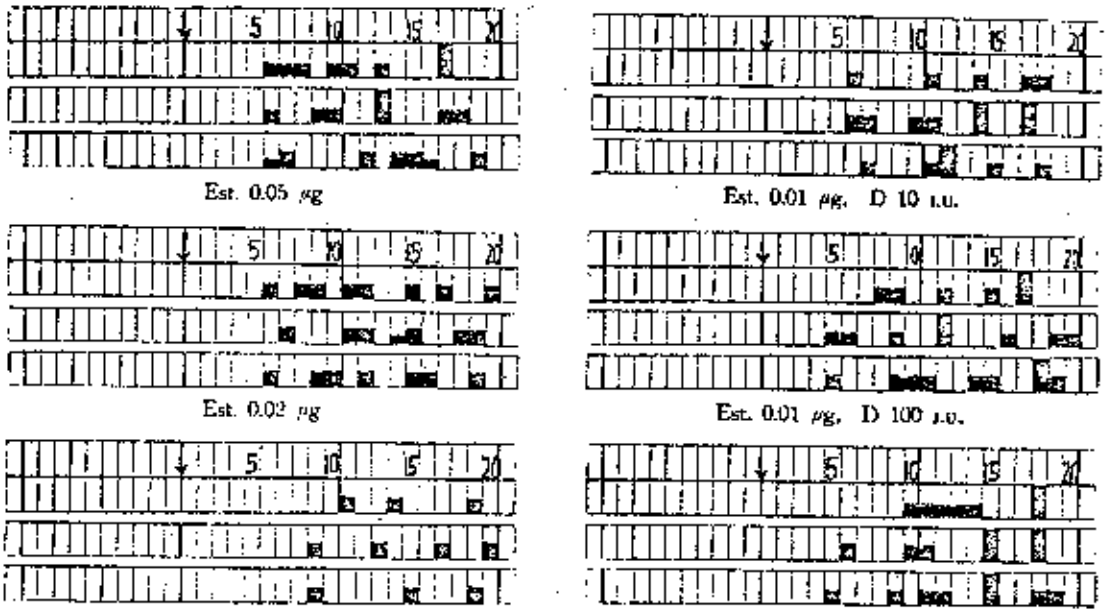
D 100 I.U.

D 1000 I.U.

↓ 投与開始 * 鞏明日日

3327

図8　去勢シロネズミにたいするDとEstrogen少量投与の影響



Est. 0.05 μg　　Est. 0.01 μg, D 10 I.U.

Est. 0.02 μg　　Est. 0.01 μg, D 100 I.U.

Est. 0.01 μg　　Est. 0.01 μg, D 1000 I.U.

↓投与開始

下垂体，副腎，卵巣および子宮におよぼすDの影響

(1)　実験材料と実験方法

前記一般状態，性周期探索後の動物を用いた．各群とも断頭屠殺後ただちに下垂体，副腎，卵巣および子宮を摘出．Torsion balanceで秤量後組織学的検索のため，それぞれの固定液で固定した．臓器重量は対体重%で比較した．組織標本の作成にあたつては下垂体はクロム明ばん液[29]固定後，臓器中央前額断についてパラフィン切片を作成し，Elftmann[30][31]のAF*3)-Schiff染色およびTrichrome染色を行ない，成熟群に行なつた各細胞数の算定には井上[32]の3視野法をもちいた．副腎，卵巣，子宮はホルマリン液で固定し，副腎ではH・E染色，Vines染色およびSudan IV染色を，卵巣および子宮ではH・E染色を行なつた．卵胞および黄体の分類は古賀[33]に準じた．

(2)　D欠乏のばあい

1)　臓器重量の変動

成熟群では下垂体が欠乏60日で有意に増加し，卵巣は90日から，子宮は欠乏初期から有意の減少をみたが副腎には変化がなかつた(表3)．幼若群も卵巣，子宮は成熟群と同時期から有意に減少したが，下垂体，副腎には有意の変化がみられなかつた(表4)．

2)　組織学的所見

下垂体前葉

対照群での好酸性細胞(α-細胞)はAcid FuchsinまたはOrange G好性で，とくに前葉中央部と側後方に密集し，数的には平均32.7%を占めていた．色素塩性細胞(γ-細胞)は62.3%と最も多く，好塩基性細胞は最も少なく4.9%で，AF-Schiff染色で淡紅色のGonadotroph(δ-細胞)と暗紫色のThyrotroph(β-細胞)とに区別される[34][35]．β-細胞は好塩基性細胞の大部分を占め(80.2%)，主として前葉中央部に散在しδ-細胞はとくに成熟雌シロネズミでは数が少なかつた(19.8%)．性周期によるこれら各細胞数の変動は平均してα-細胞3.7%，γ-細胞3.2%，β-，δ-細胞0.8%とあまり変化がなかつた．

D欠乏の成熟群ではα-細胞は欠乏60日以降は減少し胞体の萎縮，分泌顆粒の減少がみられたが幼若群では欠乏初期から減少を認めた．好塩基細胞は成熟群では欠乏30日で9.0%と著増し，60日までは，やや多かつたがその後は減少した．β-およびδ-細胞の割合は30日で

*3) AF = Aldehyde・Fuchsin　　*4) H・E = Hematoxylin・Eosin

表3　D欠乏シロネズミの体重あたり臓器重量(成熟群)

臓器	実験群 欠乏日数	例数	平均重量 (mg%)	標準偏差 (±σ)	信頼限界 (95%)	検定
下垂体	対照	8	5.0	0.25	0.37	—
	30	6	5.1	0.25	0.34	0.8<P<0.9
	60	6	4.9	0.23	0.36	0.6<P<0.7
	90	5	6.6	0.13	0.21	P<0.01
	150	6	5.5	0.11	0.23	0.3<P<0.4
	200	6	4.9	0.11	0.18	0.6<P<0.7
副腎	対照	8	21	2.1	2.7	—
	30	6	24	2.8	3.6	0.3<P<0.4
	60	6	21	2.0	2.7	0.6<P<0.7
	90	5	23	2.7	3.2	0.6<P<0.7
	150	6	21	1.5	1.8	—
	200	6	20	0.7	1.2	—
卵巣	対照	8	29	1.4	2.2	—
	30	6	30	1.0	2.0	0.6<P<0.7
	60	6	35	1.3	1.7	0.05<P<0.1
	90	5	18	2.8	3.1	P<0.001
	150	6	18	1.7	2.1	P<0.001
	200	6	13	2.3	2.6	P<0.001
子宮	対照	8	320	24	2.9	—
	30	6	195	30	3.7	P<0.001
	60	6	220	13	1.6	P<0.01
	90	5	262	20	2.2	P<0.05
	150	6	242	22	3.0	P<0.05
	200	6	172	24	3.3	P<0.01

はともに増加していたが，β-細胞の45.2%にたいしα-細胞は54.8%と後者の増加が著しかつた．60日では β-細胞はほぼ正常に復しその後は減少し小型化したが分泌顆粒は減少せず増殖の傾向をみせ，α-細胞は150日までは軽度増加のままで経過したが，胞体は萎縮性，分泌顆粒は減少した．幼若群では欠乏初期からβ-細胞は減少し，α-細胞は欠乏後期となつたときに減少した(図9，10，表5．6)．

副腎皮質

H-E染色所見　球状帯では欠乏末期に層帯の短縮，細胞質充実度の低下，核濃縮をみた．束状帯は成熟群で90日より層帯の短縮，細胞索の乱れ，細胞質充実度の低下，核濃縮および核崩壊をみ，幼若群は30日で内束状帯細胞の好酸性がやや増加したが，60日以降は成熟群と同様の退行変化を呈した．網状帯は成熟群60日までは胞体が軽度に肥大し，細胞質の好酸性が増し，90日以降は萎縮にうつり，その後は高度の萎縮を示した．幼若群ではやや早く60日から萎縮が始まつた．髄質は早期に萎縮しはじめ90日以降ではほとんど消失した(図10)．

Vines染色所見　両群とも欠乏早期から各所帯で顆粒の減少がみられた(表7)．

Sudan IV染色所見　両群とも欠乏初期には内束状帯，網状帯で脂肪顆粒が減少したが，90日以降では逆に増加し，しだいに粗大病状となつた(表8)．

卵巣

卵巣の変化は2つに分けられる．1つは黄体が欠除または著減し，ほとんど巨大ないし大卵胞のみとなるもの，他は大卵胞がなく小卵胞と小型の残留黄体のみのもので，前者は成熟群に，後者は両群にみられた．すなわち成熟群では60日までは大卵胞が減少し，黄体は対照と差がなかつたが，90日以降では直径1000μ以上の巨大卵胞が増殖し，大中小卵胞が卵巣の大部分を占め，黄体は少なく細胞性で小型化し，いわゆる多

表4　D欠乏シロネズミの体重あたり臓器重量(幼若群)

臓器	実験群 欠乏日数	例数	平均重量 (mg%)	標準偏差 (±σ)	信頼限界 (95%)	検定
下垂体	対照	5	4.9	0.15	0.28	—
	30	5	5.2	0.12	0.23	0.7<P<0.8
	60	5	5.7	0.27	0.47	0.5<P<0.6
	90	5	4.1	0.21	0.36	0.3<P<0.4
	120	5	4.1	0.20	0.26	0.3<P<0.4
副腎	対照	5	21	1.4	1.9	—
	30	5	23	1.6	2.0	0.6<P<0.7
	60	5	28	1.7	2.8	0.4<P<0.5
	90	5	20	1.4	1.9	0.6<P<0.7
	120	5	18	1.6	2.0	0.5<P<0.6
卵巣	対照	5	29	2.4	3.4	—
	30	5	28	2.0	2.9	0.1<P<0.2
	60	5	26	2.3	3.1	0.1<P<0.2
	90	5	18	2.6	3.4	P<0.001
	120	5	14	2.8	4.1	P<0.001
子宮	対照	5	320	15	25	—
	30	5	221	18	32	P<0.02
	60	5	143	11	20	P<0.001
	90	5	148	18	23	P<0.001
	120	5	135	20	31	P<0.001

表5　D欠乏と下垂体前葉各細胞の変動

実験群 (欠乏日数)	好酸性細胞(%)	好塩基性細胞(%)	色素嫌性細胞(%)	計
対照	32.8(1999)	4.9(298)	62.3(3803)	(6100)
30	30.4(1284)	9.0(380)	60.6(2562)	(4226)
60	26.3(1314)	5.3(266)	68.4(3425)	(5005)
90	23.1(1487)	3.2(205)	73.7(4752)	(6444)
150	22.9(1630)	4.3(304)	72.8(5185)	(7119)
200	25.4(1099)	3.2(138)	71.4(3091)	(4328)

(　)内は実測細胞数

表6　D欠乏と下垂体前葉好塩基性細胞の変動

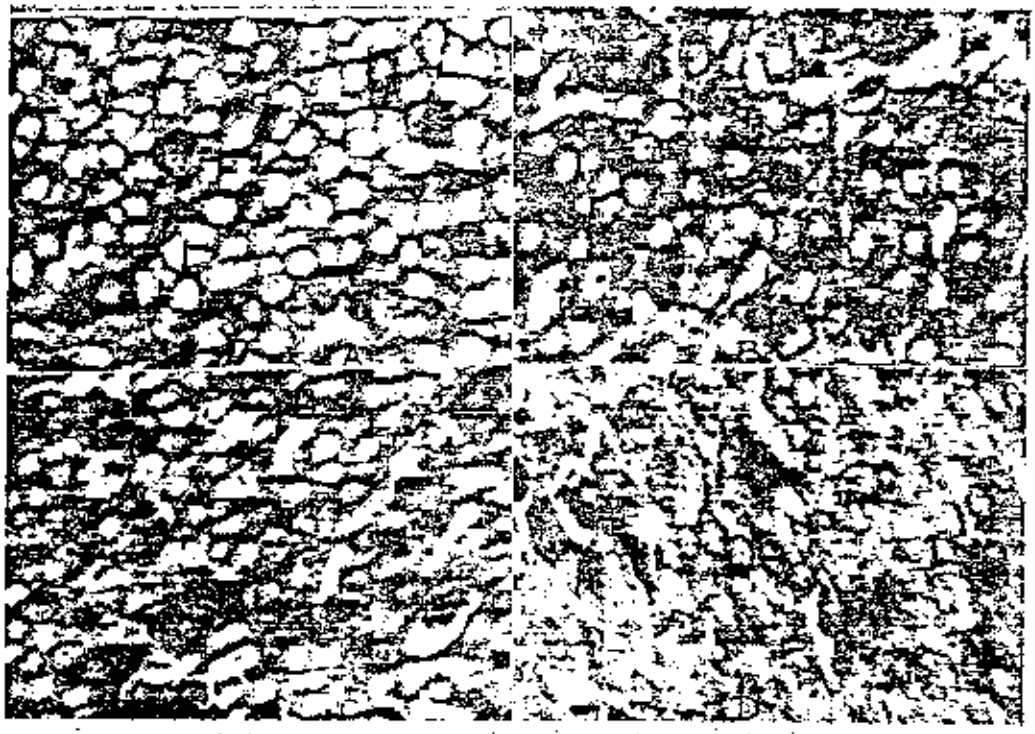
実験群 (欠乏日数)	β-細胞(%)	δ-細胞(%)	計
対照	80.2(239)	19.8(59)	(298)
30	45.2(225)	54.8(155)	(380)
60	66.9(178)	33.1(88)	(266)
90	68.2(140)	31.8(65)	(205)
150	64.8(197)	35.2(107)	(304)
200	74.6(103)	25.4(35)	(138)

(　)内は実測細胞数

表7　D欠乏飼育による副腎皮質Vinic陽性類型の変動

欠乏日数		球状帯	束状帯 外束状帯	束状帯 内束状帯	網状帯	傍髄皮質
成熟非去勢	対照	−	+	+	+	+〜++
	30	(±)	+	+	+	+〜++
	60	(±)	+〜±	+〜±	+〜±	+〜±
	90	(±)	±〜++	±〜+	±	±〜+
	150	−	±	±	±	±〜(+)
	200	−	±	±	±	±〜(+)
幼若非去勢	対照	±	+	+	+〜++	++
	30	±	+〜±	+〜±	+〜±	+〜±
	60	±	±〜+	±	±	±
	90	(±)	±	±	±	±
	120	(±)	±	±	±〜+	+〜±

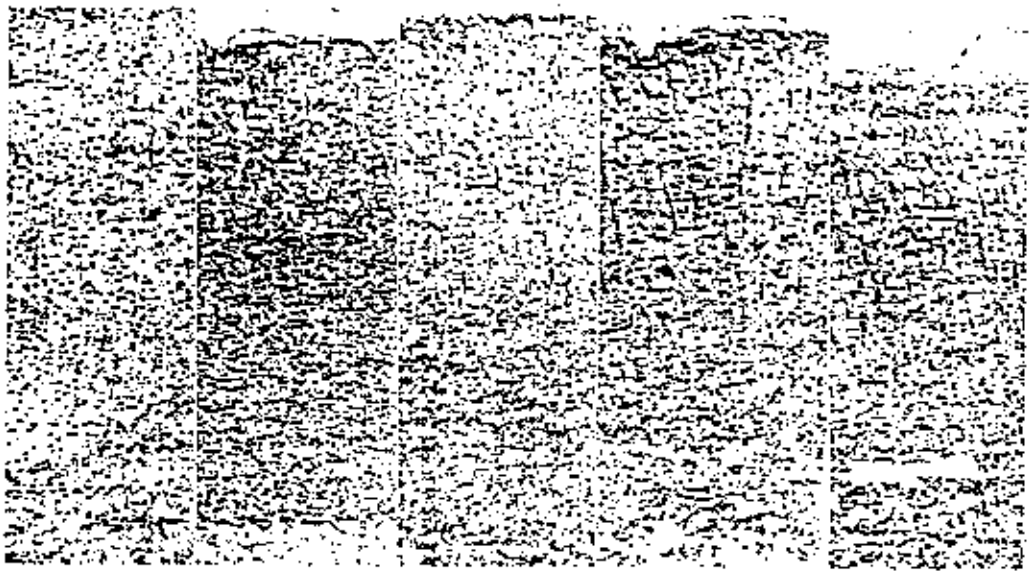
図9　D欠乏下垂体前葉細胞の変化(成熟群)



(AF-Schiff染色　20×40)

A：対照
B：欠乏30日，β，δ-細胞の増加
C：欠乏90日，β-細胞は著減なくδ-細胞は減少
D：欠乏200日，β-，δ-細胞減少

図10　D欠乏副腎皮質(成熟群)



(H・E染色)

A 対照　B 欠乏30日　C 欠乏60日　D 欠乏150日　E 欠乏200日

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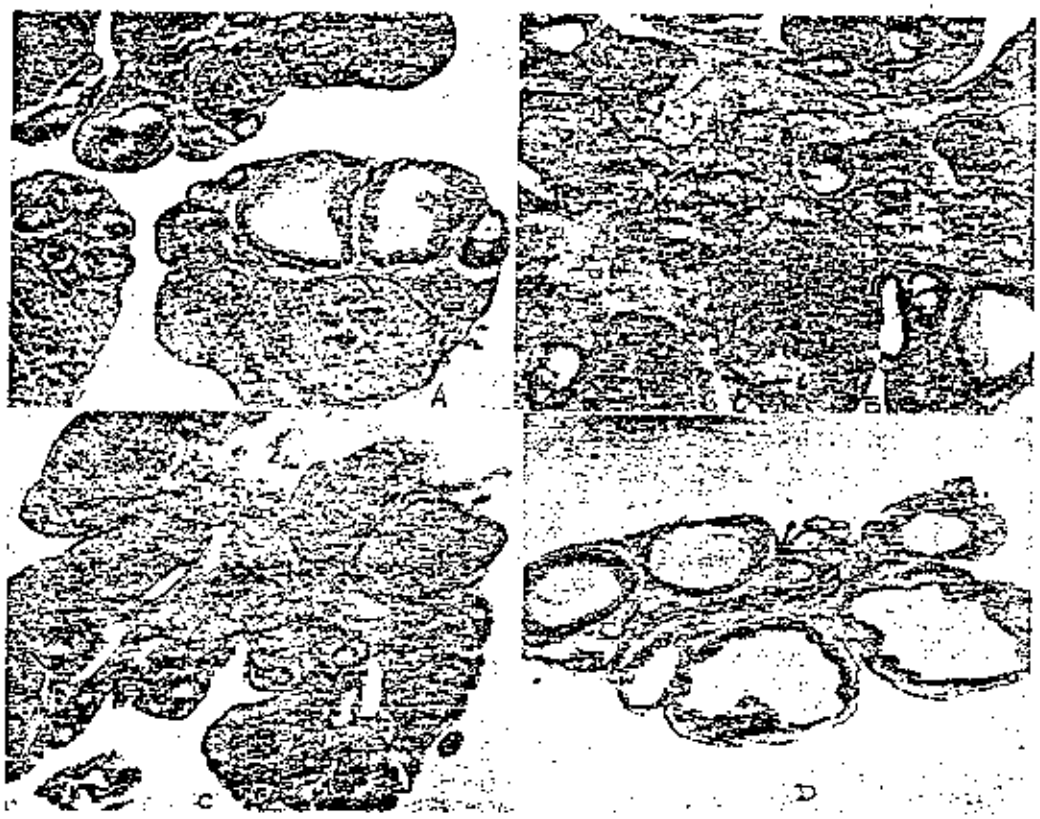
褐胞変性を示した。これら卵巣では顆粒膜細胞層が萎縮し胞体は暗調小型で核濃縮を示したが、夾膜細胞層はむしろ肥厚し、間質は疎となつていた。黄体には結合織の侵入が著明で細胞境界不鮮明。細胞質の空胞化

図11　D欠乏卵巣(成熟群)



A：対照
B：欠乏60日
C：欠乏150日、成熟卵胞の欠如
D：欠乏150日、多褐胞変性を呈し萎縮

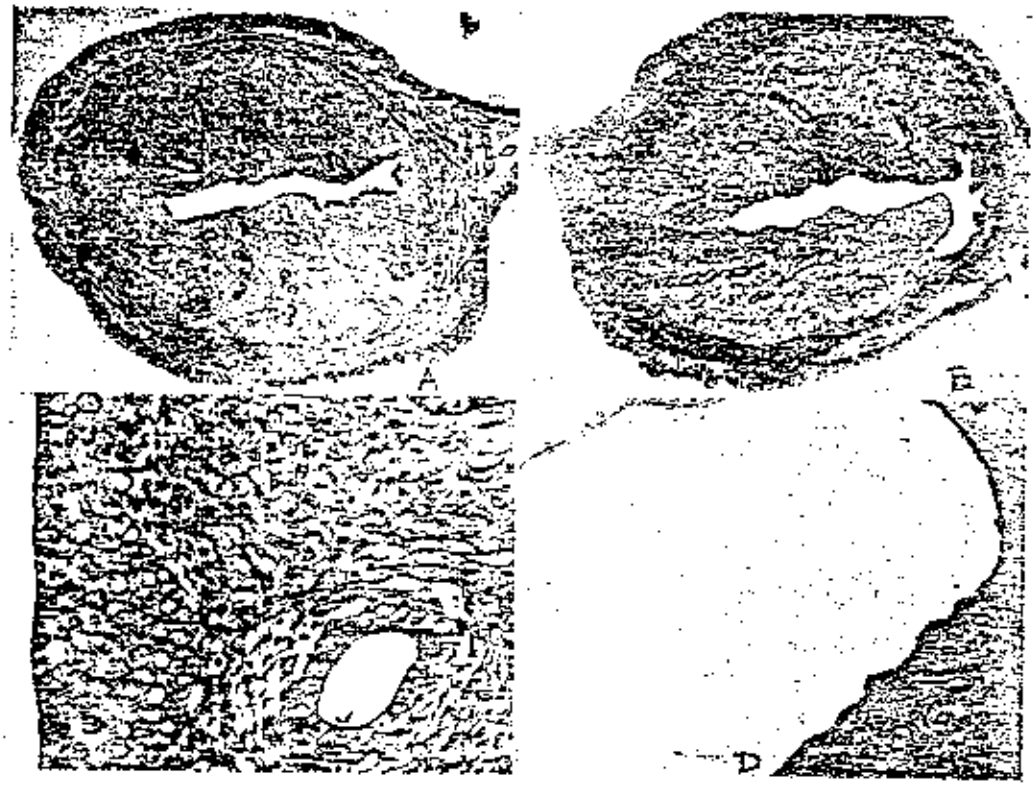
表8　D欠乏飼育による副腎皮質Sudan IV陽性顆粒の変動

欠乏日数		球状帯	中間帯	束状帯		網状帯	傍髄皮質
				外束状帯	内束状帯		
成熟非去勢	対照	⧻	—	⧺	⧺	⧺	±～(+)
	30	⧺～⧻	—	⧺～⧻	⧺～⧻	±～(+)	±
	60	⧺	—	⧺～⧻	⧻～⧻	+～(±)	±～+
	90	⧻	—	⧺	⧺	⧻～(±)	±～+
	150	⧻	±	⧺～⧻	⧺	+～(±)	±～+
	200	⧻	±	⧺～⧻	⧺	+	+
幼若非去勢	対照	⧻～⧺～(±)	—	⧺	⧺	±～(+)	±
	30	⧺～(±)	⧺～(—)	⧺～⧻	⧺～⧻	±～(+)	±
	60	⧻～(±)	⧺～(—)	⧺～⧻	⧺～⧻	±～(+)	±～(+)
	90	(⧺)～(±)	—～(+)	⧻	⧺～⧻	±～+	±～(+)
	120	(⧺)～(±)	—～(+)	⧻	⧺～⧻	±～(+)	±～(+)

表9　D欠乏飼育時の卵巣所見

欠乏日数		発育卵胞 巨大	大	中	小	原始卵胞	黄体 巨大	大	中	閉鎖卵胞
成熟	対照	−	+	+	+	+	+	+	+	散
	30	−	±	+	±〜+	++	+	+	+	散
	60	−	+	++	+〜++	+	+	+〜++	+	散
	90	+	+	+	+	+	−	++	±	散
	150	+〜++	+	+	±〜+	+	−	−	−	稀
		−	±	±	±	+〜±	−	+〜++	+	稀
	200	+	+	+	±〜+	±〜+	−	−	−	散
		±	+	+	±〜+	±	+	+	+	散
幼若	対照	−	+	+〜++	+〜±	+	±	+	+	散
	30	−	+	+	±〜+	±	±	+	+	散
	60	−	±	+〜++	±〜+	±〜+	±	+	+〜++	散
	90	−	±	+	+〜++	+	±	+	+〜++	稀
	120	−	±	+	+〜++	+〜++	±	+	+	散

図12　D欠乏子宮(成熟群)



(H・E染色)

A：対照

B：欠乏60日

C：欠乏90日、粘膜上皮の扁平上皮化性

D：欠乏200日、粘膜固有層萎縮による子宮腺萎縮

があつた．150日以降ではこれら多嚢胞変性卵巣と萎縮閉鎖卵巣との2型がみられた．幼若群の卵巣萎縮は60日からみとめられ，成熟卵胞欠除と黄体の変性を示した(図1，表9)．

子宮

両群とも早期から萎縮し，子宮内腔は30日でやや非薄となり，90日では粘膜上皮細胞は方形状，部分的には扁平化していた．粘膜固有層も60日で非薄化し90では結合織が増し，子宮腺上皮が扁平化し，子宮腔は拡大した．筋層は成熟群では末期に萎縮するが，幼若群では初期からその発育が障害されていた．

3) 小括

D欠乏により下垂体重量は60～90日までは増し，のちに減少傾向をみせたが，副腎重量はとくに変化がみられなかつた．しかし組織学的な影響は両者ともかなり著明であつた．下垂体前葉ではα-細胞の機能低下像，好塩基細胞の細型比率の変動がみられた．初期にはβ-，δ-両細胞とも増加し，とくにδ-細胞に著明であつたが，まもなくともに減少し胞体が萎縮し機能低下像を示した．副腎皮質では欠乏初期には内束帯の好酸性の増強，脂肪顆粒の変性化など軽度機能亢進像がみられたが，著次機能低下像があらわれた．卵巣に90日降重量減少し組織学的に萎縮像を示し，1つは黄体の欠如，他は成熟卵胞の欠除を特徴とする2型に分かれた．子宮の萎縮は下垂体，副腎，卵巣の障害よりは早期から認められた．

(3) D投与のばあい

1) 臓器重量の変化

非去勢群　成熟群では著変がなく，幼若群ではD_2 1000i.u.投与のばあい卵巣に有意の低下と100i.u.，1000i.u.のD_2投与で子宮に増加がみられた(表10，11)．

去勢群　成熟群10i.u.，100i.u.で副腎が増加し，子宮では100i.u.，1000i.u.で有意に増加した．幼若群でも10i.u.で下垂体が減少，100i.u.，1000i.u.で子宮が増加した．またEstradiol単独投与に比較し，D併用例では副腎，子宮に増加の傾向をみた(表12，13)．

2) 組織学的所見

下垂体前葉

非去勢群　成熟・幼若両群ともにα-細胞の腫大，増加をみたが，1000i.u.投与では幼若群のα-細胞はかえつて萎縮していた．好塩基細胞は10i.u.ではやや減少したが，投与量の増大とともに増加した．増加は主としてβ-細胞でδ-細胞にはあまり変化がなく，投与量をますとむしろ減少傾向が認められた(図13，表14，15)．

去勢群　Placebo群ではα-細胞はやや減少していたが，胞体は軽度に腫大し分泌顆粒は減少して成熟群となつた．著明な変化は好塩基性細胞の著増と胞体の腫大

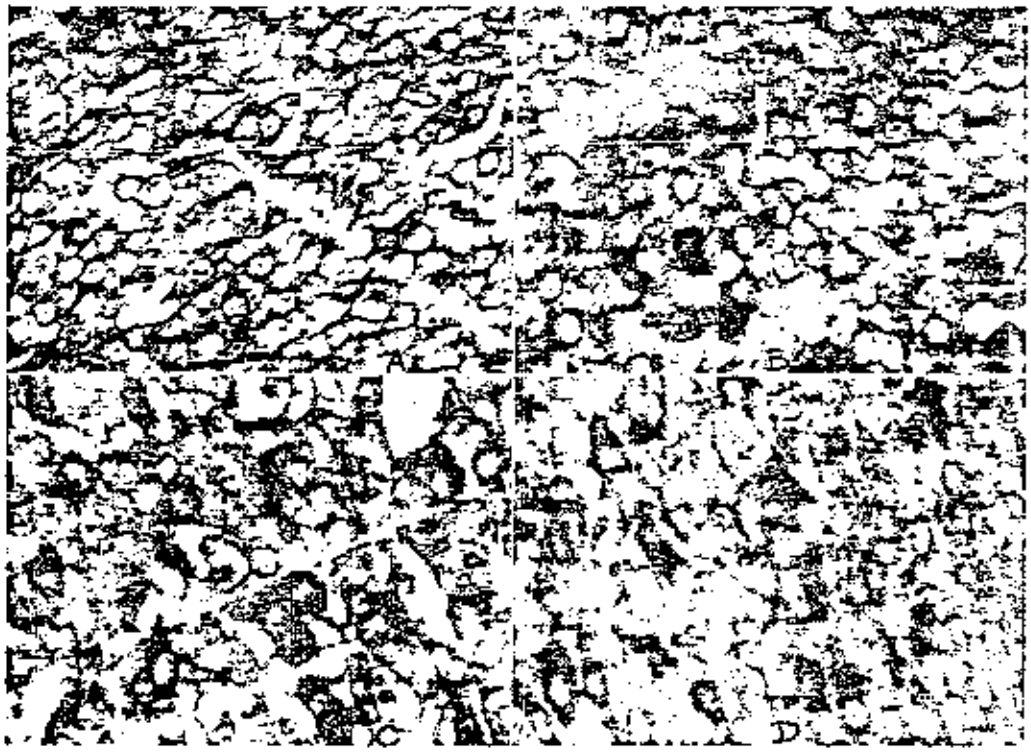
表10．D投与シロネズミの体重あたり臓器重量(成熟非去勢群)

臓器	実験群 D投与量(i.u.)	頭数	平均重量 (mg%)	標準偏差 (±σ)	信頼限界 (95%)	検定
下垂体	Placebo	5	5.1	0.21	0.32	—
	10	5	4.8	0.45	0.51	0.5＜P＜0.6
	100	5	5.2	0.36	0.42	0.7＜P＜0.8
	1000	5	5.0	0.22	0.30	0.7＜P＜0.8
副腎	Placebo	5	26	2.6	3.3	—
	10	5	23	2.0	3.0	0.4＜P＜0.5
	100	5	21	3.2	4.1	0.1＜P＜0.2
	1000	5	26	2.7	3.8	0.8＜P＜0.9
卵巣	Placebo	5	34	2.1	2.8	—
	10	5	38	3.4	4.1	0.2＜P＜0.3
	100	5	37	2.0	2.6	0.4＜P＜0.5
	1000	5	36	2.2	3.0	0.7＜P＜0.8
子宮	Placebo	5	216	23	3.2	—
	10	5	229	32	3.9	0.7＜P＜0.8
	100	5	253	27	3.8	0.2＜P＜0.3
	1000	5	261	41	4.9	0.1＜P＜0.2

表11 D投与シロネズミの体重あたり臓器重量(幼若非去勢群)

臓器	実験群 D投与量(i.u.)	例数	平均値 (mg%)	標準偏差 (±σ)	信頼限界 (95%)	検定
下垂体	Placebo	5	4.3	0.16	0.46	—
	10	5	3.6	0.12	0.22	0.1<P<0.2
	100	5	3.9	0.26	0.34	0.5<P<0.6
	1000	5	3.3	0.24	0.42	0.1<P<0.2
副腎	Placebo	5	22	1.7	2.3	—
	10	5	21	2.9	4.5	0.6<P<0.7
	100	5	23	1.3	2.5	0.6<P<0.7
	1000	5	22	3.9	5.2	0.6<P<0.7
卵巣	Placebo	5	36	2.3	3.8	—
	10	5	36	1.5	2.5	—
	100	5	33	2.4	3.2	0.3<P<0.4
	1000	5	27	2.9	3.1	P<0.02
子宮	Placebo	5	124	24	38	—
	10	5	153	16	23	0.1<P<0.2
	100	5	192	26	39	P<0.02
	1000	5	196	25	36	P<0.01

図13 D投与下垂体前葉細胞の変化(成熟非去勢群)



(AF-Schiff染色 20×40)

A : Placebo

B : D 10 i.u., β-細胞やや増加

C : D 100 i.u., β-細胞の増加, δ-細胞やや減少

D : D 1000 i.u., β-細胞増加 δ-細胞減少

表12　D投与シロネズミの体重あたり臓器重量(成熟去勢群)

臓器		実験群 D投与量(i.u.)	例数	平均重量 (mg%)	標準偏差 (±σ)	信頼限界 (95%)	検定
成熟去勢	下垂体	Placebo	5	3.5	0.21	0.28	—
		10	5	3.7	0.16	0.22	0.4<P<0.5
		100	5	3.4	0.14	0.24	0.6<P<0.7
		1000	5	4.3	0.20	0.28	0.05<P<0.1
	副腎	Placebo	5	20	1.6	3.1	—
		10	5	24	1.5	2.6	0.02<P<0.05
		100	5	27	2.1	3.1	P<0.02
		1000	5	26	2.4	3.5	0.1<P<0.2
	子宮	Placebo	5	26	1.4	1.8	—
		10	5	29	1.8	2.1	0.1<P<0.2
		100	5	38	2.0	1.6	P<0.01
		1000	5	32	2.6	2.5	0.02<P<0.05
幼若去勢	下垂体	Placebo	5	4.2	0.16	0.18	—
		10	5	3.3	0.16	0.22	P<0.0
		100	5	3.5	0.26	0.24	20.05<P<0.1
		1000	5	4.7	0.24	0.31	0.2>P<0.3
	副腎	Placebo	5	21	1.2	20	—
		10	5	22	1.0	15	0.8<P<0.9
		100	5	19	2.4	28	0.2<P<0.3
		1000	5	26	2.2	23	0.2<P<0.3
	子宮	Placebo	5	26	1.6	24	—
		10	5	28	1.2	18	0.7<P<0.8
		100	5	38	2.2	30	P<0.01
		1000	5	35	2.1	26	0.02<P<0.05

表13　Estradiol・D併用20日間投与における去勢シロネズミ体重あたり臓器重量(mg%)

投与量 Estradiol (μg)	投与量 D (i.u.)	下垂体	副腎	子宮
0.05	—	4.5	26	35
0.02	—	3.6	14	21
0.01	—	4.6	17	28
0.01	10	3.6	16	45
0.01	100	4.6	19	36
0.01	1000	4.2	35	42

で，核は偏在し胞体内にはコロイド様物質が貯留し空胞化がみとめられ，いわゆる去勢細胞が認められた．この細胞はAF-Schiff染色で淡紅色に染まり，AF陽性で暗赤色を呈するβ-細胞とは区別される．D投与によるこれら細胞の変化は表15のごとくでα-細胞は増加し染色性の増強を認め，去勢細胞は減少し胞体空胞化の改善がみられた．Estradiol 0.05～0.01μgの単独投与では去勢細胞発現率に大差はなかつたが，D併用例では10i.u.でEstradiol 0.05μg単独より去勢細胞発現率が減少し，D量をますに伴ない去勢変化の回復がみられた(図14，15)．

副腎皮質のH・E染色所見

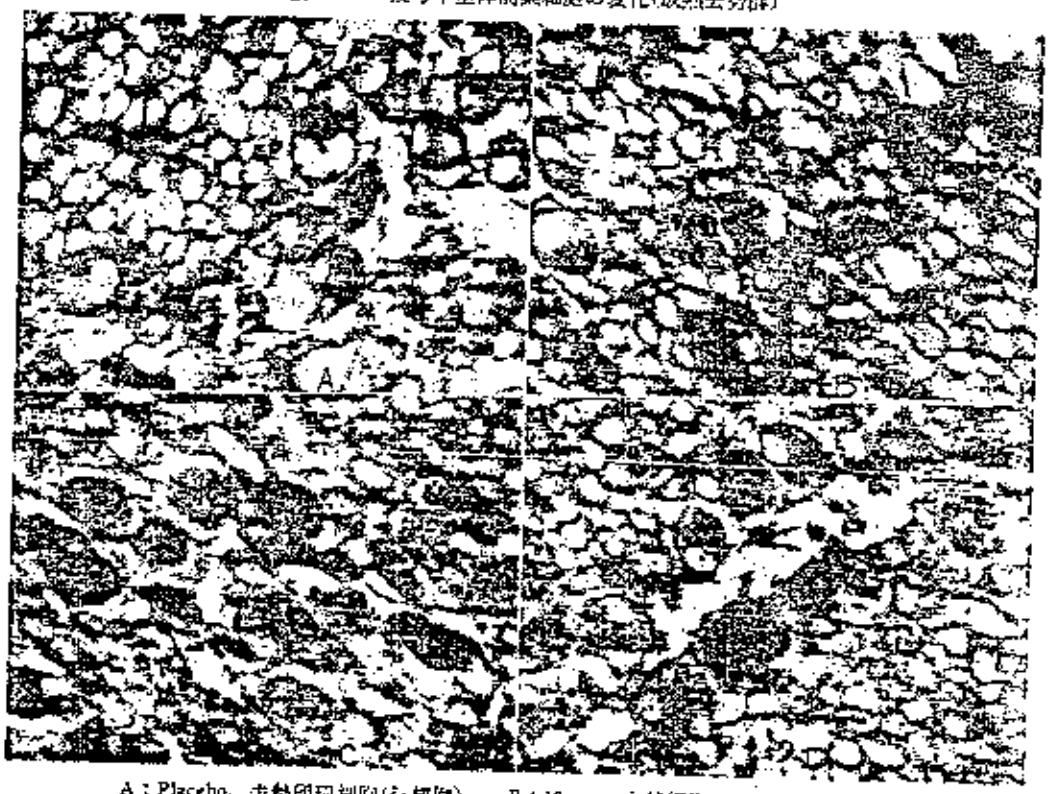
非去勢群では球状帯は層帯の短縮，細胞の小型化をしめした．連状帯ではD 100i.u.までは内束状帯細胞の好酸性がまし胞体はやや腫大し，大核，2核細胞が増加した．1000i.u.では同様の変化のほかにとくに幼若群では細胞索の乱れをみるものがあつた．網状帯，傍髄皮質でも同様に100i.u.までは機能亢進像，1000i.u.では胞体の萎縮，しよう課化を示した．去勢群では100i.u.までは去勢変化の改善がみられ1000i.u.ではまれに萎縮したものもあつた(図16，17)．

表14　D投与と下垂体前葉各細胞の変動

D投与量(i.u.)		好塩性細胞(%)	好塩基性細胞(%)	色素嫌性細胞(%)	計
非去勢	Placebo	31.5(1671)	3.6(193)	64.9(3441)	(5305)
	10	30.4(1554)	3.0(151)	66.6(3406)	(5111)
	100	37.0(2140)	3.5(20.6)	59.5(3436)	(5780)
	1000	33.9(2195)	5.4(347)	60.7(3933)	(6475)
去勢	Placebo	20.2(582)	20.1(577)	59.7(1714)	(2873)
	10	41.9(2461)	11.0(649)	47.1(2770)	(5880)
	100	48.3(1449)	13.4(401)	38.3(1149)	(2999)
	1000	35.3(1493)	15.5(653)	49.2(2079)	(4225)

(　)内は実測細胞数

図14　D投与下垂体前葉細胞の変化(成熟去勢群)



A：Placebo，去勢印環細胞(δ-細胞)　B：10 i.u.，去勢細胞はPlaceboと大差なし
C：100 i.u.，去勢細胞の減少　D：1000 i.u.，去勢細胞の減少

副腎皮質のVines染色所見

非去勢・去勢両群とも各投与量において10i.u.では細胞数の増加，100i.u.以上では減少をみた(表16，17)。

副腎皮質のSudan IV染色所見

非去勢群では100i.u.まで脂肪が減少し，1000i.u.では脂肪細胞をみとめ，去勢群では100i.u.以上で脂肪が減少していた(表18，19)。

卵巣

成熟，幼若両群とも100i.u.までは発育卵胞，黄体ともに増加したが，1000i.u.では大卵胞が減少し閉鎖卵胞の深刻がうかがわれた(図18，表20)。

子宮

非去勢群では100i.u.までは粘膜腺(有管腺)の発育，子宮腺細胞の著明な，傍腺では投与量に比例して内膜間

の肥厚を認めた。去勢Placebo群は萎縮が高度でとくに粘膜固有層，内筋層に著明であつた。D投与量100 i.u. 以上になると，いくぶんこれらの萎縮は改善され，さらにEstradiol-D併用では，それぞれの単独投与よりも有効であつた(図19)。

3) 小括

表15　D投与と下垂体前葉好塩基細胞の変動

D投与量(i.u.)		β-細胞%	δ-細胞%	計
非去勢	Placebo	68.4(132)	31.6(61)	(193)
	10	74.8(113)	25.2(38)	(151)
	100	71.1(145)	28.9(59)	(204)
	1000	78.1(271)	21.9(76)	(347)
去勢	Placebo	22.5(130)	77.5(447)	(577)
	10	35.6(231)	64.4(418)	(649)
	100	42.9(172)	57.1(229)	(401)
	1000	39.2(256)	60.8(397)	(653)

(　)内は実測細胞数

表16　D投与による副腎皮質Vines陽性顆粒の変動

D投与量(i.u.)		球状帯	束状帯 外束状帯	束状帯 内束状帯	網状帯	傍髄皮質
成熟非去勢	Placebo	(±)	+	+	+	+～++
	10	(±)	+	+～++	+～++	++～+
	100	(±)	+	+	+	+～++
	1000	±	±～+	±～+	±～+	+
幼若非去勢	Placebo	±	+	+	+～++	+～++
	10	+	+	+	++	++
	100	±	±	±～+	+～±	+
	1000	−	±～+	±～+	+	+

図15　去勢シロネズミにたいする微量EstradiolとDを併用したばあいの下垂体前葉細胞の変化

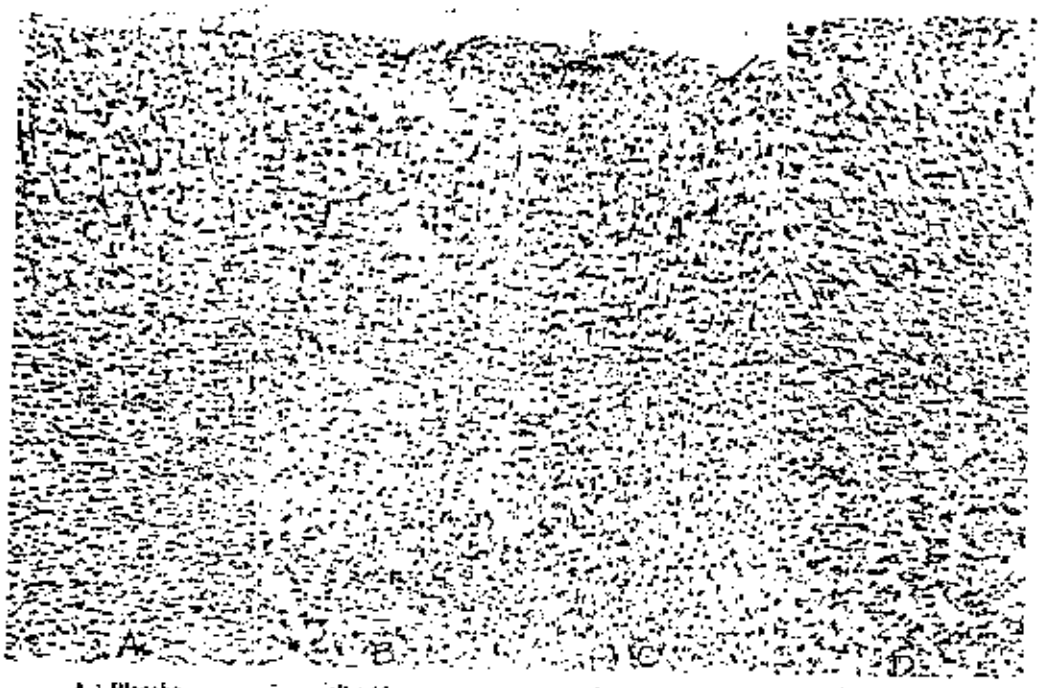
A：Estradiol 0.05 μg
去勢印環型細胞(6)および長楕型細胞(3)の著増

B：Estradiol 0.01 μg + Placebo

C：Estradiol 0.01 μg + D 100 i.u.
去勢細胞の著増はあるが同印環型細胞は減少

D：Estradiol 0.01 μg + D 1000 i.u.
去勢細胞の出現は軽度

図16 D投与副腎皮質(成熟非去勢群)



A : Placebo　B : 10 i.u.　C : 100 i.u.　D : 1000 i.u.

図17 D 投 与 副 腎 皮 質(成熟去勢群)

H・E染色

A : Placebo　B : 10 i.u.　C : 100 i.u.　D : 1000 i.u.

D投与と各内分泌腺器の関連は，D投与量100mg.までは機能亢進，1000mg.ではむしろ抑制の傾向がみられ，子宮は投与量に比例して肥大していた．D投与は下垂体，副腎，子宮の去勢による変化をある程度抑制したが，1000mg.までの投与ではいずれも不充分であった．

D欠乏の妊娠におよぼす影響

D欠乏は流早産を起こしやすく胎児の発育を障害することが知られているが，そのさいの胎盤所見につい

表17　D投与による副腎皮質Vines陽性顆粒の変動

	D投与量 (mg.)	球状帯	束状帯 外束状帯	束状帯 内束状帯	網状帯	髄質皮質
成熟去勢	Placebo	∓	∓	∓	∓	∓
	10	−〜(+)	±	±	±〜(+)	±〜+
	100	(±)	+〜±	±〜+	+	+〜++
	1000	−	±	±	±〜+	±〜+
幼若去勢	Placebo	(±)	±〜+	±〜+	±〜+	+〜++
	10	(±)	±〜+	±〜+	+〜(++)	+
	100	(±)	±〜+	±〜+	+〜++	±〜++
	1000	(±)	±	±	±〜+	±〜+

表18　D投与による副腎皮質Sudan IV陽性顆粒の変動

	D投与量 (mg.)	球状帯	束状帯 外束状帯	束状帯 内束状帯	網状帯	髄質皮質
成熟非去勢	Placebo	+++	++	++	±〜(+)	±〜(+)
	10	++〜(±)	++〜++	++〜++	−〜(±)	±
	100	+++	++	++〜++	±〜(++)	±〜(+)
	1000	+++	++	++	±〜(++)	±〜(+)
幼若非去勢	Placebo	++〜++〜±	++	++	±〜(+)	±
	10	++〜+++	++〜++	++	−〜(+)	−
	100	++	++	++	±〜(+)	±
	1000	++〜+++	++〜++	++〜++	±〜(++)	±〜(+)

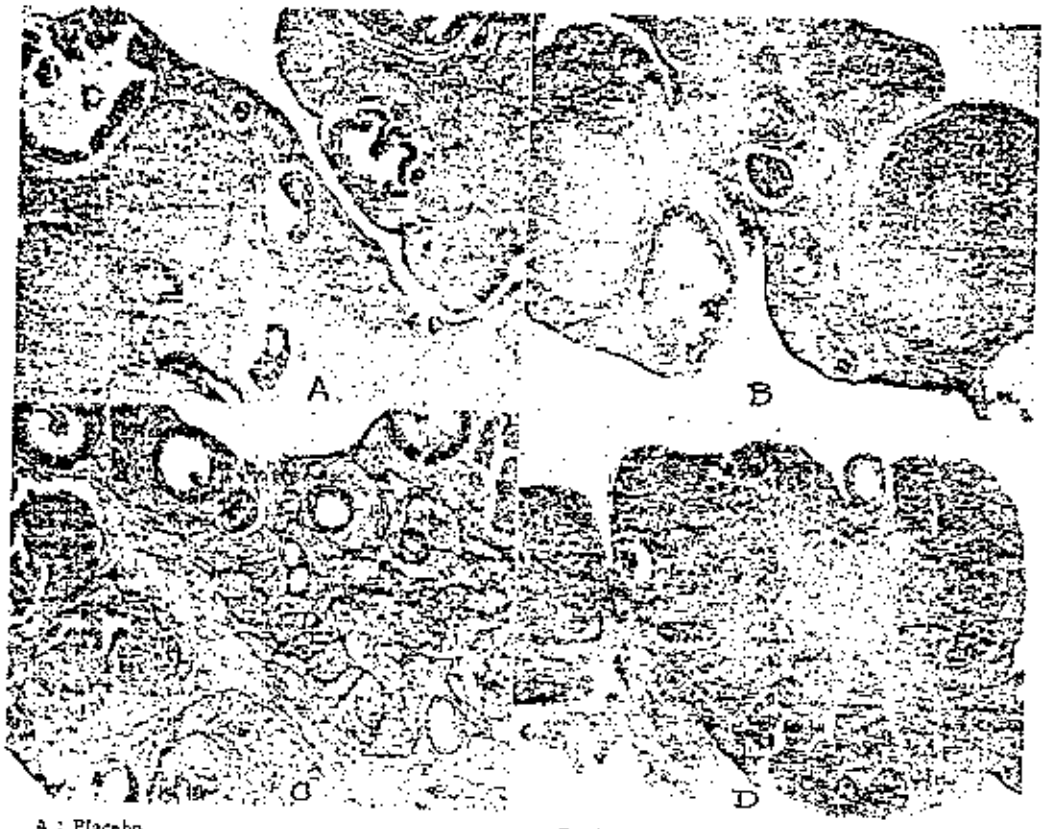
表19　D投与による副腎皮質Sudan IV陽性顆粒の変動

	D投与量(mg.)	球状帯	中間帯	束状帯 外束状帯	束状帯 内束状帯	網状帯	髄質皮質
成熟去勢	Placebo	++〜+++	±〜(+)	++	++	±〜(+)	+
	10	++〜+++	±	++	++	±〜(++)	±〜(+)
	100	++〜(±)	−	++〜(++)	++	±〜(+)	±〜(+)
	1000	++〜+++	−	++〜(++)	++〜(++)	±〜(+)	±〜(+)
幼若去勢	Placebo	++	±〜+	++〜+++	++〜++	±〜+	±〜+
	10	++〜++	±〜+	++〜++	++〜++	±〜(++)	±〜(+)
	100	++〜(±)	−	++	++〜±	±〜(+)	±〜(+)
	1000	++〜(±)	−	++〜(++)	++〜++	±〜+	±〜+

表20　D投与時の卵巣所見

	D投与量 (mg.)	発育卵胞 大	発育卵胞 中	発育卵胞 小	閉鎖卵胞	黄体 巨大	黄体 大	黄体 中	閉鎖卵胞
成熟	Placebo	+	+	+	+	+	+	+	+++
	10	+	±〜+	+〜±	+	+	+	±	+++
	100	+〜++	+	++〜++	+	+	+〜++	+	+++
	1000	±	±	+	+	±	±	∓	+++〜++++
幼若	Placebo	∓	±	±	+	∓	∓	∓	++
	10	+	±	±	+	±	+	−	++
	100	+	+	±	+	+	+	−	+++
	1000	−	+	+	+	+	+	−	+++

図18 D投与卵巣(成熟群)



A : Placebo
B : 10 I.U.
C : 100 I.U.
D : 1000 I.U.

ての報告はほとんどない。

(1) 実験方法

Wistar系シロネズミの性周期正順なものを対照妊娠群，妊娠当日からのD欠乏飼育群，欠乏飼育30日後，40日後，50日後，妊娠群の5群にわけ，それぞれ妊娠14日，20日に断頭し胎仔生存率，胎仔体重，胎盤重量および胎盤の組織学的検索を行なつた。なお欠乏30日後妊娠群では妊娠10，12，14，16，18および20日の胎盤を組織学的に検索した。妊娠の判定は発情前期に雄と同居させ，翌朝膣スミアに角化細胞と精子を認めた日を妊娠第1日とした。

(2) 実験成績

1) 妊娠率，胎仔生存率

D欠乏期間に比例して低下し，欠乏30日以降ではとくに著明となつた。妊娠14日と20日との胎仔生存率の差も欠乏30日以降では妊娠末期に著明に低下した(図20，21)

2) 胎盤重量，胎仔体重

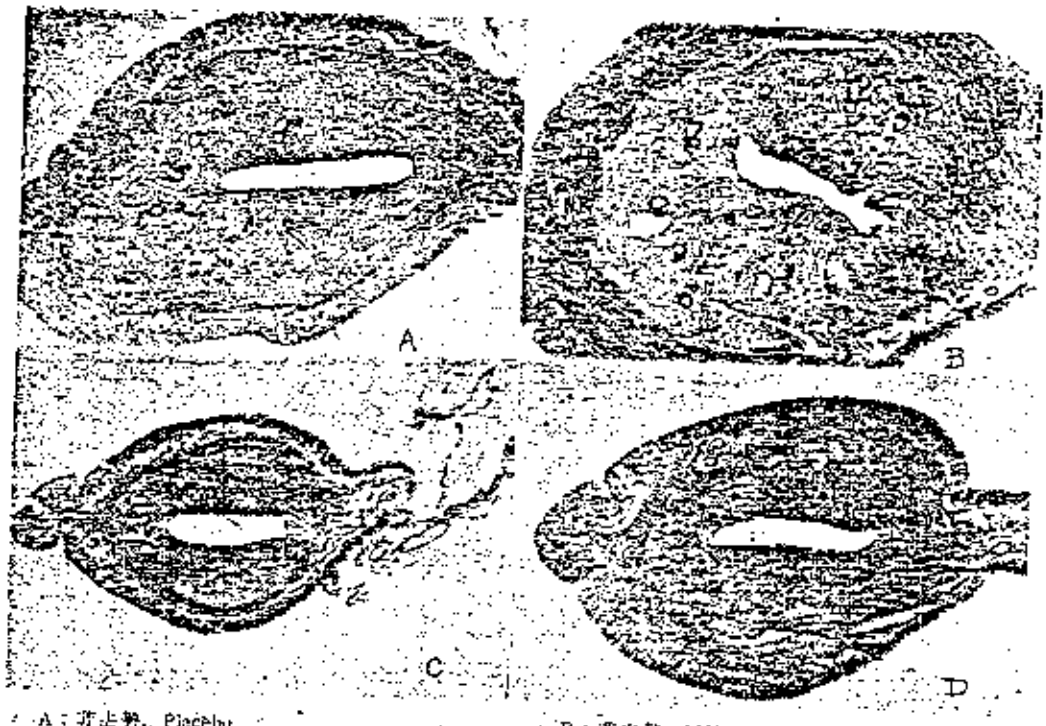
各群の生仔胎盤重量と生仔体重は表21，22のごとく妊娠20日では対照と差がなく，14日では欠乏30日以降群の胎盤重量に減少をみた。

3) 組織学的所見

組織学的検索にはホルマリン固定後着床部中央横断面のパラフィン切片を作成し，H-E染色，Van Gieson染色，PAS染色を行なつた。

シロネズミの胎盤は通常2つの部分に大別される。1つはYolk sack placentaで妊娠12日に発生し，他の1つはLabyrinthine placentaで妊娠10日の終りに発生する。妊娠12日から尿嚢血管をともなつたSyncytial trophoblastが，すでに母体血と交通するCytotrophoblast中に樹材状に入り込み妊娠14日の終りまでの間にLabyrinthを形成する。その他の部分を含めてシロネ

図19　D投与子宮(成熟群)



A：非去勢，Placebo
B：非去勢，1000 I.U.
C：去勢，Placebo
D：去勢，1000 I.U.

図20　D欠乏30日後妊娠(14日)

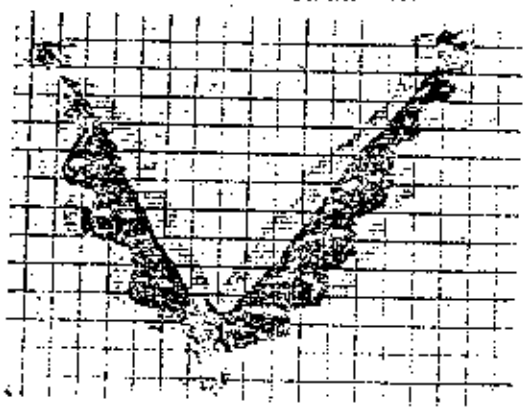
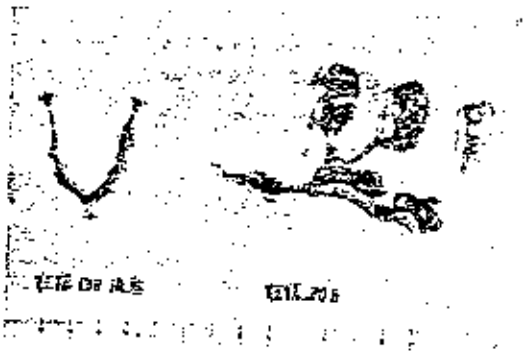


図21　D欠乏30日後妊娠(20日)



ズミ胎盤の基本的形態は妊娠14日で完成する。

図22，23に示すようにD欠乏群の胎盤はすべての時期を通じて発育が悪かつた。妊娠10日ではEctoplacental cone(胚外外胚葉由来のCytrophoblastで床脱落膜に円錐状に侵入し妊娠7日の終りに脱落膜血管がこの部分に開口する)の発育不全と変性がみられ，胚板部には部分的に出血がみられた。妊娠14日ではLabyrinthの全幅は薄く，Syncytial trophoblastは萎縮性で，所々に核濃縮を示した。尿嚢血管の血球充実度は正常であつたがIntravillous space(Maternal blood space)は狭少，貧血性でときに全く血球をみなかつた。これに反しCytotrophoblast，巨細胞層，床脱落膜の発育は正常でむしろ充血性であつた。これらLabyrinthではSyncytial trophoblastの壊死，Allantoic villiの破壊による出血がみられ，これがさらにLabyrinthの広範な出血性壊死を招き，なかにはLabyrinthの形成初期にすでにSyncytial

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図22 D欠乏胎盤(妊娠14日)

A
対照胎盤全層

B
D欠乏胎盤全層
Labyrinthの形成不全，Intravillous spaceの虚血

C
D欠乏胎盤
Allantoic villi の早期萎縮，Cytotrophoblast 床脱落膜は正常

D
Labyrinth に起こった壊死性出血
巨細胞層は正常

trophoblastの壊死を起こしているものもあつた．妊娠20日の所見も基本的には14日と同様でLabyrinthの形成不全，壊死出血が胎児死亡の原因となるもののようである．

(3) 小括

D欠乏が長期にわたると妊娠に障害をおよぼす．妊娠率，胎仔生存率が低下し，14日目の胎盤重量は低下した．しかし妊娠20日では胎仔生存率の低下のみで，生存胎仔体重，生存胎盤重量には対照と差がなかつた．胎盤の組織学的変化はLabyrinthの発育不全で，これはAllantoic villiの形成不全すなわち早期に起こるSyncytial trophoblastの退行変性に起因すると考えられる．

考 察

Schulze[3]はD欠乏時下垂体のThyrotropic potencyの減少を認め，Neuweiler[8]も性器萎縮，卵巣の嚢胞変性，下垂体細胞の変化を報告している．私の実験では下垂体前葉のα-細胞はD欠乏初期から数の減少，胞体萎縮などの機能減退像を示し，好塩基細胞は欠乏初期にはとくにGondotrophsのδ-細胞の増加が著明で，D欠乏の間脳-下垂体-卵巣系にたいする影響が推測された．欠乏が長期におよぶとβ-，δ-両細胞ともに萎縮，減少して機能減退像がみられた．副腎では欠乏初期には内臭状態，傍痛皮質に軽度の機能亢進像を認めたが卵巣，子宮では欠乏初期から萎縮が起こるから，下垂体の変化は卵巣機能低下による二次的なものと考えられるが，欠乏後期の萎縮像からみてD欠乏の直接作用も否定しえないであろう．Agduhr[5]はエルゴステロール投与により下垂体の重量増加と好酸性細胞の著増，好塩基性細胞の軽度増加をみており，伊藤[4]はα-細胞の減少萎縮と核濃縮，好塩基性細胞の腫大と増加を報告している．私の実験では非去勢群のα-細胞はD_2 100 i.u.までの投与で腫大，増加し，好塩基細胞は10i.u.で一時減少したがその後1000i.u.まで増加した．変動の主役はβ-細胞で，δ-細胞は不変あるいはやや減少した．去勢群ではD投与量を増すと去勢細胞発現率が減少した．したがつてDの下垂体前葉あるいは視床下部性中枢にたいする直接作用の存在も推定される．

副腎皮質のコレステロールはステロイドホルモンの

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図23　D欠乏胎盤(妊娠20日)

A　対照胎盤Labyrinth

B　D欠乏胎盤 Labyrinthine trophoblastの萎縮と破綻後出血

C　D欠乏胎盤 Labyrinthに始まつた出血が胎盤全層に波及

能脈物質であり，Sudan脂肪，アスコルビン酸も副腎の機能状態に応じて消長を示し，さらにZimmer[illegible]のVines陽性物質もコレステロール，アスコルビン酸，Sudan脂肪とは逆比例的関係で皮質機能の指標となるという[illegible]。D欠乏時の副腎皮質は前述のごとく，欠乏初期には軽度の機能亢進所見を示し，のち欠乏が長期となつて皮質全層ことに束状帯以下の内層に萎縮が著明となり，去勢時の変化と似た所見を呈した。これらの変化はD欠乏によるストレス反応と見るよりは下垂体，卵巣の所見から性腺の機能低下にたいする代償反応[illegible]と考えた方が妥当と思われる。

伊藤[illegible]はDの大量投与では皮質の萎縮と脂肪減少が認められると報告している。私のばあいには非去勢群で組織学的にD 100i.u.までの投与は皮質機能亢進を，1000i.u.では逆に機能減退を認め，ことに幼若群では萎縮がめだつていた。去勢群では100i.u.投与の成熟群に有意な重量増加と組織学的にもSudan脂肪の減少，微細化など去勢変化の改善がみられた。投与Dの分布についてCruickshank[illegible]らは高濃度に副腎にみとめたといい，Kodecek[illegible]はAutoradiographによりD_2-^{14}Cは大部分束状帯にあつたと報告し，[illegible]も組織化学的に同様の所見を得ている。Quarterman[illegible]はD投与シロネズミの副腎D量を経時的に観察し，副腎には30分で高濃度に達したが，その物質は化学的にも抗クル病作用からもDとは違つた物質であつたといつている。

卵巣および子宮はD欠乏により著しい障害をうけ，卵巣は欠乏90日以降は有意に萎縮し，子宮は30日ですでに重量低下をみた。組織学的に卵巣は2つの特徴ある所見を呈し1つは黄体が欠除し大卵胞のみのもの，他は小卵胞と陳旧黄体しか認められないものである。前者は成熟群に，後者はとくに幼若群に多くみられた。茅根[illegible]，[illegible]らによると卵巣は後者の所見を呈し，欠乏60～90日で腟スミアは静止期が持続したという。私の実験ではその時期はむしろ発情期像が頻発しており，この違いは被我の飼育条件の差異から，本実験ではより緩慢にD欠乏が起こつたためと思われる。

表21 D欠乏飼育がシロネズミ妊娠におよぼす影響(妊娠14日)

実験群	動物番号	着床数	生存胎仔数	平均生存率(%)	生存胎仔の胎盤重量(mg) 湿重量	乾燥重量
対照	206	12	12	98.3	143.8±2.4	14.7±2.9
	207	14	14			
	208	13	13			
	209	12	11			
	210	10	10			
妊娠直後欠乏飼育	216	11	11	87.5	135.3±3.6	14.8±3.4
	217	14	13			
	218	11	9			
	219	10	9			
	220	10	7			
欠乏飼育30日後妊娠	226	11	8	70.9	119.7±3.4	13.0±2.2
	227	10	7			
	228	17	13			
	229	10	6			
	230	—	—			
欠乏飼育40日後妊娠	236	11	5	45.5	126.2	12.1
	237	—	—		92.4	7.3
	238	—	—		98.6	8.2
	239	—	—			
	240	—	—			

Goetigens[4], NeuweilerもD欠乏シロネズミ卵巣に多嚢胞変性を認め，Parkes[7]はそれらの古い嚢スミアは持続角化を呈したという．この多嚢胞変性の原因は欠乏初期のδ-細胞の萎縮から，Gonadotropin の過剰によるとも考えられるが，子宮では早期から萎縮の起こることから，むしろ卵巣におけるEstrogen 活性が早期から低下し視中枢にたいする Feed back 作用の減弱と考えた方がよいと思われる．Parlow[8]は去勢シロネズミについて，下垂体LH[*5]にたいしては，いかなる量のEstradiolも放出を抑制させるが，FSH[*6]にたいしてはごく微量(0.005mg) の Estradiol は分泌を増加させると報告している．また去勢失敗の残存卵巣にしばしば多嚢胞変性をみることもこれらの実験けとなろう．

D投与・非去勢群の卵巣，子宮重量にはあまり変化をみないが(D 1000i.u. 投与幼若群では卵巣重量の低下と子宮重量の増加，去勢子宮では100i.u. 以上の投与で有意の重量増加をみた．組織学的には卵巣は100i.u. 投与で発育卵胞，黄体ともに増加したが，1000i.u. では卵胞発育は阻害された．子宮ではD投与は筋層の発育促進，粘膜固有層の軽度肥厚をみた．Urist[9]はD欠乏ニワトリの著明な卵巣萎縮の原因はリンたんぱくCaまたはリポたんぱく合成の阻害にあり，これらはEstrogen投与で改善されると報告している．Aizawa[10]はEstrogenがネズミ子宮底で無機リンのリン脂質へのとり込みを増加させ，これが子宮発育にきわめて有効で，リン脂質代謝にはDも促進的に作用するといつている[11]．DeLucaら[52]~[55]によれば適量のDは副甲状腺ホルモンとともにミトコンドリアからのCaの放出やATPの代謝に関係し細胞呼吸を促進させるが，過量ではこれを抑制するという．

妊娠時のD欠乏は妊娠率を低下させ，胎仔生存率も同様低下するが，妊娠14日と20日では後者に障害が大である．生存胎盤重量は妊娠20日では対照との間に差がないが，14日のそれは対照に劣る．すなわち妊娠後半の胎仔死亡原因には胎盤発育障害を重視すべきである．D欠乏妊娠では胎盤形成不全がみられ，変化の最

*5) LH = Luteinizing hormone　*6) FSH = Follicle stimulating hormone

表22　D欠乏飼育がシロネズミ妊娠におよぼす影響(妊娠20日)

実験群	動物番号	着床数	生存胎仔数	平均生存率(%)	平均生存胎仔体重(g)	生存胎仔の胎盤重量(mg) 湿重量	乾燥重量
対照	201	13	13	98.2	2.58±0.61	326.8±91.6	50.1±3.5
	202	11	11				
	203	12	11				
	204	11	11				
	205	10	10				
妊娠直後欠乏飼育	211	14	10	86.2	2.54±0.31	292.3±27.8	48.2±2.4
	212	11	11				
	213	11	10				
	214	12	10				
	215	10	9				
欠乏飼育30日後妊娠	221	7	3	48.3	2.64±0.40	295.2±19.6	49.0±3.1
	222	8	4				
	223	9	7				
	224	(10)	(0)				
	225	—	—				
欠乏飼育40日後妊娠	231	12	4	30.4	2.50±0.48	307.1±11.9	48.1±2.6
	232	11	3				
	233	—	—				
	234	—	—				
	235	—	—				
欠乏飼育50日後妊娠	241	7	0	0	—	—	—
	242	—	—				
	243	—	—				
	244	—	—				
	245	—	—				

(　)内は妊娠13日流産

も大きい場はPlacental labyrinthでAllantoic villiの発育不全，Syncytial trophoblastの退行変性のため，Labyrinth形成が不充分でMaternal blood spaceは狭少，貧血性を呈した．ここに壊死と出血がはじまり，やがて胎盤全体の壊死，胎仔死亡をひきおこすもののようである．Syncytial trophoblastの萎縮の原因は不明であるが，シロネズミではいかなる妊娠時期でも去勢により妊娠は中絶し，妊娠継続には卵巣ホルモンが必要である．したがつてD欠乏によるホルモン環境の変調を無視できないが，去勢のさいにはD欠乏のそれとは異なり胎盤全体に速やかな退行変性と壊死をひきおこす．

阿部[9]はK欠乏妊娠シロネズミにEstradiolを投与すると妊娠が継続することをみ，黒田[10]はA欠乏妊娠時の内分泌腺について詳細な研究を行ない，卵巣その他の内分泌腺の妊娠性変化はA欠乏動物では対照より軽微であつたと報告している．しかしこれらA，K欠乏のばあいの胎盤障害部位は床脱落膜，巨細胞層に始まり，Dのばあいとは所見を異にしている．妊娠時の絨毛組織はそれ自体および胎児の発育のため旺盛な代謝を要求されている．D欠乏がその代謝機構を阻害するものと思われるが，この点についてはさらに今後の追求を要する．

結　　論

雌性性機能とDとの関係を，実験的D欠乏およびD投与シロネズミの一般状態，性周期の変化，内分泌腺

得，性器の組織学的検索およびD欠乏の妊娠におよぼす影響について検討し，つぎの結果を得た．

1）発育ならびに性周期への影響

D欠乏は幼若群の発育を阻害するがD₂投与によりすみやかに回復する．D投与群の体重増加率は対照と差がない．性周期はD欠乏50日ごろから乱れてくる．成熟群ではまず発情周期が頻発し，その後静止期像となるものがおおく，幼若群では始めから持続静止期像となるものが多い．D 1000I.U. 投与群で希発発情像をみたほかはほぼ対照群ではD投与による性周期の変化はみられない．去勢群ではEstrogen 併用例にのみ性周期の回復をみた．

2）内分泌腺，性器への影響

D欠乏のばあい下垂体前葉では欠乏初期からα-細胞の萎縮，減少をみた．好塩基細胞は欠乏初期に著増したが，欠乏が長期となると萎縮などの機能低下像をみせた．副腎皮質では欠乏初期に一時機能亢進像を，のちに機能低下像を示した．卵巣は欠乏90日以降とくに萎縮がみられ，組織学的には多発卵胞性と成熟卵胞欠除の所見の2型がみられた．子宮は欠乏初期から萎縮した．

D投与群では非去勢群の分泌腺は100I.U.までは亢進傾向が，1000I.U.では萎縮の傾向がみられた．子宮は投与量に比例して肥大した．去勢群への投与は，上皮変化をいくぶん改善することが観察された．

3）妊娠におよぼす影響

D欠乏は妊娠率，胎仔生存率の低下と胎盤の発育不全をきたす．組織学的にはPlacental labyrinth 形成不全，Syncytial trophoblastの退行変性，壊死がみられ，Labyrinthに起こる壊死と出血が胎仔死亡の原因となる．

以上の如くシロネズミ雌性性機能にたいし，Dは Estrogen に類似した作用をおよぼすことがわかった．

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AN EFFECT OF VITAMIN D UPON THE UPTAKE AND RELEASE OF ZINC BY BONE

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HUXLEY and LEAVER (1966) demonstrated that the bone and dentine of rats receiving 4240 ppm of dietary zinc accumulated large amounts of the element. The effect was enhanced when this zinc excess was associated with low dietary calcium, levels of 4444 and 961 ppm being found in bone and dentine respectively, after 8 weeks on the diets.

In view of this great capacity of bone, in particular, to accumulate zinc, it seemed to be of interest to try to gain some insight into the relative rapidity of this uptake and also to see whether the level of the element fell when the animals were transferred to diets containing normal levels of zinc. Though there does not appear to be any available information relating the uptake of trace elements by bone to the presence or absence of vitamin D in the diet, it was decided to investigate the possibility that some such relationship might exist.

Three groups, each of twenty-five black and white hooded weanling rats from our own colony, were maintained for twelve weeks on diets based on those described in detail by HARTLES and LEAVER (1961), namely diets HS7, HS8 and HS10. During the first 6 weeks of the study all diets were modified by incorporating a supplement of zinc carbonate calculated to adjust the level of the element to 2000 ppm. The actual concentrations of zinc found in the diets by analysis were 2042 ppm and 46 ppm at the high and normal levels respectively. After the initial 6-week period all animals received the corresponding diets but with no zinc supplement.

The main features of the diets are summarized below:

	Calcium	vitamin D	Zinc	
Group A	Normal	Normal	High (0–6 weeks)	Normal (6–12 weeks)
Group B	Low	Normal	High (0–6 weeks)	Normal (6–12 weeks)
Group C	Low	Low	High (0–6 weeks)	Normal (6–12 weeks)

Five animals from each group were killed after 4, 6, 8, 10 and 12 weeks respectively. The femora were dissected out and prepared for analysis as described by LEAVER

(1966). The concentration of zinc in the bone samples was determined by the direct polarographic method described by HUXLEY and LEAVER (1966).

Table 1 summarizes the zinc concentration in the bone of all three groups at the five time intervals and includes the mean zinc concentration in the bone of five weanling animals sacrificed prior to the start of the experiment.

TABLE 1. ZINC CONCENTRATION OF WHOLE BONE
(Mean value of five analyses, expressed as ppm of weight of dried tissue, ± standard error of the mean)

	0 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks
Group A	323·0 ± 24·1	1579 ± 97·1	1683 ± 134·0	1204 ± 88·1	965 ± 48·3	461 ± 24·6
Group B		3118 ± 117·2	3737 ± 181·2	2696 ± 112·4	2069 ± 89·9	1136 ± 84·4
Group C		2445 ± 101·6	2802 ± 170·0	2775 ± 174·2	1847 ± 101·1	1634 ± 95·1

Though certain apparent differences between bone zinc concentrations at adjacent time intervals within the individual groups are not significant, certain overall trends are obvious. The bones in group A (normal calcium and vitamin D) acquired zinc at a rapid rate and subsequently released it slightly more slowly. Though the levels of zinc in the bones of animals on both low calcium diets reached high maxima, the group without added dietary vitamin D accumulated significantly less of the element ($p < 0{\cdot}01$). The loss of zinc, when animals were transferred to the diets containing normal levels of the element, was more rapid in the presence of vitamin D, the difference between the final levels being highly significant ($p < 0{\cdot}01$).

It appears, therefore, that when the exposure to zinc excess and subsequent transfer to normal dietary levels is carried out within the growing period (0–12 weeks after weaning) both the uptake and release of zinc by bone are rapid processes. There appears to be a significant effect whereby absence of vitamin D slows down both the uptake and subsequent release of zinc when the animal is maintained throughout on a low calcium regime.

FERGUSON and HARTLES (1963) showed that the long bones of animals fed low calcium diets with and without added vitamin D exhibited marked histological differences. In the presence of the vitamin, low calcium diets produced a condition resembling osteoporosis, whereas in its absence the condition was more akin to osteomalacia. The presence of vitamin D thus produced a more dynamic response to calcium deprivation.

In the present study the uptake of zinc from diets low in calcium might be considered to give some indication of the general "dynamic capability" of bone in the presence or absence of vitamin D. If this is so, then the effect noted would lend some support to the view that the absence of the vitamin does, in fact, lower this dynamic capability.

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INCREASED SENSITIVITY TO VITAMIN D AFTER VITAMIN-D POISONING

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PATIENTS with parathyroid deficiency usually tolerate doses of vitamin D that would poison normal people. They generally need these large doses to mobilise calcium from the bone and maintain its concentration in the plasma. Two of our patients had severe tetany from parathyroid deficiency, one postoperative, the other idiopathic; they had to have large doses of vitamin D, but the dose was increased too rapidly and they were both poisoned. Consequently one had acute pancreatitis, and the other renal failure from which he did not fully recover. After the episode of acute poisoning, both patients responded to small doses of vitamin D and have been maintained with these for over a year.

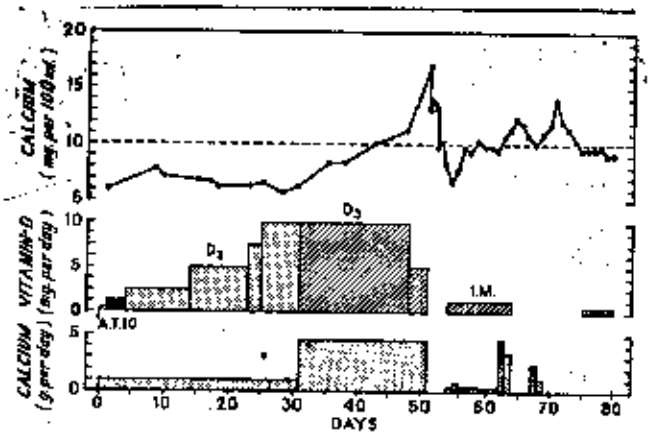

Fig. 1—Case 1: Abnormally frequent increases in dose of vitamin D leading to hypercalcaemia.

Case-reports

Case 1

We have described (Leeson and Fourman 1966) how this woman of 40 years, who had severe hypoparathyroidism after her second thyroidectomy, was poisoned with vitamin D. The excessively rapid changes in our treatment which led to hypercalcaemia are shown in fig. 1. The hypercalcaemia, though treated with ethylenediamine tetra-acetic acid (E.D.T.A.), was complicated by acute pancreatitis, and the patient underwent laparotomy. 48 hours after the operation her serum-calcium fell to 6·6 mg. per 100 ml. and her tetany returned. The hypercalcaemia returned after 10 days on calcium and 1·2 mg. of vitamin D_2 a day, and then on calcium alone. 3 weeks after the initial onset of hypercalcaemia, the patient's serum-calcium was controlled by 0·5 mg. of vitamin D_2 a day by mouth, without additional calcium. She was satisfactorily maintained on this dose for 4 months.

During the past year her diet has been supplemented with 3·8 g. of calcium a day and her daily dose of vitamin D_2, given by mouth, has varied from 0·5 to 2 mg. Fig. 2 shows that the response of her serum-calcium to changes in the dose of vita-

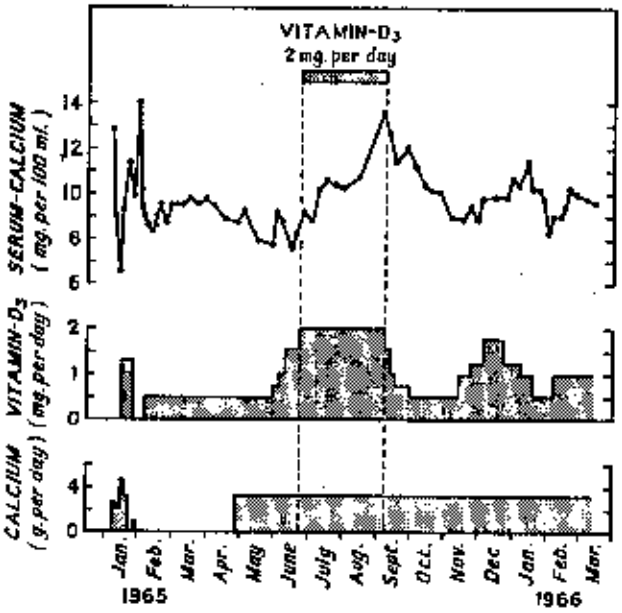

Fig. 2—Case 1: Response of serum-calcium to 0.5 mg. of vitamin D_2 daily and effect of increases in dose to 2 mg. daily after first poisoning with vitamin D.

min D_2 lags. An increase of the dose to 2 mg. twice produced hypercalcæmia. The optimum dose appears to be about 1 mg. a day, yet before she was poisoned 5 mg. was not enough.

Case 2

Since idiopathic hypoparathyroidism is uncommon, we report this case in detail. A 50-year-old English electrical fitter had rheumatoid arthritis, which had begun in 1948 with arthritis of the knee. The disease had been quiescent until 1963 when it began to affect the knees, elbows, and one finger; this was easily controlled with prednisone 5 mg. b.d. In 1958, at the age of 42, the patient had bilateral cataracts. In 1960 he complained of disabling attacks of painful cramp in his left arm and leg, unassociated with any disturbance of consciousness or abnormal physical signs. No diagnosis was made, and the attacks ceased spontaneously. The cramps returned in 1964 when the patient was taking prednisone for his rheumatoid arthritis. He had not had any operation on his neck. He was referred to us as a case of idiopathic hypoparathyroidism with hemitetany (Jesserer 1958) and cataracts.

Clinical Findings

The patient was a small cheerful man in good general health. Height 61 in. span 65 in., weight 64·5 kg. His nails were flecked with white, brittle, and ridged; the fourth metacarpal was short; he had bilateral subcapsular cataracts; the optic fundi were normal; and the left knee was slightly flexed with wasting of the quadriceps, crepitus, and a small effusion. Movements of the right knee and hip were painful but full; other joints were normal. There were no tophi. The blood-pressure was 120/80 mm. Hg. Trousseau's sign was present on both sides, and Chvostek's sign was absent. The clinical findings in other systems were negative, and the urine was normal.

The patient was having ten or more attacks a day in which his left leg and arm went into very painful tetanic spasm for 10–15 seconds and then recovered spontaneously. These attacks were more frequent at the end of the day; at home the patient noticed they were brought on by cold and fatigue. In hospital the attacks culminated twice in a major epileptic fit when the patient was having magnesium and his serum-calcium was particularly low.

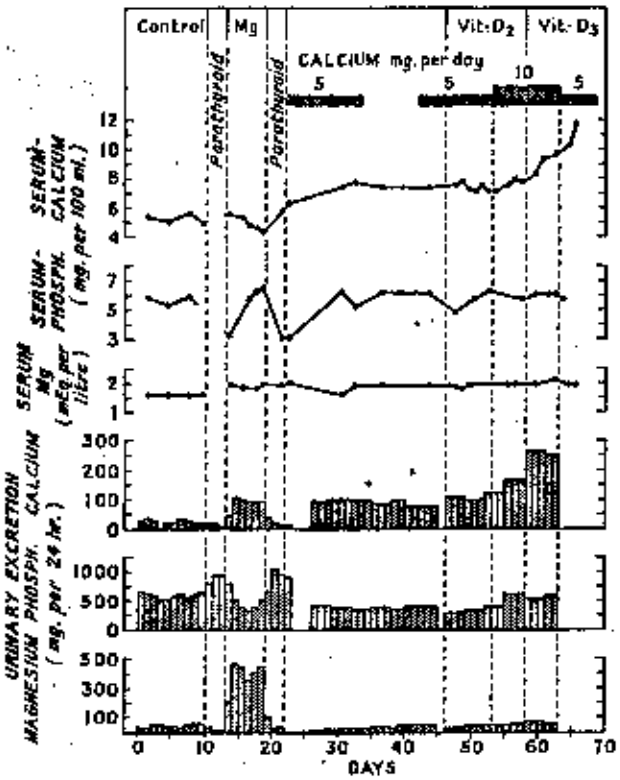

Fig. 3—Case 2: Effect of parathyroid hormone, magnesium, calcium, and vitamin D on serum and urinary calcium, phosphorus, and magnesium whilst on a constant diet.

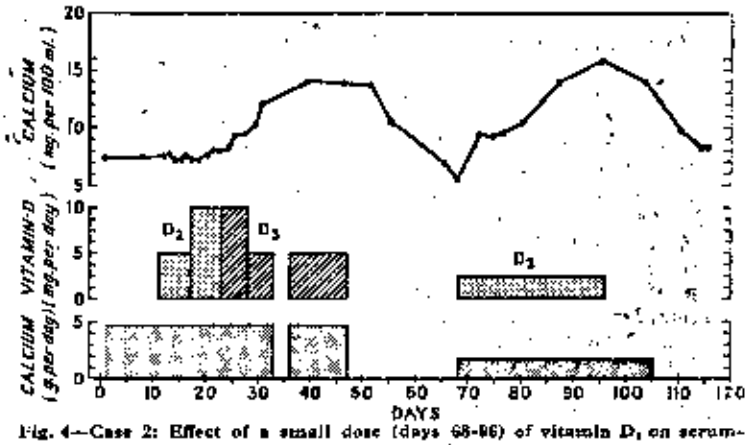

Fig. 4—Case 2: Effect of a small dose (days 68–96) of vitamin D_2 on serum-calcium after poisoning with a large dose (days 10–47).

Investigations

The laboratory findings were: serum-calcium 4·9–5·3 mg. per 100 ml.; serum-phosphorus 5·3–6·3 mg. per 100 ml.; serum-magnesium 1·65–1·7 mEq. per litre; serum-alkaline-phosphatase 6–9 King-Armstrong units per 100 ml.; blood-urea 35–40 mg. per 100 ml.; serum-creatinine 1·4 mg. per 100 ml.; serum sodium, potassium, bicarbonate, and chloride normal; total plasma-proteins 7 g. per 100 ml., serum-albumin 4·6 g. per 100 ml., serum-globulin 2·4 g. per 100 ml. Hæmoglobin 14 g. per 100 ml.; leucocytes 6800 per c.mm.; differential count normal; erythrocyte sedimentation-rate 28 mm. in the 1st hour (Westergren); no L.E. cells; Rose-Waaler test negative; urine normal and sterile on culture. Urinary excretion per 24 hours: calcium 19–26 mg.; phosphorus 494–662 mg.; magnesium 27–41 mg. Chest X-ray normal. X-ray of the abdomen, no renal calcification. Skull X-ray, flecks of calcium above the pituitary.

Electromyography, typical patterns of tetany obtained from both hands on arterial occlusion.

Electroencephalography was carried out when the serum-calcium was 5·3 mg. per 100 ml. The slow activity sometimes associated with hypocalcæmia was not seen, and, even during spontaneous attacks of hemitetany, there was no evidence of seizure discharge.

Fæcal fat excretion was 32 g. in 6 days.

Bone biopsy from iliac-crest trephine.—Some of the bone surfaces were covered by osteoid seams. There was no excess of osteoblasts or osteoclasts.

We tested the responses to parathyroid extract ('Parathormone', Lilly), to intravenous magnesium, and to oral calcium before and during treatment with oral vitamin D. Fig. 3 shows the changes in blood and urine. Fig. 4 shows the response to vitamins D_2 and D_3. These tests were carried out in collaboration with Dr. F. W. Heaton in metabolic ward of the M.R.C. mineral metabolism unit; the patient received a constant diet throughout. Prednisone 5 mg. b.d. was continued throughout the tests and treatment.

Response to Parathyroid Extract

2·5 ml. parathormone was given intramuscularly twice daily for 3 days. The urinary excretion of phosphorus increased, and the serum-phosphorus fell to 3·1 mg. per 100 ml. Although the serum-calcium was unchanged, the tetany diminished. The urinary excretion of magnesium fell to 7 mg. a day; the serum level rose slightly.

Response to Magnesium

42 mEq of magnesium as 50% magnesium sulphate was

injected intravenously over 10 minutes, daily for 6 days. There was a fourfold increase in the small urinary excretion of calcium and the serum-calcium was slightly reduced. The serum-magnesium and serum-phosphorus did not alter significantly. The urinary excretion of magnesium increased. During this period the frequency and severity of the patient's tetany increased, and he had two epileptic convulsions. This may have been due to hypokalæmia (serum-potassium 2·9 mEq. per litre) associated with acidosis (serum-bicarbonate 13 mEq. per litre) (Fourman and McCance 1955).

Response to Oral Calcium

6 g. of calcium as calcium gluconate (Sandoz) was given daily for 3 weeks. The serum-calcium rose to 7·5 mg. per 100 ml. and the tetany diminished. The urinary excretion of calcium increased from 25 to 95 mg. a day. The serum concentration of phosphorus and magnesium and the urinary excretion of magnesium did not change. The urinary excretion of phosphorus fell from 900 to 420 mg. a day.

Response to Oral Vitamins D_2 and D_3

The patient was given vitamins D_2 and D_3 with 6 g. of calcium by mouth. First he was treated with vitamin D_2 (ergocalciferol). There was no change after 6 days on 5 mg. a day; on 10 mg. a day the serum-calcium and serum-magnesium rose slightly; the urinary excretion of calcium increased from 100 to 160 mg. a day; and the urinary excretion of phosphorus increased from 350 mg. to the initial value of 600 mg. a day. At the time these changes seemed unimpressive; consequently vitamin D_3 (cholecalciferol) was substituted for vitamin D_2 in the same dose. The serum-calcium rose steadily to 9·5 mg. per 100 ml. on the 5th day, and the urinary excretion of calcium increased to 260 mg. a day. At the same time the blood-urea increased from 35 to 69 mg. per 100 ml. This adverse effect must be attributed to a direct effect on the kidney, since the serum-calcium was not abnormally raised. After 5 days the dose of vitamin D_3 was reduced from 10 to 5 mg. a day, and the patient left hospital. He was readmitted 20 days later; his serum-calcium was 13·8 mg. per 100 ml. and his blood-urea was 145 mg. per 100 ml. Vitamin D_3 and calcium were discontinued, and serum-calcium fell to 10·4 mg. per 100 ml. on the 4th day and 5·3 mg. per 100 ml. on the 15th day. The blood-urea fell to 49 mg. per 100 ml.

Since then the patient has had vitamin D_2, which has poisoned him twice. The first time (fig. 4) was after he took 2·5 mg. a day for a month; the serum-calcium rose to 15·8 mg. per 100 ml. and the blood-urea rose to 150 mg. per 100 ml. After a month without vitamin D or calcium, the serum-calcium was 8·3 mg. per 100 ml. The second time was after the patient took 0·5 mg. a day for 15 days; the serum-calcium rose to 11·7 mg. per 100 ml. After a further 3 weeks without treatment the patient has been satisfactorily maintained on 0·5 mg. of oral vitamin D_2 a day. He has always had the same preparation of vitamin D_2; the calcium supplement, 2 g. daily, has not varied. His blood-urea has not returned to normal.

Discussion

Both these patients had severe parathyroid insufficiency with incapacitating tetany which could not be controlled with calcium alone. Treatment with vitamin D was unavoidable but it was also disastrous, causing pancreatitis in one patient and renal damage in the other.

In both patients, the initial episode of vitamin-D poisoning happened shortly after the treatment was changed from D_2 to D_3. This could be interpreted as evidence that they were more sensitive to D_3. We think this is unlikely and we attribute the poisoning to a simple accumulation of vitamin D. We have not had an opportunity to make further comparisons of the response to these two forms of vitamin D.

The well-documented toxic effect of vitamin D on the kidney (Anning et al. 1948) depends on the size of the dose and the duration of exposure. Brief exposure may cause only a transient disturbance of tubular function, characterised by impaired conservation of water potassium and reduced capacity to excrete acid (Eps 1962). Our first patient, who was only hypercalcæmic 5 days, probably had impaired tubular function. (admission to hospital she had intense thirst and polyur and her serum-potassium was only 2·7 mEq. per litr Her blood-urea was never raised and she has no evidenc of permanent renal damage. A lengthy exposure to vitamin-D intoxication causes nephrocalcinosis. This deposi of calcium, which is often microscopic, can impair glomerular as well as tubular function. Our second patient, who was hypercalcæmic for 26 days the first time and at least 10 days the second time, has glomerular damage. The first time his blood-urea rose from 35 to 145 mg. per 100 ml. and fell only slowly, reaching 49 mg. per 100 ml. on the 16th day. The second time it rose to 150 mg. per 100 ml. and was still 77 mg. per 100 ml. 2 months later. The initial rise in his blood-urea, from 35 to 69 mg. per 100 ml. happened without hypercalcæmia. His persistently high serum-phosphorus (6·0–6·6 mg. per 100 ml.) may well have contributed to his severe renal damage, since it has been shown in rat that an excess of phosphate alone will induce nephrocalcinosis (Fourman 1959).

Both patients responded to much smaller doses of vitamin D after they had been poisoned by it. Before the poisoning neither of them responded to 5 mg. a day; afterwards they were maintained on 0·5 mg. This change cannot have been due to the pancreatitis or the renal failure. Vitamin-D activity may persist in the serum long after treatment has been discontinued (Thomas et al. 1959), but this would not explain the change in our patients who became hypocalcæmic shortly after vitamin D was withdrawn and were then maintained on a consistently small dose of vitamin D for over a year. We suggest that a massive dose of vitamin D has in some way increased the sensitivity of the bone to a small dose.

These patients illustrate two interesting aspects of magnesium metabolism. The effect of parathyroid extract in the second patient, who responded by increasing the renal conservation of magnesium as well as calcium, confirms in man the observations by MacIntyre et al. (1963) in parathyroidectomised rats. In the first patient, vitamin-D poisoning was associated with a fall in the serum-magnesium from 1·6 to 0·88 mEq. per litre and epileptic fits. The vitamin D may have caused this fall in the serum-magnesium (Silverman and Gardner 1954, Hanna 1961, Richardson and Welt 1963), and this in turn may have caused the fits (Hanna et al. 1960). The serum-magnesium reverted to normal spontaneously, and the patient has not had any further fits.

Summary

Two patients with parathyroid deficiency and tetany were accidentally poisoned with vitamin D. Afterwards their sensitivity to vitamin D increased and they were maintained on about a tenth of their former dose. One had idiopathic hypoparathyroidism which began with cataracts and hemitetany at the age of 48. The other patient had generalised tetany and epilepsy after her second thyroidectomy.

In one patient, the hypercalcæmia caused by vitamin-D poisoning was associated with a low serum-magnesium and epilepsy. In the other patient, parathyroid extract reduced the urinary excretion of magnesium.

These cases illustrate the cumulative effect of vitamin D and the dangers of increasing the dose too frequently.

We are indebted to Dr. E. W. Jackson for referring the first patient, and to Dr. T. G. Reah for referring case 2; to Prof. G. A. Smart and Dr. Percy Smith, who undertook the care of the second patient in Newcastle after he returned home from Leeds; to Dr. K. A. Exley for the electroencephalographs; and to the members of the department of chemical pathology, Sister V. Walker, Dr. F. W. Heaton, Dr. D. B. Morgan, Dr. C. R. Paterson, and Miss Judith Metcalfe for their collaboration. P. F. is in receipt of a grant from the Medical Research Council.

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Iatrogenic rickets in low-birth-weight infants

This report describes 4 premature infants, all with very low birth weights, who developed nutritional rickets in a neonatal unit of a pediatric hospital. All infants were fed a proprietary milk formula containing vitamin D but no vitamin supplement. Because of their small size, the amount of formula ingested was small, resulting in a low daily vitamin D intake. A comparable group of infants who received a daily vitamin D supplement of 400 I.U. in addition to the enriched formula did not develop rickets. It is concluded that the rickets developed because the vitamin D intake was inadequate.

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MOST FLUID MILK, evaporated milk, and proprietary infant feedings marketed in the United States and Canada are enriched with vitamin D. This measure has caused a dramatic reduction in the incidence of rickets. Although the concentration of vitamin D differs somewhat from product to product, the level is usually 450 I.U. of vitamin D per liter of milk or of formula as prepared for feeding. It is now widely held that healthy babies receiving vitamin D–enriched formulas need no vitamin D supplements.

In general we agree with this opinion, provided that enough enriched formula is consumed to supply the amount of vitamin D needed for prophylaxis of rickets. Neglect of the latter provisio has caused us to regret a decision made recently regarding the feeding routine adopted for infants in the neonatal unit of The Hospital for Sick Children. In the past each infant was given vitamin D drops, supplying 400 I.U. of vitamin D per day, in addition to the proprietary milk formula containing approximately 450 I.U. of vitamin D per liter. During a 6 month period from August, 1968, until the end of January, 1969, the vitamin D supplement was discontinued in the belief that the formula alone would provide for the vitamin needs of the infant.

The following is a report of 4 premature infants, all with very low birth weights, who developed nutritional rickets during their stay in the neonatal unit during this period of time.

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CASE REPORTS

Case 1. A boy was born at 30 weeks' gestation with a birth weight of 870 Gm. During the first week of life he had severe respiratory distress associated with episodes of hypoglycemia and hypocalcemia, all suitably corrected by intravenous therapy.

Oral feedings with a vitamin D-enriched formula* were commenced at the age of 5 days and were gradually increased as the infant gained weight. He was discharged from the hospital at the age of 3 months, weighing 2,600 Gm. At the time of a routine follow-up examination 2 weeks later, he was found to have a typical rachitic rosary; roentgenographic examination of the chest and wrists confirmed the diagnosis of rickets with early healing. The serum calcium concentration at that time was 8.6 mg. per 100 ml. and that of inorganic phosphorus was 4.7 mg. per 100 ml. The alkaline phosphatase activity was not determined.

Based upon the volume of formula administered and the vitamin D content claimed by the manufacturer, the calculated total intake of vitamin D in this infant began at 50 U. and increased gradually to 200 U. per day (Fig. 1). The average daily intake of vitamin D up to the age of 3½ months was 102 I.U.

Following the diagnosis of rickets, the infant received a daily vitamin supplement containing 400 I.U. of vitamin D in addition to the enriched formula. Radiographic examination of the wrists 2 weeks later showed continued healing of the rachitic lesions.

Case 2. A girl was born at approximately 27 weeks' gestation and weighed 680 Gm. She had severe respiratory distress, hypocalcemia, and hypoglycemia during the first week of life, which were corrected by intravenous therapy. At 4 months of age, when she weighed 2,220 Gm. roentgenograms of the skull, obtained because the head seemed large, showed that calcification of the calvarium was defective; roentgenographic examination of the chest and wrists showed active rickets. The serum concentration of calcium was 7.8 mg. per 100 ml., that of inorganic phosphorus was 3.6 mg. per 100 ml., and the alkaline phosphatase activity was 119 K.A. units per 100 ml. Vitamin D intake increased gradually from 12 to 300 I.U. per day as the amount of formula was increased during her 4 month stay in the unit. The average daily intake of vitamin D up to the age of 4 months was 127 I.U. Healing of rickets was visible roentgenographically 2 weeks after beginning a daily supplemental intake of 400 I.U. of vitamin D.

*Enfalac liquid, Mead Johnson Laboratories. Stated vitamin D content: 400 International Units per 32 ounces (946 ml.) of formula, reconstituted as recommended.

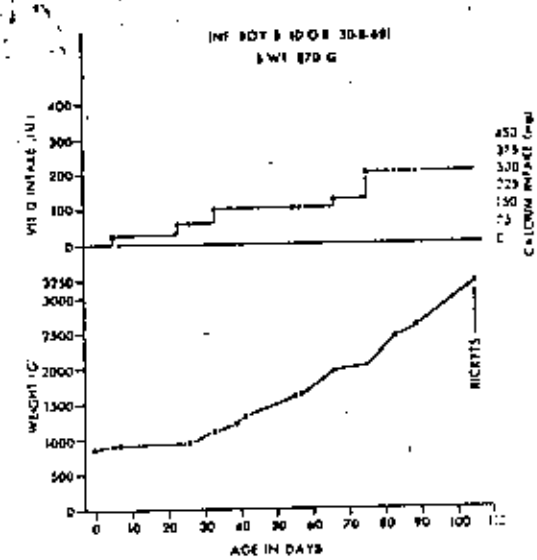

Fig. 1. Case 1. Daily vitamin D intake, calcium intake, and body weight plotted against age. Rickets was discovered by clinical examination at 105 days of age.

Case 3. A girl, born at 28 weeks' gestation and weighing 935 Gm., had moderate respiratory distress during the first 4 days of life. Recovery was uneventful. At the age of 2 months, she sustained a fracture of the shaft of the right radius; roentgenograms of the knees and wrists showed moderately severe rickets. The serum calcium concentration was 9.8 mg. per 100 ml., serum phosphorus concentration was 4.6 mg. per 100 ml., and the alkaline phosphatase activity was 90 K.A. units per 100 ml. The intake of vitamin D during the first month of life had been 50 I.U. daily and gradually increased to 200 I.U. daily by the end of the second month. Thus the average daily intake of vitamin D was 103 I.U. Two weeks after starting vitamin D supplements, healing of the bony lesions was observed.

Case 4. A girl, born at 32 weeks' gestation and weighing 1,300 Gm., had mild respiratory distress during the first 48 hours of life. Recovery was uneventful. At the age of 2 months, when she weighed 2,100 Gm. roentgenographic study of the wrists showed changes in the metaphyses consistent with early rickets. The serum concentration of calcium was 8.5 mg. per 100 ml.,

and of phosphorus was 4.3 mg. per 100 ml.; alkaline phosphatase activity was 61 K.A. units per 100 ml. The initial daily vitamin D intake of 100 I.U. had increased to 250 I.U. by the time rickets was discovered. Averaged daily intake of vitamin D during the 2 month period was 150 I.U.

DISCUSSION

Vitamin D–deficiency rickets developed in 4 low-birth-weight infants while they were in the neonatal unit of The Hospital for Sick Children, Toronto. All had received a proprietary milk formula containing vitamin D but no additional vitamin supplements. The formula was stated by the manufacturer to contain 400 I.U. of vitamin D per United States quart.

From August, 1968, to the end of January, 1969, 547 infants were admitted to the neonatal unit. Twenty-five of these weighed between 1,000 and 1,300 Gm.; of the 12 surviving infants, one developed rickets. Eighteen infants weighed 1,000 Gm. or less; of the 7 surviving, 3 developed rickets. During the preceding 6 month period from February to the end of July, 1968, when vitamin D supplements were administered to all babies as a routine procedure, 535 infants were admitted. Of these, 21 weighed between 1,000 and 1,300 Gm. at birth, and 9 survived. Fourteen infants weighed less than 1,000 Gm. at birth, and 6 survived. None of these infants developed rickets.

Because of their small size, the 4 infants who developed rickets received only small amounts of formula, their only source of vitamin D. They usually received only about 50 I.U. of vitamin D per day during the first month of life. It was not until they weighed more than 2,500 Gm. that they took enough formula to provide 300 I.U. of vitamin D per day. In addition, 3 of the 4 infants in the series, Cases 1, 2, and 3, were small for gestational age. Because such infants usually gain weight very rapidly, conspicuous manifestations of rickets are to be anticipated when intake of vitamin D is deficient.

The minimum requirement of vitamin D for premature infants is not well defined. Early studies[1,2] demonstrated that as little as 100 to 200 I.U. of vitamin D per day prevented biochemical evidence of rickets and sustained normal skeletal growth, but at that time infants weighing less than 1,500 Gm. at birth rarely survived. Whether or not the dosages described in the early papers are valid for very small premature infants has not been tested.

Advisory committees in the United States and Canada[3-6] recommend a total of 400 I.U., from all sources, per day as the prophylactic intake for premature infants. This dose, it is indicated, should provide a margin of safety that allows for individual variations in requirement. The point has been made that within a few weeks of age, most full-term babies fed formulas enriched with appropriate concentrations of vitamin D acquire the recommended allowance from the formula, and additional vitamin D is not essential.[3] In our experience, this statement has been borne out in practice; the findings reported in the present article do not contradict the contention. Among the 150 infants admitted to The Hospital for Sick Children with vitamin D–deficiency rickets during the last 10 years, none had received vitamin D–enriched formula as the major source of nutrition. However, for premature babies, or normal-sized babies in whom food intake is appreciably curtailed for considerable periods of time, enriched formulas do not always supply the vitamin D requirements. In these children, it is important to provide additional vitamin D as an oral supplement to avoid the misfortune that befell the 4 premature infants mentioned in this article.

Because of the small amounts of formula consumed by the patients under consideration, a question arose as to whether calcium deficiency might also have contributed to development of the rachitic lesions. That it did not is demonstrated by the fact that premature infants of similar size had satisfactory skeletal development during a comparable period of time when vitamin supplements were provided.

SUMMARY

This report describes 4 infants weighing less than 1,300 Gm. who developed vitamin D–deficiency rickets while in the neonatal unit of a large pediatric hospital.

All infants were fed a proprietary milk formula containing 450 I.U. of vitamin D per liter but received no vitamin supplement. Because of their small size, the amount of formula they ingested was small, initially providing only approximately 50 I.U. of vitamin D per day.

A comparable group of low-birth-weight infants who received a vitamin D supplement of 400 I.U. per day in addition to the enriched formula did not develop rickets. It is concluded that the rickets developed because the vitamin D intake was inadequate and that low-birth-weight infants should receive a daily vitamin D supplement containing 400 I.U. of vitamin D.

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CLINICAL EXPERIENCE WITH CRYSTALLINE VITAMIN D: THE INFLUENCE OF THE MENSTRUUM ON THE EFFECTIVENESS OF THE ANTIRACHITIC FACTOR

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In 1931, Askew and his colleagues[1] in England and Windaus and his coworkers[2] in Germany, employing different chemical methods, finally isolated from irradiated ergosterol a pure crystalline substance which has marked antirachitic activity. Windaus named this crystalline product "vitamin D_2," and the English investigators called it "calciferol." It has been fairly well established that vitamin D_2 and calciferol are identical substances, and we shall refer to them in this paper as "crystalline vitamin D." Windaus and Leuttringhaus[3] have isolated a number of other substances which occur during the course of irradiation of ergosterol, and it is interesting to note that none of them has any antirachitic activity. They are all isomers of ergosterol and have been designated as "lumisterol," "tachysterol," "suprasterol 1 and 2," and "substance 248" (toxisterol).

One milligram of crystalline vitamin D has a potency of 40,000 international units (approximately 14,815 Steenbock units). Crystalline vitamin D, being an isomer of ergosterol, has the chemical formula of $C_{28}H_{44}O$. It is dextrorotatory and has a melting point of 114° to 115° C. and an intense absorption band at 265 mu. The pure vitamin is extraordinarily stable, being resistant to oxidation and showing no loss of potency after storage for five months at 20° and at 37° C. or after heating for nine hours at 115° C.

Crystalline vitamin D as well as tachysterol and substance 248 are toxic to rats and mice when given in large amounts. The margin of safety, however, is great, inasmuch as toxic effects occur only after the administration of 3,000 times the therapeutic dose of crystalline vitamin D.

Clinical tests of the antirachitic value of crystalline vitamin D have been carried out abroad by Spence[4] and by Gorter.[5] Spence observed the effect of the daily administration of 1 c.c., 2 c.c. and 3 c.c. of an oily solution of crystalline vitamin D on the course of the disease in twelve patients with active rickets, ranging in age from one to eighteen years. One cubic centimeter of this preparation contained 3,000 international units (1,111 Steenbock units). In all

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This work was planned with Dr. A. F. Hess but was carried out and written after his death.

twelve cases definite healing was noted on the roentgenograms by the end of the fourth week, and complete healing was observed within thirteen weeks. Gorter places the daily curative dose of crystalline vitamin D at 0.05 mg., in other words at 2,000 international units (740 Steenbock units).

The discrepancy between the rat unit potency and the clinical effectiveness of various antirachitic agents constitutes one of the most interesting problems in the therapeutics of rickets. For example, it has been shown that 42 rat units (Steenbock units) in the form of irradiated milk protect against or cure infantile rickets, whereas 80 units in the form of milk from cows fed irradiated yeast and from 600 to 800 units in the form of viosterol are necessary to bring about similar results. The ratio of effectiveness of irradiated milk, "yeast" milk, and viosterol from the standpoint of rat units is approximately 15:8:1.[6]

In view of the fact that irradiated milk and milk from cows fed irradiated yeast require fewer units than viosterol to protect against or cure infantile rickets, the question arose as to whether the medium of milk may allow for better utilization of the antirachitic vitamin in infants than does the medium of oil. In order to test the validity of this hypothesis, it was decided to give one group of rachitic infants daily 90 units (Steenbock units) of crystalline vitamin D dissolved in oil and another group a similar number of units of the antirachitic factor incorporated in milk and to compare the healing in both groups of infants. A potency of 90 units was chosen because it is known from experience with viosterol and from available reports in the literature concerning the curative dose of crystalline vitamin D that this dosage in units in oil would be inadequate to cure a large percentage of rachitic infants. On the other hand, 90 units incorporated in milk should prove to be effective if the supposition were correct that the menstruum of milk was responsible for the lower number of units in the form of irradiated milk or of "yeast" milk required to cure rickets.

Accordingly nine infants showing evidences of rickets on roentgenographic examination were given daily 90 units of crystalline vitamin D incorporated in 24 ounces of milk; and another group of nine infants, comparable from the standpoint of age, severity of rickets, and daily intake of milk, were given 90 units of crystalline vitamin D dissolved in six drops of corn oil. These infants were enrolled in various health stations in New York City; I am indebted to the commissioner, Dr. John L. Rice, for his cooperation in connection with this study. The rachitic infants were followed up carefully in their homes and in the clinics throughout the test period, which began early in January and ended late in April. A special nurse was assigned to this investigation and the babies were seen by her on an average of twice a

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week. It should be added that only those babies whose mothers were cooperative and could be relied upon to follow instructions were included in the study.

After the infants had received crystalline vitamin D in milk or in oil for four weeks, roentgenograms were again taken of their wrists in order to determine the degree of healing which had taken place during this period. As emphasized in previous publications, the employment of the four-week test period is a good method of evaluating the relative potencies of antirachitic agents. In order to determine the progress of healing, however, roentgenograms were taken, when possible, after the infants had received crystalline vitamin D for six, eight, and ten weeks.

If milk were found to play a rôle in bringing about better utilization of the antirachitic factor, it would be of interest to know the minimum curative dose of crystalline vitamin D incorporated in milk. Thus we might learn whether milk to which the antirachitic factor has been directly added resembles, from the point of view of the number of units required to cure rickets, irradiated milk or milk from cows fed irradiated yeast. Accordingly, eight rachitic infants were given daily 45 units of crystalline vitamin D incorporated in 24 ounces of milk, a potency which is known to be adequate for irradiated milk but inadequate for "yeast" milk.

The scope of the investigation was further enlarged to determine the effect on the course of active rickets of a larger amount of crystalline vitamin D dissolved in oil. Ten rachitic infants were therefore given daily 6 drops of an oily preparation of crystalline vitamin D totaling 900 Steenbock units.

This study therefore comprised thirty-six rachitic infants divided into four groups: (1) nine infants receiving daily 90 units of crystalline vitamin D dissolved in oil; (2) nine infants receiving 90 units incorporated in milk; (3) eight infants receiving 45 units of crystalline vitamin D incorporated in milk, and (4) ten infants receiving 900 units of crystalline vitamin D dissolved in oil.

The incorporation of crystalline vitamin D in milk was accomplished either by (1) the "vipro" process, as advocated by Laquer,[7] consisting of the addition of an oily preparation of vitamin D to dry skimmed milk which is then mixed with fluid milk, or (2) by dissolving crystalline vitamin D in propylene glycol* and adding this solution directly to milk. By means of these methods, crystalline vitamin D is uniformly distributed throughout the milk, a fact which has been demonstrated by biologic assays of various levels of milks so treated. The vipro method was used for the preparation of milk of weaker potency, namely 45 units in 24 ounces of milk, and the propylene glycol solution for the incorporation of 90 units of crystalline vitamin D in 24 ounces of milk. The practical advantage of a propylene glycol solution of crystalline vitamin D is that it mixes freely with the milk, whereas drops of an oily solution cannot be distributed uniformly through milk unless the vipro process is employed.

The preparations of crystalline vitamin D were kindly sent to us by Dr. Charles W. Hooper. The incorporation of crystalline vitamin D was carried out daily shortly before delivery of the milk. Biologic assays of the milk and of the oily preparations of crystalline vitamin D used in this investigation were made three times during the course of the investigation, and the results showed that the requisite number of units were contained therein.

*Flint (J. Indus. & Engin. Chem. 24: 361, 1932) has shown propylene glycol to be relatively nontoxic and states that it may have a true food value in the sense that ethyl alcohol does without the drug action of the latter. Young rats grew at the normal rate and reached maturity when the only liquid they received was a 5 per cent solution of propylene glycol. It was also well tolerated by animals whose livers and kidneys had been injured by various poisons.

Propylene glycol is miscible with milk and water in all proportions and forms stable solutions of crystalline vitamin D. The taste and odor of milk are not altered by the addition of therapeutic amounts of crystalline vitamin D propylene glycol solution.

RESULTS

The results of this study may be gleaned from Table I. In surveying the data, it will be noted that at the end of four weeks all of the nine rachitic infants receiving daily 90 units of crystalline vitamin D in milk showed definite healing, whereas only three of nine rachitic infants receiving 90 units of crystalline vitamin D in corn oil showed healing. It should be added that the gains in weight in both groups were approximately the same.

At the end of six weeks healing progressed in those infants receiving the antirachitic vitamin in milk, and in one instance the lower ends of the radius and ulna were normal. We were able to obtain roentgenograms of six infants who had received crystalline vitamin D in milk for eight weeks, and in four of them the bones were normal, and in the other two infants rickets was either healed or almost healed (Figs. 1, 2, 3, 4). In contrast to these favorable results with crystalline vitamin D in milk, three of the eight infants receiving daily 90 units of crystalline vitamin D in oil for six weeks showed no healing and at the end of eight weeks the rachitic process became worse in two instances (Figs. 5, 6, 7).

One rachitic infant (B. R.) is of especial interest and significance. This baby had received 90 units of crystalline vitamin D in oil for four weeks, and roentgenographic examination at the end of this period showed that the rachitic process advanced. This infant was then given the same number of units of crystalline vitamin D in milk and, as will be noted in Fig. 8, definite healing resulted in four weeks and calcification in the rachitic zone was increased two weeks later.

It is obvious that 90 units of crystalline vitamin D in the form of propylene glycol solution incorporated in milk was much more ef-

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TABLE I

COMPARISON OF HEALING, AMONG 36 RACHITIC INFANTS, BROUGHT ABOUT BY CRYSTALLINE VITAMIN D INCORPORATED IN MILK OR IN CORN OIL

CASE	AGE (MO.)	DATE BEGUN	MENSTRUUM IN WHICH CRYSTALLINE VITAMIN D WAS INCORPORATED	NO. OF RAT UNITS (STEENBOCK) GIVEN	ROENTGENOLOGIC RICKETS: AT ONSET	HEALING AFTER 4 WK.	HEALING AFTER 6 WK.	HEALING AFTER 8 WK.	HEALING AFTER 10 WK.	COMMENT
C. M.	8	1/23	Milk†	45	Moderate	+	++	+++	++++	Porto Rican
B. G.	6	1/25			Moderate	+	+±	++	+++	Porto Rican
F. S.	13	1/26			Moderate	0	0			Negro
E. D.	6	2/ 7			Slight	+	++	+++±		Italian
G. N.	8	2/18			Moderate	+	+±	++	+++	Porto Rican
C. W.	22	2/17			Marked	+±	++	+++		Negro
G. B.	8	2/21			Moderate	+±	+++	+++±		Negro
J. N.	6	2/24			Mild	0	0	0 (worse)		Negro
V. L.	0	1/13	Milk‡	90	Slight	++	+++	Normal		Italian
S. Q.	4	1/13			Moderate	+±	++±	+++±	++++	Porto Rican
J. A.	5	1/13			Slight	+++	++++	Normal		Negro
R. L.	5	1/19			Moderate	+++	Normal	Normal	Normal*	Negro
C. H.	20	1/25			Marked	+++	+++±	++++	Normal	Negro—1/25: calcium, 9.8 mg.; phosphorus, 3 mg. 2/23: calcium, 10.6 mg.; phosphorus, 5 mg.
K. S.	4	2/22			Slight	++	+++	Normal		Negro
B. C.	7	3/ 9			Moderate	+				Porto Rican
F. S.	15	3/17			Moderate	+	+±			Negro
B. R.	6	3/17			Moderate	+±	+++			This infant had received 90 units of crystalline vitamin D in oil for one month, and no healing resulted

+Denotes slight healing; ++, moderate healing; +++, marked healing; ++++, healed rickets.
* X-ray picture taken at 12 weeks.
†One liter contained 60 units.
‡One liter contained 120 units.

TABLE I—CONT'D

CASE	AGE (MO.)	DATE BEGUN	MENSTRUUM IN WHICH CRYSTALLINE VITAMIN D WAS INCORPORATED	NO. OF RAT UNITS (STEENBOCK) GIVEN	ROENTGENOLOGIC RICKETS: AT ONSET	HEALING AFTER 4 WK.	HEALING AFTER 6 WK.	HEALING AFTER 8 WK.	HEALING AFTER 10 WK.	COMMENT
T. R.	5	2/ 4	Oil§	90	Moderate	+±	++±	+++	++++	Porto Rican
S. L.	7	2/12			Slight	+	+	+±		
B. M.	4	2/14			Slight	+	++		++	Negro
B. R.	5	2/16			Slight	0 (worse)				Negro
V. B.	14	2/16			Marked	+	+±	++	+++	Negro
D. R.	7	2/21			Moderate	0	0	0 (worse)	0	Negro
C. Q.	5	2/23			Slight	+	+	+±	++±	Porto Rican
C. B.	8	2/28			Moderate	0	0	0 (worse)		Negro
T. B.	8	3/19			Moderate	0	0			Negro
J. B.	3	2/16	Oil‖	900	Slight	++		Normal	Normal	Negro
P. G.	6	2/ 9			Slight	++	++++			Italian
J. B.	6	2/16			Slight	++	++±	+++	+++±	Negro
T. B.	6	2/16			Slight	++	++++	Normal		Negro
C. M.	6	2/16			Moderate	++				Negro
R. P.	17	2/17			Marked	+				Negro
H. B.	4	2/17			Slight	++	+++	+++±	Normal	Negro
J. D.	7	2/17			Moderate	++	++±	+++	+++±	Negro
N. E.	3	2/17			Slight	+	++	++±		2/15: calcium, 7.2 mg.; phosphorus, 5.3 mg. 3/13: calcium, 10.3 mg.; phosphorus, 6.1 mg.
J. D.	24	1/30			Marked	+	+	+	++	Negro; at 12 weeks healing ++

§One drop contained 15 units.
‖One drop contained 150 units.

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fective than the same number of units in oil. These results clearly indicate that the medium of milk allows for better utilization of the

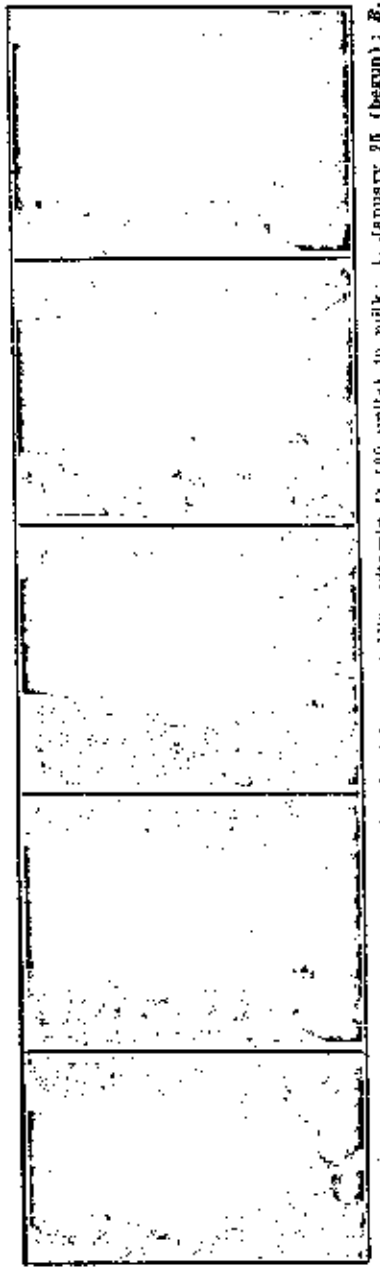

Fig. 1.—(C. H.) Healing of rickets brought about by crystalline vitamin D (90 units) in milk: A, January 26 (begun); B, February 21 (four weeks later); C, March 8 (two weeks later); D, March 21 (two weeks later); E, April 24 (five weeks later).

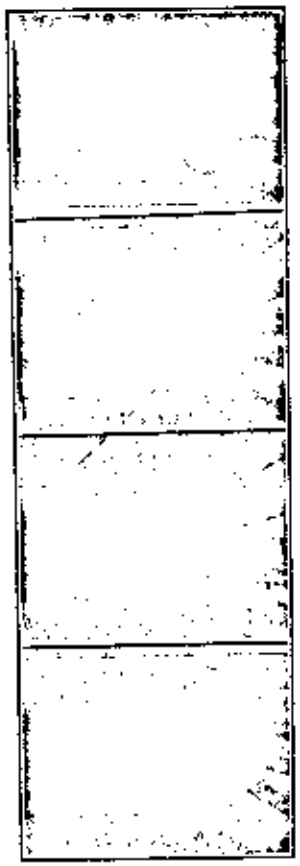

Fig. 2.—(R. L.) Healing of rickets brought about by crystalline vitamin D (90 units) in milk: A, January 19 (begun); B, February 15 (four weeks later); C, March 5 (two and one-half weeks later); D, March 29 (three and one-half weeks later).

antirachitic vitamin than does the medium of corn oil, and thus offer an explanation for the greater effectiveness, from the standpoint of rat units, of antirachitic milks as a group as compared with viosterol.

Of the eight infants who received daily 45 units of crystalline vitamin D in 24 ounces of milk, six showed definite healing at the end of four weeks. At six weeks healing progressed in these six infants, and after eight weeks roentgenographic examination revealed further

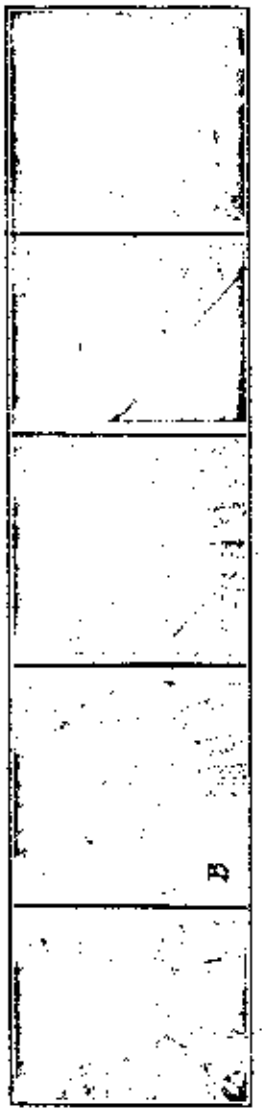

Fig. 3.—(S. Q.) Healing of rickets brought about by crystalline vitamin D (90 units) in milk: A, January 13 (begun); B, February 9 (four weeks later); C, February 27 (two and one-half weeks later); D, March 26 (two weeks later).

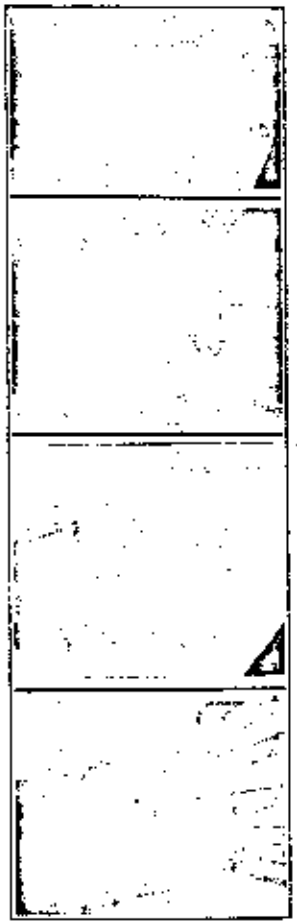

Fig. 4.—(J. A.) Healing of rickets brought about by crystalline vitamin D (90 units) in milk: A, January 13 (begun); B, February 9 (four weeks later); C, February 27 (two and one-half weeks later); D, March 14 (two weeks later).

healing; in two instances the rachitic disorder was almost healed. Two infants, however, failed to show healing at the end of four and six weeks, and in one infant followed for eight weeks the rickets became worse. One baby (F. S.), who showed no healing after having received 45 units in milk for six weeks, was given 90 units in milk for

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four weeks, and at the end of this period definite healing was noted on the roentgenogram.

It is evident that the results with 45 units of crystalline vitamin D in milk were superior to those obtained with 90 units of crystalline

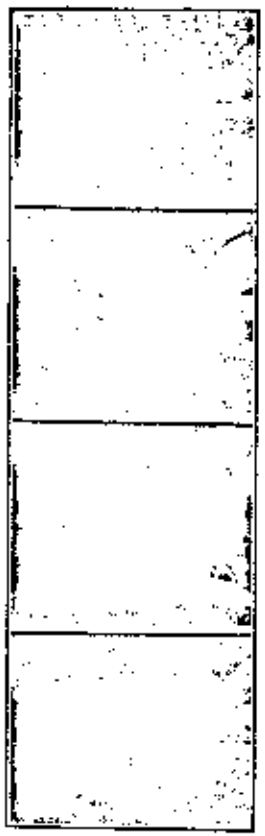

Fig. 5.—(C. B.) Absence of healing with crystalline vitamin D (90 units) in corn oil; *A*, February 28 (begun); *B*, March 29 (four weeks later); *C*, April 12 (two weeks later); *D*, April 27 (two weeks later).

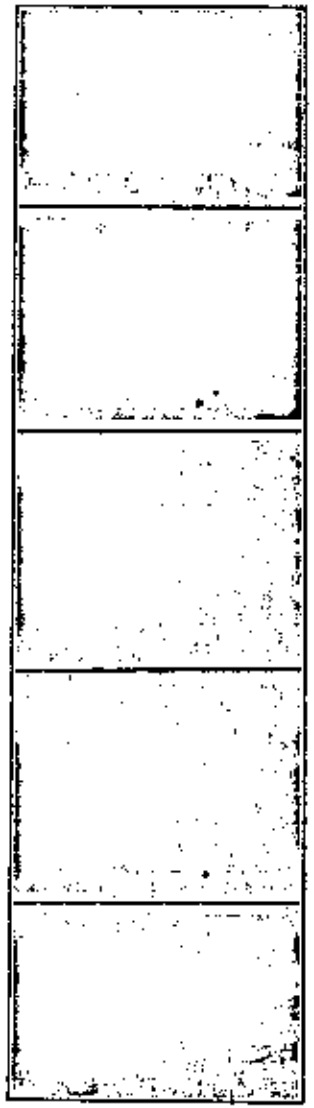

Fig. 6.—(D. B.) Absence of healing with crystalline vitamin D (90 units) in corn oil; *A*, February 21 (begun); *B*, March 21 (four weeks later); *C*, April 4 (two weeks later); *D*, April 18 (two weeks later); *E*, April 30 (two weeks later).

vitamin D in oil. However, in view of the fact that healing was not brought about in two of eight infants, this unitage in milk must be considered inadequate for curative purposes.

From these experiences it would seem that milk which had been fortified with crystalline vitamin D resembled more closely milk produced from cows fed irradiated yeast rather than irradiated milk, in-

asmuch as 45 units of crystalline vitamin D failed to cure all of the eight rachitic infants, whereas 90 units proved adequate.

At first glance, it would seem that the increased effectiveness of crystalline vitamin D when incorporated in milk as compared with its administration in oil might be due to better absorption of vitamin D in the intestinal tract when the antirachitic factor is administered in milk. However, biologic assays of the feces of two infants who had been receiving 90 units of crystalline vitamin D in milk and of the

Fig. 7.—(T. B.) Absence of healing with crystalline vitamin D (90 units) in corn oil; *A*, March 17 (begun); *B*, April 19 (four and one-half weeks later); *C*, May 1 (one and one-half weeks later).

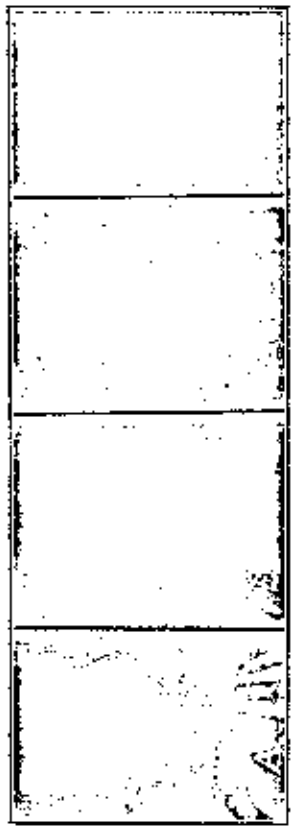

Fig. 8.—(B. K.) Absence of healing with crystalline vitamin D (90 units) in corn oil; healing brought about by crystalline vitamin D (90 units) in milk; *A*, February 18 (oil begun); *B*, March 18 (milk begun); *C*, April 17 (four weeks later); *D*, April 30 (two weeks later).

feces of two infants who had been receiving 90 units of crystalline vitamin D in oil for the same period showed approximately the same amount of vitamin D. Samples of blood were also taken from these infants for assay of vitamin D in order to determine whether there was an increase of the antirachitic factor in the blood of those receiving crystalline vitamin D in milk. The results of this experiment revealed that there was no difference in the vitamin D content of the blood in both groups of infants. The superior utilization of crystalline vitamin D when administered in the menstruum of milk does not seem, therefore, to be due to better absorption of the antirachitic factor in the intestinal tract, but a final decision concerning the role of absorption

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in connection with this interesting phenomenon must be held in reservation until assays of the blood and feces of a larger number of infants are carried out.

RESULTS WITH 900 STEENBOCK UNITS OF CRYSTALLINE VITAMIN D IN OIL

Ten rachitic infants, ranging in age from three to twenty-four months, were given daily 6 drops of crystalline vitamin D in oil, one drop containing 150 (Steenbock) units. One baby had manifest tetany; and the calcium content of the blood was 7.3 mg., and the inorganic phosphorus 6.1 mg. per 100 c.c. As will be observed in Table I, healing was noted on the roentgenograms in every instance at the end of four weeks, and the calcium content of the blood of the infant with tetany was normal, 10.3 mg. per 100 c.c. of blood. At the end of eight weeks, two of seven infants showed normal bones on roentgenographic examination and the other five infants revealed from slight to marked healing. However, one negro baby who had marked rickets was followed for twelve weeks, and at the end of this period only slight to moderate healing was observed. The impression gained from these experiences is that 900 units of crystalline vitamin D in oil is a good therapeutic dose for mild or moderate cases of rickets, but may not be adequate for severe cases. The therapeutic results with 90 units of crystalline vitamin D in milk were superior to those obtained with 900 units in oil.

We used approximately a 450-D preparation of crystalline vitamin D in oil but would advise that commercially a 250-D preparation should be supplied in order to conform with the present potency of viosterol (250-D). For mild and moderate cases of rickets from 10 to 15 drops of crystalline vitamin D in oil (250-D) should be given, and for marked cases, from 20 to 60 drops daily. It may be noted that these recommended curative doses of crystalline vitamin D in oil are the same as those generally advised for viosterol. One can readily understand the similarity in behavior of crystalline vitamin D in oil and viosterol when it is recalled that crystalline vitamin D is obtained from irradiated ergosterol and this accounts for the entire antirachitic potency of irradiated ergosterol.

The advantage of the use of pure crystalline vitamin D is that it obviates the necessity to some extent of biologic assays, as 1 mg. of crystalline vitamin D has a uniform potency of 40,000 international units. However, biologic assays should be carried out from time to time as a check on the accuracy of the technic employed in the preparation of solutions of crystalline vitamin D.

SUMMARY

In order to determine whether crystalline vitamin D in milk was more effective unit for unit than crystalline vitamin D in oil, nine rachitic infants were given 90 units of crystalline vitamin D in milk, and nine were given 90 units of crystalline vitamin D in oil and a comparison of healing in both groups was noted. The scope of the investigation was enlarged to include a study of the effect on the course of rickets of 45 units of crystalline vitamin D in milk, and 900 units in oil. Accordingly eight rachitic infants were given daily 45 units of crystalline vitamin D in milk, and ten infants, 900 units in oil.

The results of the administration of 90 units of crystalline vitamin D in milk were definitely superior to those obtained with a similar number of units in oil. Of nine infants receiving 90 units of crystalline vitamin D in milk, healing was observed in all at the end of one month, whereas only three of nine infants receiving 90 units of crystalline vitamin D in oil showed healing during the same period of observation. Roentgenograms were also taken after crystalline vitamin D had been given for six, eight, and ten weeks, and the results revealed that the group of infants receiving the antirachitic vitamin in milk reacted more favorably. These results indicate that the medium of milk allows for better utilization of the antirachitic vitamin than does the medium of corn oil and thus offer an explanation for the greater effectiveness, from the standpoint of rat units, of antirachitic milks as compared with viosterol.

The therapeutic results with 45 units of crystalline vitamin D in milk were superior to those obtained with 90 units of crystalline vitamin D in oil. However, two failures were noted among eight infants receiving the 45-unit milk, so that this dosage of units in milk must be regarded as below the therapeutic level.

With 900 units of crystalline vitamin D in oil, satisfactory healing resulted in nine of ten rachitic infants. We would recommend that from 10 to 15 drops (800 to 1,200 Steenbock units) of a 250-D preparation of crystalline vitamin D in oil should be used as a curative dose for mild and moderate cases of rickets and that from 20 to 60 drops be given for marked cases. However, if a solution of crystalline vitamin D in propylene glycol 250-D is employed by incorporation in 24 ounces of milk, only one drop (83 Steenbock units) is necessary to cure infantile rickets.

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FURTHER OBSERVATIONS ON THE COMPARATIVE ANTIRACHITIC VALUE OF CRYSTALLINE VITAMIN D ADMINISTERED IN MILK, CORN OIL, OR IN PROPYLENE GLYCOL

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In a previous communication[1] on the comparative antirachitic value of crystalline vitamin D incorporated in milk and in corn oil, it was shown that the daily administration of 243 U.S.P. X (revised, 1934) units (90 Steenbock units) of crystalline vitamin D distributed in 24 ounces of milk brought about excellent healing in all of nine infants with active rickets, whereas a similar number of units of crystalline vitamin D administered in 6 drops of corn oil failed to bring about satisfactory results in a comparable group of rachitic infants. The curative effect of the daily administration of 2,430 U.S.P. units (900 Steenbock units) of crystalline vitamin D in an oily solution was satisfactory but not quite so good as that which was observed with 243 U.S.P. units in milk. The results, therefore, of this study indicated that crystalline vitamin D is more effective, from the standpoint of rat units, when it is given in the daily ration of milk than when administered in a few drops of oil. An explanation was thus offered for the greater effectiveness, unit for unit, of irradiated milk and yeast milk as compared with viosterol (a solution of irradiated ergosterol in oil).

The previous study involved infants having active rickets, and consequently only the curative properties of crystalline vitamin D could be tested. The present investigation was undertaken (1) to determine the dosage of crystalline vitamin D necessary to prevent infantile rickets and (2) to investigate, from a prophylactic point of view, the question of the influence of the menstruum on the effectiveness of the antirachitic factor. This study differed from the previous investigation also in that the antirachitic effectiveness of a propylene glycol solution of crystalline vitamin D was determined in order to find out whether this solvent brought about better utilization of crystalline vitamin D than did corn oil. In the previous study, as well as in this investigation, a propylene glycol solution of crystalline vitamin D was added to milk to bring about a uniform distribution of this antirachitic substance in milk. The question arose as to whether propylene glycol rather than milk was responsible in the previous study for the more favorable results obtained when crystalline vitamin D was incorporated in milk.

Accordingly, it was decided to distribute, at the beginning of the winter, a large number of infants among the following groups, and to compare at the end of the winter, the incidence of rickets in these various groups:

Group 1, infants receiving 145 U.S.P. units of crystalline vitamin D in milk.

Group 2, infants receiving 145 U.S.P. units of crystalline vitamin D in corn oil.

Group 3, infants receiving 145 U.S.P. units of crystalline vitamin D in propylene glycol.

Group 4, infants receiving 290 U.S.P. units of crystalline vitamin D in milk.

Group 5, infants receiving 290 U.S.P. units of crystalline vitamin D in corn oil.

Group 6, infants receiving 290 U.S.P. units of crystalline vitamin D in propylene glycol.

Group 7, infants receiving 1,450 U.S.P. units of crystalline vitamin D in corn oil.

Group 8, infants serving as controls.

The study was carried out in the Department of Health Baby Health Stations of New York City, and I am indebted to the Commissioner, Dr. John Rice, for his cooperation in this project. During the month of December, 441 infants, ranging in age from two weeks to five months, were examined and placed among the aforementioned groups. The wrists of these infants manifesting moderate or marked enlargement of the costochondral junctions were roentgenographed to determine whether rickets was demonstrable by roentgen ray examination. If roentgenologic changes characteristic of rickets were observed, such infants were excluded from the study which, as stated above, was essentially a preventive one.

It was necessary to utilize the roentgenologic criterion in establishing the diagnosis of rickets because it is most difficult, and often impossible, to make a diagnosis of active rickets merely by physical examination. It is common experience to observe infants who have moderate, or even marked, beading of the ribs but nevertheless reveal no evidences of rickets by chemical examination of the blood or even by roentgenologic examination of the long bones. This point of view has also been emphasized recently by Tisdall and by Eliot.

The groups receiving crystalline vitamin D in oil or in propylene glycol included many nursing infants. In view of the fact that breast-fed infants do not develop rickets as frequently as artificially fed infants, it was felt that, if the groups of infants receiving crystalline vitamin D in these solvents showed higher incidences of rickets than the groups receiving crystalline vitamin D in milk, one could be more convinced that with the administration of this antirachitic substance in milk, a greater degree of utilization of crystalline vitamin D is brought

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about. In addition, it was thought advisable to obtain some information concerning the prophylactic dosage of crystalline vitamin D for breast-fed infants.

The incorporation of crystalline vitamin D in milk was made in the laboratory shortly before the delivery of the milk to the homes of the infants. As stated previously, a propylene glycol solution of crystalline vitamin D was used in order to bring about a uniform distribution of this antirachitic substance in milk. Mothers were given formulas for milk mixtures which did not contain more than 28 ounces of milk, as the number of units of crystalline vitamin D in this quantity of milk was comparable to the number of units given in oil or in propylene glycol.* Throughout the greater part of the experiment, a large proportion of infants receiving crystalline vitamin D in milk took less than 28 ounces of milk. This was compensated for by the fact that toward the end of the experiment, in spite of instructions to the contrary, several babies were given one quart of milk daily.

TABLE I

THE DISTRIBUTION OF NEGRO INFANTS AS WELL AS BREAST-FED, BREAST- AND BOTTLE-FED, AND BOTTLE-FED INFANTS AMONG THE VARIOUS GROUPS

GROUP	TOTAL NUMBER OF INFANTS IN EACH GROUP	NUMBER OF NEGRO INFANTS	PERCENTAGE OF NEGRO INFANTS IN EACH GROUP	BREAST-FED INFANTS	BREAST- AND BOTTLE-FED INFANTS	BOTTLE-FED INFANTS
1	58	33	57	—	—	58
2	41	24	58	11	17	13
3	44	24	55	16	17	11
4	51	27	53	—	—	51
5	52	30	58	20	20	12
6	45	26	58	19	14	12
7	42	21	50	14	7	21
8	22	13	59	8	6	8

The mothers were instructed to give 7 drops of the oily or of the propylene glycol solution in ½ teaspoonful of orange juice or water. These preparations were well taken by the infants. The infants were followed closely at the Baby Health Stations and also in their homes where they were seen on an average of twice a month by a nurse. If there was any doubt that a mother could not be relied upon to carry out instructions, her infant was excluded from the study.

The investigation was terminated at the end of April, during which month, as well as during the latter part of March, roentgenograms were taken of the wrists of all infants to determine the presence of rickets. Although we began with 441 infants in December, only 355 were followed through to the end of the investigation, the remaining 86 having been excluded for various reasons, as for example, lack of cooperation,

*I am indebted to Dr. Charles W. Hooper for supplying the preparations of crystalline vitamin D and also for carrying out biologic assays of these preparations.

moving out of the district, illness, etc. Of the 355 infants, 188 or 55 per cent were negroes, and it may be [illegible] that they were quite evenly distributed among the various [illegible] majority of the other infants were of Puerto Rican or Italian extraction. Table I also illustrates the number of breast-fed and artificially fed infants in the various groups.

RESULTS

The results of this investigation may be gleaned from Table II. It may be noted that the infants who received approximately 145 U.S.P. units of crystalline vitamin D in milk developed rickets less frequently than those who received a similar number of units in corn oil or in propylene glycol. Three of the 58 infants receiving 145 U.S.P. units in milk developed rickets, whereas 6 of the 41 infants receiving 145 units in corn oil and 6 of the 44 infants receiving 145 units in propylene glycol showed roentgenologic evidences of rickets at the end of the study. The incidence of rickets in the "milk group" was 5.1 per cent, in the "oil group," 14.6 per cent, and in the "propylene glycol group," 13.6 per cent. None of the infants receiving crystalline vitamin D in milk developed moderate or severe rickets, whereas 2 in the "oil group" and 1 in the "propylene glycol group" manifested the more severe form of this disorder.

TABLE II

COMPARISON OF THE INCIDENCE OF RICKETS AMONG INFANTS RECEIVING "CRYSTALLINE VITAMIN D" INCORPORATED IN MILK, CORN OIL, OR PROPYLENE GLYCOL

GROUPS	NUMBER OF UNITS (U.S.P.) OF CRYSTALLINE VITAMIN D	MEDIUM IN WHICH CRYSTALLINE VITAMIN D WAS INCORPORATED	NUMBER OF INFANTS IN EACH GROUP	AVERAGE AGE AT BEGINNING OF STUDY (MONTHS)	AVERAGE GAIN IN WEIGHT PER MONTH DURING STUDY (LBS.)	NUMBER OF INFANTS WHO DEVELOPED RICKETS	DEGREE OF RICKETS: MILD	MODERATE	SEVERE	PERCENTAGE OF INFANTS WHO DEVELOPED RICKETS
1	145	Milk*	58	3.3	1.1	3	3	0	0	5.1
2	145	Oil†	41	3.4	1.3	6	4	1	1	14.6
3	145	Propylene glycol‡	44	3.1	1.3	6	5	0	1	13.6
4	290	Milk§	51	3.2	1.1	1	1	0	0	1.9
5	290	Oil‖	52	3.6	1.1	5	2	2	1	9.6
6	290	Propylene glycol¶	45	3.0	1.2	5	4	1	0	11.1
7	1,450	Oil#	42	3.1	1.3	1	1	0	0	2.4
8	Controls		22	2.6	1.1	8	3	1	1	36.3

*One quart contained 186 U.S.P. (revised) units.
†One drop contained 20.8 U.S.P. (revised) units.
‡One quart contained 532 U.S.P. (revised) units.
§One drop contained 41.6 U.S.P. (revised) units.
‖One drop contained 208 U.S.P. (revised) units.

On surveying the data concerning 290 units of crystalline vitamin D, it will be seen again that the infants receiving crystalline vitamin D in milk showed a lower incidence of rickets than those receiving a similar unitage of crystalline vitamin D in oil or in propylene glycol. Of 51 infants in the "milk group," 1 developed rickets, whereas of 52 infants in the "oil group," 5 manifested roentgenologic signs of rickets at the end of the investigation. Of 45 infants receiving 290 units of crystalline vitamin D in propylene glycol, rickets developed in 5 instances. The incidence of rickets in the "milk group" was 1.9 per cent; in the "oil group," 9.6 per cent; and in the "propylene glycol group," 11.1 per cent. No infant receiving crystalline vitamin D in milk developed moderate or marked rickets, whereas 3 receiving crystalline vitamin D in oil, and 1 receiving crystalline vitamin D in propylene glycol developed more advanced forms of rickets.

Of the 42 infants who received 1,450 U.S.P. units of crystalline vitamin D in corn oil, 1 infant developed rickets, an incidence of 2.4 per cent. This single exception was a negro infant who developed a mild form of rickets. This dosage of crystalline vitamin D in oil is comparable to 7 drops of viosterol (250D).

Twenty-two infants served as controls, having received no antirachitic agent from December until April. Of these infants, 8, or 36.3 per cent, developed rickets. It is interesting to mention that 13 of these 22 infants were negroes, and that none of the white infants manifested rickets, whereas 8 of the 13 negro infants developed evidences of rickets as noted by roentgen ray examination. In other words, the incidence of rickets among the negro infants was 62 per cent. This experience emphasizes the importance of including negro infants in studying the preventive value of an antirachitic agent.

It should also be mentioned in this connection that of the 27 infants who received crystalline vitamin D in various dosages (Groups 1 to 7) and developed rickets, 21, or 78 per cent, were negro infants. It may be recalled that 55 per cent of the infants in this study were negroes.

COMMENT

The results of this investigation indicate that crystalline vitamin D incorporated in the daily ration of milk is much more effective from the standpoint of rat units in preventing rickets than is crystalline vitamin D administered in corn oil or in propylene glycol. These results are in accordance with those reported in a previous study, in which the curative test was employed, and offer an explanation for the greater effectiveness, from the standpoint of rat units, of irradiated milk and yeast milk as compared with viosterol.

The prophylactic dosage of crystalline vitamin D depends, therefore, on whether this antirachitic substance is administered in the daily ration of milk, or whether it is given in a few drops of oil. If it is given in oil

the dosage should be comparable to that which is generally advised for viosterol, namely, 8 to 10 drops, or in terms of U.S.P. X (revised, 1934) units, 2,000 to 2,500. This dosage is recommended in order to conform with the prophylactic dosage of viosterol, as there seems to be no difference in the antirachitic effect of viosterol and crystalline vitamin D in oil. The results obtained with 1,450 units of crystalline vitamin D in oil were satisfactory, only 1 of 41 infants having developed rickets. It is possible that had we used 2,000 or 2,500 units, rickets might have been prevented in this infant also. However, it must be remembered that occasionally slight rickets will develop in spite of the fact that a generally effective amount of vitamin D had been given. For one reason or another, some infants require excessively large amounts of vitamin D to protect them against this nutritional disorder. This holds true also in regard to the treatment of rickets; cases of so-called "resistant rickets" yield to treatment only after huge doses of an antirachitic agent have been given. The converse situation also obtains not infrequently, namely, that many infants require comparatively small doses of an antirachitic agent to protect them against rickets or to cure them of this disorder. For this reason it is important that large groups of infants be employed in studying the preventive or curative dosage of an antirachitic agent. It is probable that the considerable confusion which has arisen in regard to the relative effectiveness of antirachitic agents, from the viewpoint of rat units, might have been avoided if the curative dosage had been tested in from 8 to 10 rachitic infants, rather than in from 2 to 4 infants.

When crystalline vitamin D in propylene glycol is administered in the daily ration of milk, the incorporation of 332 U.S.P. units in one quart of milk should be sufficient to protect 98 per cent of infants against rickets, inasmuch as only 1 of 51 infants receiving this unitage in milk developed rickets.

The question arises as to why the antirachitic factor should be more effectively utilized when it is administered in milk. I have no explanation based on scientific proof for this interesting phenomenon. It is possible that the combination of vitamin D and calcium and phosphorus may play a rôle in the superior effectiveness of vitamin D milk. On the other hand, the increased effectiveness of the antirachitic factor when incorporated in milk may be due to the manner of administration of crystalline vitamin D when milk is used as the vehicle, rather than to any inherent quality of milk itself. Crystalline vitamin D, when given in milk, was distributed in approximately 28 ounces of fluid and was given several times during the day, depending upon the number of bottles which the baby was receiving. On the other hand, crystalline vitamin D in oil or in propylene glycol was given in ½ teaspoonful of fluid and only once during the day. It is possible, therefore, that the difference in effectiveness of crystalline vitamin D among the various groups of infants

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was due to (1) the quantity of solvent employed, many ounces in the case of milk, as compared with ½ teaspoonful of fluid when oil or propylene glycol was employed as the vehicle of crystalline vitamin D, or (2) the frequency of administration of vitamin D during the day, several times when milk was used as a carrier of crystalline vitamin D, as compared with a single daily administration when the oil or the propylene glycol solution was employed. These aspects of this interesting problem are now under investigation, and in this connection the following experience is of interest:

A negro boy, four months of age, exhibited moderate rickets on roentgenologic examination in December. He was given 290 U.S.P. units of crystalline vitamin D in ½ teaspoonful of milk. At the end of seven weeks no healing was noted on the roentgenogram. This infant was then (February 13) given 290 U.S.P. units of crystalline vitamin D incorporated in 28 ounces of milk. In two weeks definite healing was noted on the roentgenogram and in two months almost complete healing.

In this particular instance, when crystalline vitamin D was given to a rachitic infant in a small quantity of milk once a day, no healing resulted; on the other hand, when given in a large quantity of milk, 28 ounces, several times during the day, prompt healing occurred.

SUMMARY

In order to determine the prophylactic dosage of crystalline vitamin D, as well as the influence of the menstruum on the effectiveness of this antirachitic substance, 441 young infants were given, at the beginning of the winter, 145, 290, or 1,450 U.S.P. X (revised, 1934) units of crystalline vitamin D incorporated in 28 ounces of milk, in 7 drops of corn oil, or in 7 drops of propylene glycol. At the end of the winter the incidence of rickets among these various groups of infants was determined by roentgenologic examination of the wrists.

The results of this investigation demonstrated that in infants receiving crystalline vitamin D in the daily ration of milk, rickets developed less frequently than in those receiving a comparable number of units of this antirachitic agent in 7 drops of corn oil or of propylene glycol. An explanation was thus offered for the greater effectiveness, from the standpoint of rat units, of irradiated milk and yeast milk, as compared with viosterol.

It was found that 1,450 units of crystalline vitamin D in oil daily protected 41 of 42 infants against rickets, and this may be regarded as a satisfactory protective dose.

The addition of 332 U.S.P. units of crystalline vitamin D to the quart of milk provided a highly satisfactory antirachitic milk inasmuch as only 1 of 51 infants receiving milk of this unitage developed rickets.

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Response to Vitamin D of Magnesium Deficient Rats.* (P2123)

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The changes of plasma calcium concentration during magnesium deficiency have been extensively studied in ruminants. Blaxter and Sharman(1) fed calves a low magnesium diet and found that plasma magnesium concentrations fell progressively from 2.0 mg/100 ml to about 1.0 mg/100 ml without change in the plasma calcium concentrations. When plasma magnesium levels fell below 0.8 mg/100 ml plasma calcium concentrations decreased rapidly. McAleese and Forbes(2) obtained similar findings in lambs that were kept indoors and fed a low magnesium diet. They also showed that addition of calcium and vit. D to the ration had no influence on the hypomagnesemia and hypocalcemia. L'Estrange and Axford(3) in 3 separate experiments in which lactating ewes were fed a low magnesium, normal calcium diet found that hypocalcemia developed when the plasma magnesium concentrations decreased below 1 mg/100 ml. Supplementary dietary magnesium increased the concentrations of both calcium and magnesium in plasma to normal within a few days. Smith(4) reported that efficiency of calcium utilization and plasma calcium concentrations in hypomagnesemic calves were restored to normal by the addition to the diet of large amounts of vit D (70,000 i.u./day) whereas physiologic amounts were ineffective.

The above observations suggest that magnesium deficiency in calves and sheep interferes with calcium homeostasis and that the vit D requirement in the magnesium deficient animal is greater than in the normal (4). In order to test whether this is also true in the rat, measurements of concentrative calcium transport by the rat small intestine *in vitro* were made in preparations from vit D deficient and vit D fed magnesium deficient and control rats. The effect of vit. D treatment on serum calcium and citrate concentrations of hypomagnesemic and control rats were also measured.

Material and methods. Male Sprague-Dawley rats at weaning were divided into groups at random and given a vit. D free diet‡ that differed only in the magnesium content as described previously(5).

The following analytical methods were used: Serum magnesium, Schachter(6) serum calcium, Harrison and Harrison(7), inorganic phosphate, Fiske and Subarrow(8) and serum citrate, Natelson, Pincus and Lugovoy (9), statistical treatment, Natrella(10).

The concentrative calcium transport studies were made on everted intestinal loops as previously described(11). Duodenal loops from magnesium deficient and control animals both vit. D deficient and vit. D treated were studied. The intestinal loop was filled with 0.6 ml of modified magnesium-free Krebs-Henseleit bicarbonate buffer(12) containing 0.02 M glucose. The loop was immersed in 5 ml of the same buffer solution. The initial

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‡ The basal diet consisted of casein (180 g), dextrose (231 g), corn starch (450 g), peanut oil (80 g), vit. D free vitamin mixture (30 g), choline chloride (0.5 g), magnesium free salt mixture (8 g), and $CaHPO_4$ (16 g). A magnesium salt mixture (3.0 g/kilo) was added to the diets of the control groups.

The vit. D free vitamin mixture consisted of thiamine HCl (4 mg), riboflavin (5 mg), pyridoxine (5 mg), nicotinamide (10 mg), calcium pantothenate (28 mg), p-amino benzoic acid (200 mg), inositol (200 mg), alpha tocopherol (110 mg), glucose (19.438 g). In addition a supplement of beta carotene (10 mg) and 2-methyl,1-4-napthoquinone (25 mg) were added in the peanut oil.

The "magnesium free salt mixture" consisted of NaCl (138 g), KCl (224 g), $FePO_4 \cdot H_2O$ (41 g), KI (0.16 g), $MnSO_4$ (0.70 g), and $CuSO_4$ (0.13 g). The magnesium salt mixture consisted of $MgCO_3$ (50 gm) + $MgSO_4$ (32 g).

TABLE I. Effect of a Single Dose of Vitamin D on Concentrations of Calcium, Phosphorus and Magnesium in Serum of Magnesium Deficient and Control Rats Previously Depleted of Vitamin D. In this experiment they were also fed a low-calcium, low-phosphorus, diet 72 hr before they were bled. The vitamin D treated animals received 500 i.u. of vitamin D_2 P.O. 18 hr before they were bled.

Serum (mg/100 ml)	Vitamin D deficient: Mg deficient (14)		Vitamin D deficient: Control (9)		Vitamin D treated: Mg deficient (6)		Vitamin D treated: Control (6)	
Ca	5.5 ± .45		5.8 ± .09		7.3 ± .70	*	8.3 ± .42	
Mg	.33 ± .01	‡	1.99 ± .15		.11 ± .02	‡	1.86 ± .14	
P	7.5 ± .58	*	8.9 ± 1.22		7.0 ± 1.60	†	9.1 ± .90	

Code:

Data are means ± 95% confidence intervals. ( ) indicates no. of animals.

* Differences significant at $P < .05$ level.

† " " " $P < .01$ "

‡ " " " $P < .005$ "

The statistical comparison is between magnesium deficient and control rats of similar vitamin D status.

concentrations of stable Ca and Ca^{45} in the solution within the loops (serosal fluid) and outside bathing the mucosal surface (mucosal fluid) were the same *i.e.*, 0.2 mM Ca/l and 0.05 μc Ca^{45}/ml. Concentrative transport of calcium was measured as the ratio of the concentration of Ca^{45} in serosal fluid to that in mucosal fluid (Cs/Cm) at the end of a 90 minute incubation at 37°C.

Results. Table I depicts the results of the first experiment. The magnesium depleted and control rats were studied after 18 days on a diet deficient in vit. D. In this experiment, they were placed on a low calcium, low-phosphorus diet (basal diet without $CaHPO_4$) for 72 hours and half of the animals were given 500 i.u. vit. D_2[a] orally 18 hours before all were bled. Magnesium deficient animals showed a diminished response to vit. D as measured by the degree of elevation of the serum calcium concentration ($P < .05$).

The effect of a small dose of vitamin D on concentrative transport of calcium by everted duodenal loops *in vitro* was measured in magnesium deficient and control rats that had been on a vit. D deficient diet for two weeks. The mean body weight (± 95% confidence intervals) for the control animals was 90 ± 6.1 g and for the magnesium deficient rats 70 ± 4.2 g. Fig. 1 depicts the response to 3 i.u. of vit. D_2[a] given by stomach tube 72 hours before the animals were killed. The concentration ratios between the serosal and mucosal fluid (Cs/Cm) achieved by the everted duodenal loops were the same for the magnesium deficient and control rats when vitamin D deficient. Following treatment with vit. D the intestinal preparations from control rats showed a significantly greater increase in concentrative calcium transport than the preparations from the magnesium deficient rats ($P < 0.005$). The rise of serum calcium concentration following this small dose of vit. D was also greater in control rats than in magnesium depleted animals ($P < 0.005$).

Since severe magnesium deprivation might have caused changes in the intestine which allowed less vit. D to be absorbed, thus accounting for the diminished response seen in the magnesium deficient animals, a group of rats was studied after the intramuscular administrations of the same dose (3 i.u.) of vit. D_2 72 hours before they were killed. This experiment demonstrated the same lessened response to vit. D of the magnesium deficient rats as measured by increase in serum calcium and by the *in vitro* transport of calcium against a concentration gradient by everted duodenal loops.

The effect of vit. D on serum citrate is shown in Fig. 2. Serum citrate was measured 72 hours after vit. D_2 was given intramuscularly. The vitamin D deficient animals are grouped together since we have shown that the serum citrate concentration of magnesium deficient vitamin D deficient rats is the same

[a] Vitamin D given as crystalline calciferol (Nutritional Biochemicals Corp.) dissolved in propylene glycol.

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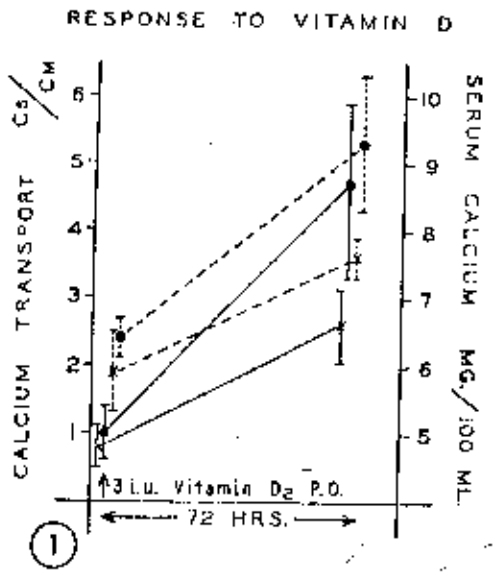

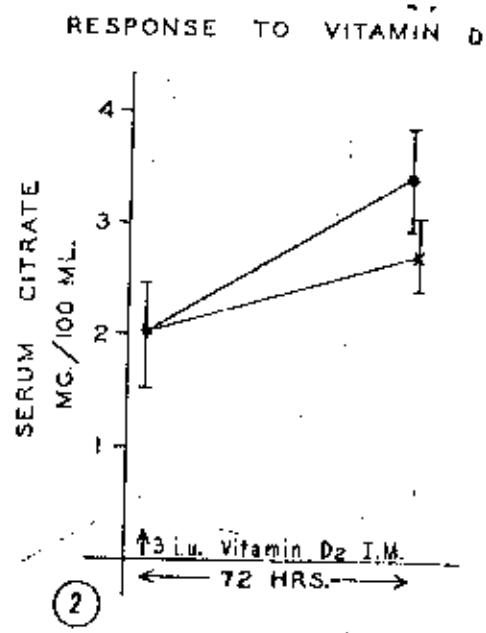

FIG. 1. Effects of 3 i.u. vit. D on concentrative transport of calcium *in vitro* and on serum calcium concentrations of magnesium deficient and control rats. Differences in concentration ratios between the serosal and mucosal fluid (Cs/Cm) after vit. D treatment are significant (P. <0.005). Differences in serum calcium concentration following vitamin D are also significant (P. <0.005). Code: Data are means from 6 observations in each group. ×, Magnesium deficient; ●, Control; ——— ---, Concentrative transport of calcium; - - - - - , Serum calcium; ⊥ ⊥ 95% confidence intervals.

FIG. 2. Serum citrate concentrations before and after intramuscular administration of 3 i.u. of vit. D. Differences in serum citrate levels after vit. D treatment are significant (P<0.005) in control animals only. Code: Data are means from 6 observations in each group. ×, Magnesium deficient; ●, Control; - ——, Serum citrate; ⊥ 95% confidence intervals.

as that of vitamin D deficient rats with normal magnesium content(5). An effect of a small dose of vit. D (3 i.u.) on the serum citrate concentration was evident in the control animals (P<0.005) but the magnesium deficient rats showed no significant response.

Discussion. The degree of response to vit. D was measured in these experiments by increase of serum calcium and citrate concentrations and of concentrative transport of calcium *in vitro* of intestinal loops prepared from control rats and rats given vit. D. The effective dose of vit. D differs with time between administration of vitamin and the determination of response. When a moderately large dose of vit. D_2, 500 i.u., is given and the serum calcium response tested 18 hours later the effect is much less than maximum and is analogous to the results obtained 72 hours after a small dose of vit. D_2, 3 i.u. In both types of experiments the magnesium deficient rats show significantly less effect of vit. D than the control rats. In the second series of experiments all of the physiologic effects of vit. D measured including augmentation of concentrative transport of calcium *in vitro* by the small intestine and increase of serum calcium and citrate concentrations were less in the magnesium depleted animals. Magnesium deficiency did not alter intestinal transport *in vitro* in the vitamin D-deficient rats indicating that the action is on the effect of vit. D in enhancing calcium transport rather than on the transport system itself. The effect of vit. D in increasing serum calcium in the vitamin D deficient rats was probably not due entirely to increased intestinal uptake of calcium since the animals had been placed on a low calcium diet during the 72 hours between administration of the

vit. D and the taking of the blood samples. The poor response of the serum calcium concentration of the magnesium deficient rats suggests that the effect of vit. D in increasing calcium mobilization from the skeleton is also reduced by magnesium depletion.

The partial refracteriness of magnesium deficient rats to small doses of vit. D as measured by intestinal transport of calcium *in vitro* was previously observed in rats given 100 i.u. per week during the 3 week period of magnesium depletion(13). No difference between magnesium deficient and control rats was noted, however, when a larger dose of vit. D was fed, 1000 i.u. per week. The diminished response to vit. D. of magnesium deficient rats can, therefore, be overcome by increased vit. D dosage. It has been shown in hypomagnesemic calves that pharmacologic doses of vit. D restored calcium utilization and serum calcium concentrations to normal whereas physiologic amounts were ineffective (4). Previously we have also shown that the serum citrate concentration of magnesium deficient rats did not rise 72 hours after oral administration of a single dose of 5,000 i.u., but a significant increase resulted in rats given 20,000 i.u. of vit. D(5).

This dose response can explain the failure of demonstration of hypocalcemia in magnesium deficient rats in the great number of studies which have been done, although in ruminants(2,14) and man(15,16) an association between hypomagnesemia and hypocalcemia has been found. The serum calcium concentrations of hypocalcemic and hypomagnesemic calves did not rise when calcium salts and vit. D were given but increased to normal values following magnesium administration(14). Similar findings have been reported in patients with malabsorption secondary to resection of portions of the small intestine(17). In addition there have been reports of patients with hypomagnesemia associated with hypoparathyroidism who were refractory to treatment until given magnesium salts(18,19). The concept of partial vit. D unresponsiveness in magnesium depleted subjects may explain these findings in animals and patients. In the many studies of magnesium deficiency in rats hypercalcemia has been a significant finding(20). The magnesium deficient rat shows many of the findings of hyperparathyroidism including hypercitricemia(21,5) increased calcium absorption (22) and hypophosphatemia with increased phosphate clearances(5). The rats have in all of these studies been given sufficient quantities of vit. D to obscure the partial insensitivity to vit. D which in this animal can only be shown by measurement of the response to small doses of vitamin D.

The mechanism whereby magnesium deficiency reduces the response to low concentrations of vit. D is unknown. There is a lag period between vit. D administration to an animal and detection of a physiologic effect. This lag period is decreased when large doses of vit. D are given(23). This time interval may be the period necessary for incorporation of vit. D into the cell structure, for the metabolic transformation of the steroid to an active molecule or for induction of a specific protein molecule through activation of DNA directed synthesis of mRNA. Whatever the role of magnesium may be in facilitating the physiologic response to vit. D the effects of magnesium deficiency on this response in the rat are only evident at low concentrations of vit. D.

Summary. Measurements of concentrative calcium transport by the rat small intestine *in vitro* were made in preparations from vitamin D deficient and vitamin D fed magnesium deficient and control rats. The effects of vitamin D treatment on serum calcium and citrate concentrations of hypomagnesemic and control rats were also measured. These studies indicate that magnesium deficient rats are less responsive to physiologic amounts of vitamin D than control rats.

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Endocrinology, 81:(4) 849, 1967

Effects of Vitamin D on Magnesium Metabolism in Rats[1]

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ABSTRACT. The effect of a single dose of 5000 IU of vitamin D on the concentrations of magnesium in serum and bone and the excretion of magnesium in urine of vitamin D-deficient rats fed either a magnesium-deficient or control diet was determined. Vitamin D treatment caused a more rapid decrease of bone and serum magnesium concentrations of rats placed on a low magnesium diet. Urinary magnesium excretion was higher in the vitamin D-treated rats. This effect was seen in rats fed the magnesium-containing control diet as well as the magnesium-deficient ration. The effect of vitamin D in increasing the renal clearance of magnesium is opposite to that of parathyroid hormone. This may indicate a separate action of vitamin D on renal tubular function or an inhibition of parathyroid hormone output by vitamin D. (*Endocrinology* 81: 849, 1967)

IN 1930, McHargue and Roy (1) found that the carcass magnesium content of rats subjected to ultraviolet irradiation was less than that of control animals. Subsequently, it has also been observed that vitamin D administration reduces serum magnesium concentrations in the rat (2–5), in the dog (6) and in man (7).

The mechanism whereby vitamin D reduces the concentration of serum magnesium was studied by Hanna (3), who found that intramuscular administration of large doses of vitamin D increased urinary magnesium excretion despite hypomagnesemia. Richardson and Welt (4), however, did not find a significant effect of vitamin D on urinary or fecal magnesium excretion, nor was there a significant change in their experiments in the concentrations of total carcass and muscle magnesium after the subcutaneous injections of large amounts of vitamin D_2.

In the above studies the effects of large doses of vitamin D on the magnesium metabolism of rats were measured in comparison with findings in control animals fed a normal vitamin D ration. The serum magnesium concentration of rats given physiologic doses of vitamin D is less than that of vitamin D-deficient rats (5). In order to determine the effects of physiologic amounts of vitamin D on magnesium metabolism, the serum, bone and urine magnesium concentrations of vitamin D-deficient rats were studied after the administration of a single dose of vitamin D in conjunction with either a low magnesium or a control magnesium-containing diet.

Materials and Methods

Male Sprague-Dawley rats from dams deprived of vitamin D during lactation were randomly divided into groups at weaning. They were given a vitamin D-free, magnesium-containing diet that has been described in detail elsewhere (5). After 2 weeks on this diet half the rats were given 5000 IU of vitamin D_2 orally. Twenty-four hr after this vitamin D treatment the vitamin D-deficient and the vitamin D-treated groups of rats were each divided into 2 subgroups: one was fed a magnesium-deficient diet and the other continued on the control magnesium-containing diet. There were then 4 groups of animals: vitamin D-treated control and magnesium deficient, and vitamin D-deficient control and magnesium deficient. Rats from each group were bled and killed at 24-hr intervals following vitamin D administration.

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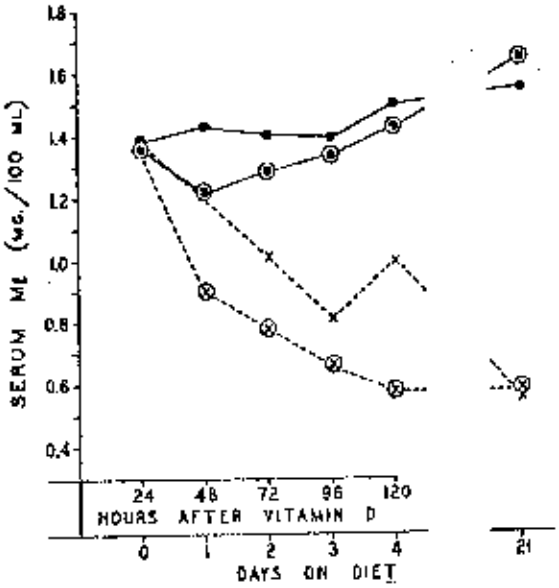

FIG. 1. Effects of vitamin D on serum Mg concentrations in rats fed normal and Mg-deficient diets. The effect of vitamin D in reducing serum magnesium concentration was significant ($p < 0.01$) in the Mg-fed rats 48 hr after its administration. In the Mg-deficient animals the effect was highly significant ($p < 0.005$) at the same interval, which was also following the first day of Mg deprivation, and the differences remained significant throughout the first 4 days of Mg-deficient diet. Data are means of 5–7 rats in each group at each time interval. The 4 groups studied are designated by the following code: ⊙——— ———⊙ = Mg-fed vitamin D-treated; ⊗- - - - - - ⊗ = Mg-deficient, vitamin D-treated; ●——— ———● = Mg-fed, vitamin D-deficient; X- - - - - X = Mg-deficient, vitamin D-deficient. Vitamin D-treated animals received 5000 IU of vitamin D_2 p.o.

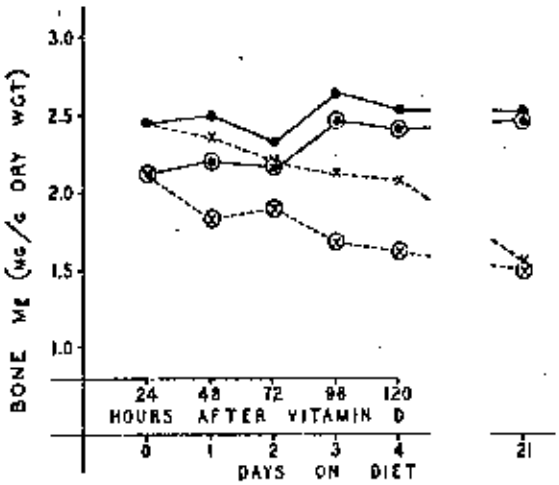

FIG. 2. Effects of vitamin D on bone Mg concentrations in rats fed normal and Mg-deficient diets. The effect of vitamin D in reducing bone Mg concentration was significant ($p < 0.05$) in the Mg-fed rats at 24 and 48 hr after its administration. In the Mg-deficient animals the effect was highly significant ($p < 0.005$) after the first day of Mg deprivation, and significant differences persisted throughout the first 4 days of Mg-deficient diet. Code as in Fig. 1.

The 24-hr urine excretion prior to bleeding was collected under toluene in metabolic cages to avoid contamination with feces or spilled food. Blood was drawn from the abdominal aorta and the left tibia was removed and dried at 105 C for 48 hr. Chemical analysis of the bone was performed after ashing in a muffle furnace at 500 C.

The following analytic methods were used: serum and bone magnesium (8); urine magnesium, atomic absorption (9); serum calcium (10); calcium in the urine and bone ash, Clark-Collip modification of the Kramer Tisdall method (11); inorganic phosphate in serum, bone and urine (12). Experiments were designed in a factorial manner and the data were analyzed statistically according to Natrella (13).

Results

Fig. 1 and 2 depict the serum and bone magnesium concentrations of the four groups studied. All animals placed on the magnesium-deficient diet showed a progressive decrease in concentrations of serum and bone magnesium. Following 24 hours of this diet the serum magnesium concentrations of the vitamin D-treated rats had fallen below 1 mg/100 ml and the bone concentrations were also significantly diminished ($p < 0.01$). By the fourth day of magnesium depletion the serum and bone magnesium concentrations were reduced to minimum values. The vitamin D-deficient rats fed the magnesium-deficient diet also developed hypomagnesemia and diminished bone magnesium content, but the rate of fall was considerably slower than that of the vitamin D-treated animals and the bone concentrations did not show a significant decrease until the third day of magnesium depletion. An effect of vita-

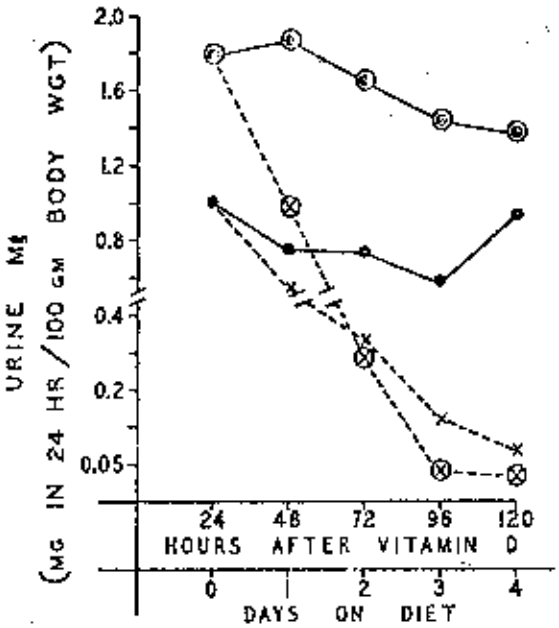

FIG. 3. Effects of vitamin D on urinary excretion of Mg of rats fed normal and Mg-deficient diets. The effect of vitamin D in increasing the urinary excretion of Mg was highly significant ($p < 0.005$) in the control rats starting at 24 hr after vitamin D administration. In the magnesium-deficient rats renal excretion of magnesium was significantly greater in the vitamin D-treated group in the first 24-hr period after reduction of magnesium intake. Code as in Fig. 1.

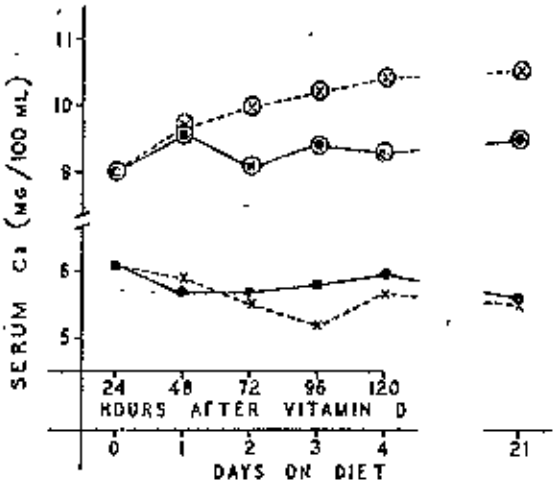

FIG. 4. Effects of vitamin D on serum Ca concentrations of rats fed normal and Mg-deficient diets. A highly significant ($p < 0.005$) effect of Mg deficiency in increasing the serum calcium concentration was seen in the vitamin D-treated rats after 48 hr of Mg deprivation. The vitamin D-deficient animals did not develop hypercalcemia. Code as in Fig. 1.

min D on serum and bone magnesium concentrations was also seen in the rats continued on the control diet. The vitamin D-treated animals showed a diminished bone magnesium content ($p < 0.05$) 24 hours after vitamin D was given and diminished serum concentrations ($p < 0.01$) by 48 hours in comparison with vitamin D-deficient rats. Between 48 and 72 hours following vitamin D administration serum and bone magnesium concentrations increased again so that after 72 hours there was not a significant difference between treated and deficient rats.

Fig. 3 shows the changes in urine magnesium concentrations. Vitamin D-treated rats showed an increased excretion of magnesium in comparison with the vitamin D-deficient animals ($p < 0.005$). The calculated 24-hour clearances of magnesium of the vitamin D-treated rats were greater than those of the vitamin D-deficient rats (Table 1). This effect was seen within

TABLE 1. Magnesium clearance (ml/24 hr)

Days on diet	Hr after* vitamin D	Magnesium-containing diet		Magnesium-deficient diet	
		Vitamin D deficient	Vitamin D treated	Vitamin D deficient	Vitamin D treated
0	0–24	6.16	11.24	—	—
1	24–48	3.71	10.65	2.66	8.35
2	48–72	2.58	9.00	1.70	4.35
3	72–96	3.12	6.63	1.65	0.47
4	96–120	6.62	7.04	0.49	0.41

* Vitamin D-treated groups only.

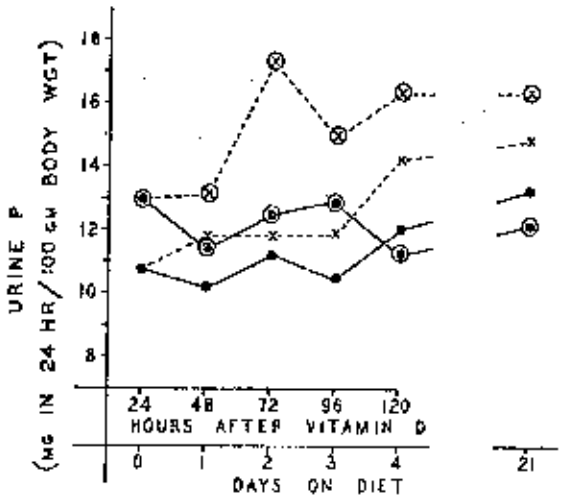

FIG. 5. Effects of vitamin D on urinary excretion of phosphorus of rats fed normal and Mg-deficient diets. The effect of Mg deficiency in increasing the urinary excretion of phosphorus was evident in the period between 1 and 2 days of Mg deprivation ($p < 0.005$) in vitamin D-treated rats. Vitamin D-deficient animals also developed a significantly greater ($p < 0.05$) increase of urine phosphate between 3 and 4 days in comparison with magnesium-fed controls. Code as in Fig. 1.

the first 24-hour period in both the magnesium-fed and the magnesium-deficient groups. Thereafter, the vitamin D-treated animals fed the magnesium-containing diet continued to show a greater urinary excretion of magnesium than the vitamin D-deficient rats. The magnesium-deficient rats showed decreasing amounts of magnesium in the urine and the renal clearances of magnesium approached the same low level in vitamin D-treated and vitamin D-deficient rats.

Other effects of magnesium deficiency in rats were also seen earlier in the vitamin D-treated animals. Hypercalcemia (Fig. 4), hypophosphatemia and hyperphosphaturia (Fig. 5) were evident ($p < 0.005$) after two days of magnesium depletion. In agreement with previous studies (5) the vitamin D-deficient rat did not develop hypercalcemia, which is in accord with the concept that the development of hypercalcemia in the hypomagnesemic rat is dependent on parathyroid hormone (14), and depletion of vitamin D results in parathyroid hormone unresponsiveness (2). The vitamin D-deficient, magnesium-deficient rat did develop hypophosphatemia and hyperphosphaturia but only after four days of magnesium depletion. These findings are consistent with previous experiments showing that the phosphate transport system which controls the concentration of phosphate in extracellular fluids is influenced by the concentration of extracellular magnesium in the absence of vitamin D or parathyroid hormone (15).

Discussion

An effect of vitamin D in enhancing magnesium excretion in the urine occurred even in rats on a low magnesium intake. This vitamin D effect on urinary magnesium was less marked in Hanna's studies (3) and was not seen in those of Richardson and Welt (4). In the experiments of these investigators the comparison was between rats receiving "ordinary" amounts of vitamin D and those given massive doses. The control animals may have received sufficient vitamin D to develop the maximum effect on renal magnesium clearances. It has been suggested that the increased urinary excretion of magnesium following vitamin D administration is secondary to the increased concentration of calcium in the urine (6, 16). In the present experiments magnesium excretion in the urine increased 2-fold within the first 24-hour period after the administration of vitamin D at a time when urine calcium excretion had not yet risen.

The mechanism whereby vitamin D increases magnesium excretion in the urine is not entirely dependent on parathyroid hormone action since similar although quantitatively smaller changes were observed by Heaton (14) in parathyroidectomized rats. Parathyroid hormone does alter renal tubular reabsorption of magnesium as shown by MacIntyre *et al.* (17) and Arnaud *et al.* (18), who found a decrease in urinary magnesium excretion following

parathyroid hormone administration to parathyroidectomized rats. The action of vitamin D on magnesium clearances is thus opposite to that of parathyroid hormone, and might result from inhibition of its output. Alternatively, there may be a direct action of vitamin D on renal tubular function. The initial negative balance of magnesium following vitamin D is only temporary, as shown by the return to normal of serum and bone magnesium levels in the rats on the magnesium-containing diet. This recovery could be due to increased gastro-intestinal absorption of Mg induced by vitamin D, which has been observed (19–21). It was suggested by Richardson and Welt (4) that the hypomagnesemia induced by vitamin D was due to a redistribution of this ion between extracellular fluid and other compartments. Wallach *et al.* (6) similarly interpreted their findings of an increased magnesium concentration in liver, pancreas and bone following vitamin D. In their studies pharmacologic doses of vitamin D were given for prolonged periods. Although they did not determine magnesium balances, magnesium retention could have resulted from increased intestinal absorption.

Magnesium deprivation leads to compensatory renal conservation of magnesium (22). In our studies urinary excretion of magnesium persisted following reduction of magnesium intake despite appreciable decreases in serum magnesium. Only after serum magnesium levels were markedly reduced did the magnesium excretion drop to minimal values. This presumably represents the effect of a decrease in the filtered load of magnesium presented to the tubules rather than an increased rate of tubular reabsorption of magnesium.

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STUDIES ON THE EFFECTS OF OVERDOSAGE OF VITAMIN D.*

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(From the Fleischmann Laboratories, New York.)

(Received for publication, July 12, 1929.)

The discovery of irradiated ergosterol as the specific antirachitic substance makes available a concentrated form of vitamin which can be administered to animals in doses of 100 to 500,000 times the daily curative dose. This provides a means for studying the effects of massive overdosages on the body functions and tissues.

Kreitmair and Moll (6), Pfannenstiel (7), Harris and Moore (3), and more recently, Klein (5) have found that massive overdosages of irradiated ergosterol produce anorexia, impairment of growth, and a decline in the general physical condition of white rats. Following the administration of extremely high dosages death frequently ensued. Dixon and Hoyle (2) report that the general pathological features mentioned above did not occur when similar dosages were fed to rats on normal diets, but they did find calcium phosphate concretions in the urinary tract.

In view of the results mentioned, it seemed of interest to study the calcium, phosphorus, and ash balances of animals fed varying dosages of irradiated ergosterol for a short time. The present investigation includes the calcium, phosphorus, and ash balances, blood serum calcium and phosphorus, the ash and Ca:P ratio of bones, hearts, and kidneys, and the weight curves of groups fed varying amounts of vitamin D.

Methods.

Four litters of six rats each were divided into six groups. Each group contained a rat from the respective litters, making four

* Read before the meeting of the American Chemical Society at Columbus, May, 1929.

rats in a group. The animals weighed between 40 and 60 gm. These animals were taken at the time of weaning and placed on the following diets with the indicated dosage of vitamin D per day for the last 14 days of the experiment. The duration of the experiment was 4 weeks. Balances were made on the 1st, 3rd, and 4th week of the period.

Group No.		Times daily curative dose.
I	Stock ration* + 0.4 gm. L.F.X.†	40
II	" " " " " + 0.001 mg. ergosterol	50
III	Stock ration + 0.4 gm. L.F.X. + 0.1 mg. ergosterol	1,040
IV	Stock ration + 0.4 gm. L.F.X. + 10.0 mg. ergosterol	100,040
V	Steenbock Ration 2965‡ + 0.4 gm. L.F.X. + 0.001 mg. ergosterol	50
VI	Steenbock Ration 2965 + 0.4 gm. L.F.X. + 10.0 mg. ergosterol	100,040

* Bills' modification of Steenbock formula:

Ground yellow maize	76
Linseed oil meal	16
Crude casein	5
Ground alfalfa	2
Calcium carbonate	0.5
Iodized sodium chloride	0.5

To 3 parts of the above add 1 part of powdered whole milk.

† An irradiated yeast preparation which gave a ++ line test in doses of 10 mg.

‡ Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 274 (1925).

The balances were obtained by determining the difference between the total ash of the food offered and the ash of the urine, feces, and the food unconsumed. The serum calcium was determined by the Kramer-Tisdall (8) method, and the serum phosphorus by the Briggs (1) modification of the Bell-Doisy method. The bone ash determinations were made on extracted fat-free tibia and femur.

Results.

Daily doses of vitamin D up to 1000 times the curative dosage for a period of 2 weeks have no deleterious effect upon the growth of young white rats fed a normal diet. Feeding dosages of 100,000

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times the curative dosage has a pronounced effect on the animals. Growth stops in less than a week and loss in body weight ensues. The animals in Group IV lost an average of 11 gm. during the 2 week period in which they received the vitamin D. Groups I, II, and III during the same period gained an average of from 40 to 46 gm. Excessive overdosage has a pronounced effect on the amount of food consumed. During the last week of this experiment the animals in Group IV consumed an average of only 8.25 gm., while those in Groups I, II, and III consumed an average of 65 gm. In appearance, the animals in Group IV were small, emaciated, and had greasy skin and hair. The region around the genitals and anus was stained yellow, a condition previously reported by Harris and Moore. The animals which died from this excessive dosage of vitamin D usually exhibited symptoms of labored breathing, extreme weakness, and recurrent convulsive tremors for 24 hours before death occurred. The animals in Group III, receiving daily 1000 times the curative dosage, appeared normal in all respects.

The results of other work now being carried on indicate that 10,000 times this amount of vitamin D administered daily over a period of 6 months does not affect the growth or general physical condition of the animals. These rats were fed the high calcium-low phosphorus Steenbock Ration 2965.

A similar dosage of vitamin D when administered to nursing mothers fed a modified Steenbock stock ration[1] does not affect the health of the mother or the young, except in those cases in which conditions resembling pellagra appeared. We were able to control this condition by the addition of large dosages of yeast. It has been found that the acute symptoms of vitamin D overdosage can be counteracted at certain levels by liberal feedings of yeast. Experiments now in progress indicate that a continuation of this dosage of vitamin D to second generation animals has no ill effects on their health and reproduction.

Dosages of 50,000 times the normal daily dose of vitamin D do have an effect on the health and reproduction of the mother and young. First generation mothers usually raised several litters, whereas in some cases total failure to reproduce resulted. But the second generation animals on this level of vitamin D failed to

[1] Bills' modification of the Steenbock formula.

reproduce. Some second generation animals maintained on this level of vitamin D showed, at the age of 3 to 4 months, excessive growth of incisors and calcification of some of the organs and tissues, particularly the kidneys, lungs, and aorta.

The effect on the animals fed the rachitogenic diet is not so pronounced. Group VI gained an average of 1 gm., while Group V gained an average of 34 gm. during the 2 week period in which the vitamin D preparation was administered. The animals in Group VI, receiving 100,040 times the curative dosage, showed the same symptoms as those in Group IV. The average food intake during the 4th week was 24 gm. for Group VI as compared

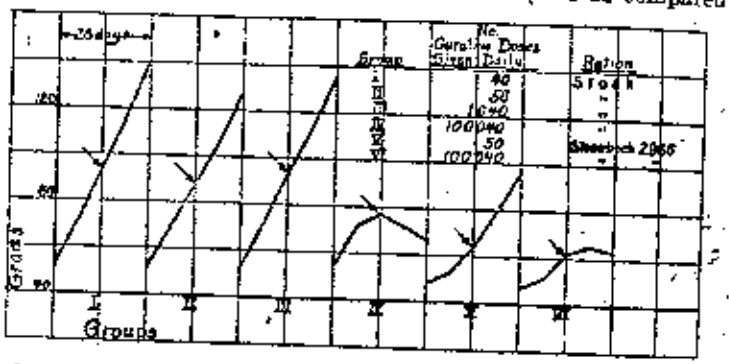

Chart I. The effect of varying dosages of vitamin D on the growth of white rats. Dosages were started at the points indicated by the arrows.

with 85 gm. for Group V which received only 50 times the curative dosage (daily).

The growth curves for the rats in Groups I to VI are shown in Chart I.

The blood serum calcium shows a definite increase over the normal in Groups IV and VI. Blood serum phosphorus shows an increase with increasing dosage of vitamin D in Groups I, II, and III. In Group IV, contrary to expectations, the blood serum phosphorus is lowered. Hess and Lewis (4) have obtained in a few cases an increase in blood serum calcium and a decrease in phosphorus when they administered large dosages of irradiated ergosterol to rachitic children. In Group VI the blood serum calcium and phosphorus are both higher than in Group V, which

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substantiates the findings of Klein and other investigators using the high calcium-low phosphorus diet.

The bone analyses (Table I) show a progressive decrease in the percentage of ash and a corresponding increase in the Ca:P ratio with increasing dosage of vitamin D. The percentage of calcium in the ash increases very slightly, while the percentage of phosphorus shows a slight decrease. Consequently the loss of phosphorus is relatively greater than that of calcium, as indicated by the increased Ca:P ratio in the bones of the animals in Group IV. These results indicate a loss of ash from the bone when excessive amounts of vitamin D are administered. Some experiments performed recently corroborate in general the results reported in this paper. The effect of a limited food intake on the depletion of the ash is also being investigated.

TABLE I.

Blood and Bone Analyses.

Group No.	No. of curative doses given daily.	Blood serum.		Bones	
		Calcium.	Phosphorus.	Ash based on extracted dry weight.	Ratio, Ca:P.
		mg. per 100 cc.	*mg. per 100 cc.*	*per cent*	
I	40	11.9	9.2	58.4	1.92
II	50	12.3	9.9	57.0	1.93
III	1,040	12.1	10.3	55.7	1.97
IV	100,040	14.6	8.1	47.5	2.06
V	50	11.7	7.1	51.2	2.00
VI	100,040	15.0	8.0	46.3	2.03

The ash determinations on the kidneys and hearts of the animals show a decreasing percentage of ash with increasing vitamin D intake. One exception to this result was in the kidneys of one rat in Group IV, which contained 6.6 per cent ash consisting of 38.2 per cent calcium and 12.1 per cent phosphorus, and having a Ca:P ratio of 3.2. This high ash content was undoubtedly due to calcium phosphate and other concretions containing calcium. Table II gives the average analyses.

In the calcium and phosphorus balance, calcium retention tends to increase through the 3 weeks of the balance experiment in all

groups except Groups IV and VI, which received the very large dosages, and in these two groups a marked decrease is shown. The phosphorus retention, however, does not change much except in Groups IV and VI, where a marked decrease occurs. In Group IV during the 3rd week there was an actual loss of phosphorus, and during the 4th week a loss of both calcium and phosphorus, indicating a drainage of these elements from the body when massive dosages are administered. In Group VI the Ca:P ratio of the retained calcium and phosphorus increases from 4.0 during the control period to 6.2 and 7.3 respectively for the 2 weeks of the high vitamin D administration. The ratios for Group V are 3.5 for the control week and 4.7 and 4.6 for the last 2 weeks. The relative retention of calcium is about 30 per cent higher than the phosphorus when excessive amounts of vitamin D are administered. It is interesting to note that the positive balance of calcium in Group IV during the third week is greater than the total ash balance which is 24 mg. per animal. These results are given in Table III.

TABLE II.

Heart and Kidney Analyses.

Group No.	No. of curative doses given daily.	Heart. Ash based on dry weight.	Kidneys.			
			Ash based on dry weight.	Ash.		Ratio, Ca:P.
				Ca	P	
		per cent	*per cent*	*per cent*	*per cent*	
I	40	2.3	3.0	13.1	2.1	6.2
II	50	2.4	3.2	11.0	1.8	6.1
III	1,040	1.7	2.5			
IV	100,040	1.8	3.7*	38.2†	12.1	3.2
V	50	1.8	2.3	12.8	2.1	6.1
VI	100,040	1.0	2.2	21.5	3.6	6.0

* Average of three rats, individuals being 2.7, 6.6, and 1.7 per cent.
† Per cent of Ca and P given is for kidneys having 6.6 per cent ash.

The ash balance shows an increased ash retention throughout the experiment, except in Groups IV and VI in which a decrease is shown, and an actual loss in Group IV for the 4th week. These results are given in Table IV.

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Dixon and Hoyle (2), since they found calcium phosphate concretions in the urinary tract, postulate that large overdosages of vitamin D cause an increased absorption of calcium and phosphorus from the intestinal tract and their subsequent elimination through the kidneys. The present study shows that with increasing dosages of vitamin D the percentage of ash in the bones, heart, and kidneys, and the total ash of the entire body decrease.

TABLE III.

Calcium and Phosphorus Balance (Mg. per Rat).

Group No.	1st wk.		3rd wk.		4th wk.	
	Ca	P	Ca	P	Ca	P
I	197.1	148.2	252.9	138.9	277.9	158.8
II	188.6	135.7	218.4	115.5	1152.2	64.7
III	204.0	151.4	237.8	123.3	259.9	147.4
IV	194.1	143.7	67.3	−1.3	−16.8	−31.4
V	293.4	84.7	389.9	82.7	472.4	101.4
VI	358.8	88.7	231.3	37.3	266.3	36.4

TABLE IV.

Ash Balance (Mg. per Rat).

Group No.	1st wk.	3rd wk.	4th wk.
I	860.4	943.7	1183.0
II	899.0	804.0	455.0
III	970.0	843.0	1109.0
IV	1029.0	24.0	−214.0
V	1034.0	1266.0	1723.0
VI	1224.0	711.0	859.0

The blood serum calcium and phosphorus and the Ca:P ratio of the bone ash show a progressive increase, with the exception of blood phosphorus in Group IV. The calcium and phosphorus and ash retention decrease with the high dosage. These results indicate that there is a drainage of mineral constituents from the body when excessively high dosages of vitamin D are administered and that there is a relatively greater elimination of phosphorus than of calcium from the body. If phosphorus, as suggested by Dixon and Hoyle (2), is eliminated through the urinary tract, it must be

in a soluble form, possibly as secondary calcium phosphate or combined with sodium or potassium. Should this be true, then the excessive elimination of phosphorus through the kidneys might account for the loss of minerals other than calcium and phosphorus.

These experiments indicate that excessively large dosages of irradiated ergosterol must be administered before any ill effects are noted. Other experiments show that amounts as high as 10,000 times the daily curative dose given over a long period of time have no effect on the growth of white rats and no apparent effect on their body functions.

The wide range between the effective amount of vitamin D required for the health of the animal and the daily intake which has deleterious effects on the growth of white rats is indicated by these results. If it were possible to apply these relative figures of the toxic dose of vitamin D for white rats to the infant, the harmful dose would be 1.5 liters per day of a solution of irradiated ergosterol in oil 100 times as potent as cod liver oil.

CONCLUSIONS.

1. Excessively large dosages of irradiated ergosterol must be administered daily before any ill effects are noted.
2. Dosages as high as 10,000 times the daily curative dose over a period of 6 months have no effect on the growth of white rats, and no apparent effect on their body functions.
3. Excessive amounts of vitamin D cause a drainage of mineral constituents from the body, with a relatively greater elimination of phosphorus than of calcium.
4. Dosages of 100,000 times the daily curative dose produce anorexia, emaciation, greasy hair, labored breathing, and eventually death.

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Skin-Pigment Regulation of Vitamin-D Biosynthesis in Man

Variation in solar ultraviolet at different latitudes may have caused racial differentiation in man.

W. Farnsworth Loomis

Vitamin D mediates the absorption of calcium from the intestine and the deposition of inorganic minerals in growing bone; this "sunshine vitamin" is produced in the skin, where solar rays from the far-ultraviolet region of the spectrum (wavelength, 290 to 320 millimicrons) convert the provitamin 7-dehydrocholesterol into natural vitamin D (*1*) (Fig. 1).

Unlike other vitamins, this essential calcification factor is not present in significant amounts in the normal diet; it occurs in the liver oils of bony fishes and, in very small amounts, in a few foodstuffs in the summer (see Table 1). Almost none is present in foodstuffs in winter.

Chemical elucidation of the nature of vitamin D has made it possible to eradicate rickets from the modern world through artificial fortification of milk and other foods with this essential factor. Before this century, however, mankind resembled the living plant in being dependent on sunshine for his health and well-being, a regulated amount of vitamin D synthesis being essential if he were to avoid the twin dangers of rickets on the one hand and an excess of vitamin D on the other.

Unlike the water-soluble vitamins, too much vitamin D causes disease just as too little does, for the calcification process must be regulated and controlled much as metabolism is regulated by the thyroid hormone. The term *vitamin D* is, in fact, almost a misnomer, for this factor resembles the hormones more closely than it resembles the dietary vitamins in that it is not normally ingested but is synthesized in the body by one organ—the skin—and then distributed by the blood stream for action elsewhere in the body. As in the case of hormones, moreover, the rate of synthesis of vitamin D must be regulated within definite limits if both failure of calcification and pathological calcifications are to be avoided.

Synthesis of too little vitamin D results in the bowlegs, knock-knees, and twisted spines (scoliosis) associated with rickets in infants whose bones are growing rapidly. Similar defects in ossification appear in older children and women deprived of this vitamin; puberty, pregnancy, and lactation predispose the individual toward osteomalacia, which is essentially adult rickets. In osteomalacia the bones become soft and pliable, a condition which often leads to pelvic deformities that create serious hazards during childbirth. Such deformities were common, for example, among the women of India who followed the custom of purdah, which demands that they live secluded within-doors and away from the calcifying power of the sun's rays (*2*). Cod-liver oil or other source of vitamin D is a specific for rickets and osteomalacia, the usual recommended daily dosage being 10 micrograms of 400 international units (1 I.U.=0.025 microgram of vitamin D).

Ingestion of vitamin D in amounts above about 100,000 I.U. (2.5 milligrams) per day produces the condition known as hypervitaminosis D, in which the blood levels of both calcium and phosphorus are markedly elevated and multiple calcifications of the soft tissues of the body appear. Ultimate death usually follows renal disease secondary to the appearance of kidney stones (*3*). Although this condition has been described only in patients given overdoses of vitamin D by mouth, similarly toxic results would probably follow the natural synthesis of equal doses of vitamin D by unpigmented skin exposed to excessive solar radiation. The body appears to have no power to regulate the amount of vitamin D absorbed from food and no power to selectively destroy toxic doses once they have been absorbed. These facts suggest that the physiological means of regulating the concentration of vitamin D in the body is through control of the rate of photochemical synthesis of vitamin D in the skin.

It is the thesis of this article that the rate of vitamin-D synthesis in the stratum granulosum of the skin is regulated by the twin processes of pigmentation and keratinization of the overlying stratum corneum, which allow only regulated amounts of solar ultraviolet radiation to penetrate the outer layer of skin and reach the region where vitamin D is synthesized. According to this view, different types of skin—white (depigmented and dekeratinized), yellow (mainly keratinized), and black (mainly pigmented)—are adaptations of the stratum corneum which maximize ultraviolet penetration in northern latitudes and minimize it in southern latitudes, so that the rate of vitamin-D synthesis is maintained within physiological limits (0.01 to 2.5 milligrams of vitamin D per day) throughout man's worldwide habitat.

Figure 2 provides evidence in support of this view, for it is apparent that there is a marked correlation between skin pigmentation and equatorial latitudes. In addition, the reversible summer pigmentation and keratinization activated by ultraviolet radiation and known a suntan represents a means of maintaining physiologically constant rates of vitamin-D synthesis despite the great seasonal variation in solar ultraviolet radiation in the northern latitudes.

Ultraviolet Transmission and Vitamin-D Synthesis

In 1958 Beckemeier (*4*) reported that 1 square centimeter of white human skin synthesized up to 18 I.U. of vitamin D in 3 hours. Using this figure, we calculate that an antirachitic preventive dose of 400 I.U. per day can

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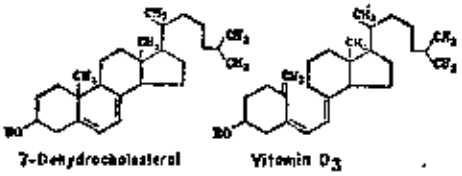

Fig. 1. Chemical structures of 7-dehydrocholesterol and vitamin D_3.

be synthesized by daily exposure of an area of skin approximately equal to that of the nearly transparent pink cheeks of European infants (about 20 square centimeters). Perhaps this explains why mothers in northern climates customarily put their infants out of doors for "some fresh air and sunshine" even in the middle of winter.

From this high rate of synthesis by only a small area of thin unpigmented skin, one can calculate the daily amount of vitamin D that would be synthesized at the equator by the skin of adults who exposed almost all their 1½ square meters (22,500 square centimeters) of body surface during the whole of a tropical day. Such a calculation shows that the skin of such individuals would synthesize up to 800,000 I.U. of vitamin D in a 6-hour period if the stratum corneum contained no pigment capable of filtering out the intense solar ultraviolet radiation.

Direct evidence that pigmented skin is an effective ultraviolet filter was provided by Macht, Anderson, and Bell (5), who used a spectrographic method to show that excised specimens of whole skin from Negroes prevented the transmission of ultraviolet radiation of wavelengths below 436 millimicrons, while excised specimens of white skin allowed radiation from both the 405- and the 365-millimicron bands of the mercury spectrum to pass through.

These early studies with whole skin were refined by Thomson (6), who used isolated stratum corneum obtained by blistering the skin with cantharides. He found that the average percentage of solar radiation of 300- to 400-millimicron wavelength transmitted by the stratum corneum of 22 Europeans was 64 percent, while the average for 29 Africans was only 18 percent. There was no overlapping of values for the two groups (Fig. 3), but there was considerable variation within each group, the values for the Europeans varying from 53 to as high as 72 percent and those for the Africans (who were mainly Ibos but also included men from most of the Nigerian tribes) varying from 36 to as low as 3 percent.

In his careful studies, Thomson measured skin thickness as well as pigmentation and found that the former was a minor variable. Studies on the degree of blackness of the various African specimens were made by skin-reflectance measurements. These showed that the darker the skin is, the lower is the percentage of ultraviolet radiation transmitted. One specimen from an albino African showed transmission of 53 percent—a value within the range for the European group. Thomson concluded from these studies that skin pigmentation was mainly responsible for protecting the African from excessive solar ultraviolet radiation, the thickness of the horny layer in Africans playing only a minor role. Thomson did not mention the fact that skin pigmentation and thickening of the horny layer in Africans would protect against excessive vitamin-D synthesis as well as sunburn.

Thomson's results indicate that African stratum corneum filters out solar ultraviolet radiation equivalent to between 50 and 95 percent of that which reaches the vitamin-D-synthesizing region of the skin of Europeans. This explains "the fact, agreed to by all, that of all races the Negro is most susceptible to rickets" (7). It is clear from Thomson's figures that exposure of the face of Negro infants to winter sunlight in Scandinavia would result in synthesis of too little vitamin D to meet the infant's body requirements.

It was Hess who first proved that sunlight could cure rickets (8). Seeking

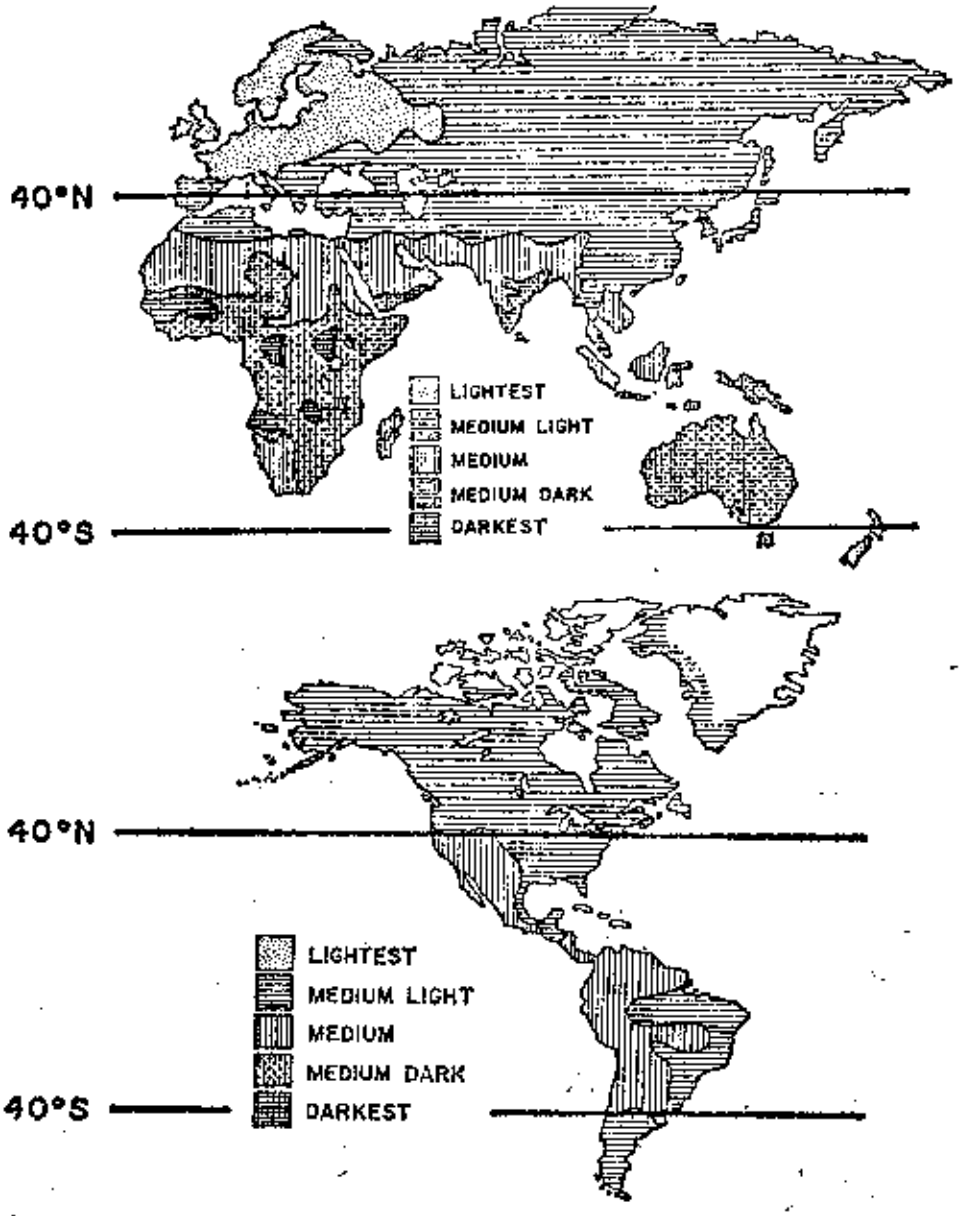

Fig. 2. Distribution of human skin color before 1492. [Adapted from Brace and Montague, *Man's Evolution* (Macmillan, New York, 1965), p. 272]

experimental proof of a relationship between skin pigmentation and rickets, he took six white and six black rats and placed them on a rachitogenic diet containing low amounts of phosphorus. Exposing both groups to a critical amount of ultraviolet light, he found that all the white rats remained healthy while all the black rats developed rickets. He concluded (9), "It is manifest that the protective rays were rendered inert by the integumentary pigment."

To return now to Thomson's results and consider their bearing on hypervitaminosis, they explain why deeply pigmented Africans living near the equator and exposing almost all their body surface to the ultraviolet of the tropical sun do not suffer from kidney stones and other evidences of hypervitaminosis. Under conditions where untanned Europeans would synthesize up to 800,000 I.U. per day, deeply pigmented Africans would synthesize 5 to 10 percent as much; thus their daily production would fall within the acceptable range.

In this connection it is significant that Reinertson and Wheatley (*10*) found that the 7-dehydrocholesterol content of human skin does not vary significantly between Negroes and Whites. Skin from the back, abdomen, and thigh of adults of both races averaged 3.8 percent (standard deviation, 0.8 percent), the lowest result in their series being obtained in a specimen of the epidermis of the sole, an area that receives no radiation at all, while the highest result among adults was from a Negro. The highest content of all was found in a specimen from a 2-week-old infant that showed 3.8 percent of the provitamin, a fact that correlates well with the especially high need for vitamin D during the first 2 years of life.

In their paper and ensuing discussion, the above workers emphasize that 7-dehydrocholesterol is found almost entirely beneath the stratum corneum, thus establishing the fact that in man it is not present in the secretions of the sebaceous glands as it is in birds and some northern fur-covered animals which, respectively, obtain their vitamin D by preening or by licking their fur after the provitamin has been converted into the vitamin on the surface of the body. It would appear that vitamin D is made in man solely by the irradiation of the provitamin in the layers underneath the stratum corneum, a mechanism that would allow efficient regulation of the biosynthesis of this essential factor by varying the degree of ultraviolet penetration through differing amounts of pigmentation in the overlying stratum corneum.

Origin of White Skin

Having originated in the tropics where too much sunlight rather than too little was the danger, the first hominids had no difficulty in obtaining sufficient amounts of vitamin D until they extended their range north of the Mediterranean Sea and latitude 40°N (Fig. 2), where the winter sun is less than 20 degrees above the horizon (*11*) and most of the needed ultraviolet is removed from the sun's rays by the powerful filtering action of the atmosphere through which the slanting rays have to pass. Before the present century, for example, there was a very high incidence of rickets among infants in London and Glasgow, because in these latitudes the midday sun is less than 35 degrees from the horizon for 5 and 6 months, respectively, of the year; in Jamaica and other southern localities, on the other hand, the sun's midday altitude is never less than 50 degrees and rickets is almost unknown (*2*). The farther north one goes, the more severe becomes this effect of latitude on the availability of winter ultraviolet radiation, an effect compounded by cloudy winter skies.

Table 1. Vitamin-D content of two fish-liver oils and of the only foodstuffs known to contain vitamin D. [From K. H. Coward, *The Biological Standardization of the Vitamins* (Wood, Baltimore, 1938), p. 223]

Fish-liver oil or foodstuff	Vitamin-D content (I.U./gram)
Halibut-liver oil	2000–4000
Cod-liver oil	60–300
Milk	0.1
Butter	0.0–4.0
Cream	0.5
Egg yolk	1.5–5.0
Calf liver	0.0
Olive oil	0.0

Having evolved in the tropics, early hominids were probably deeply pigmented and covered with fur, as are most other tropical primates. The first adaptation one might expect therefore to lowered availability of ultraviolet light as they moved north of the Mediterranean would be a reduction of fur, for Cruikshank and Kodicek have shown (*12*) that shaved rats synthesize four times more vitamin D than normal rats do.

As early hominids moved farther and farther north, their more deeply pigmented infants must have been especially likely to develop the grossly bent legs and twisted spines characteristic of rickets, deformities which

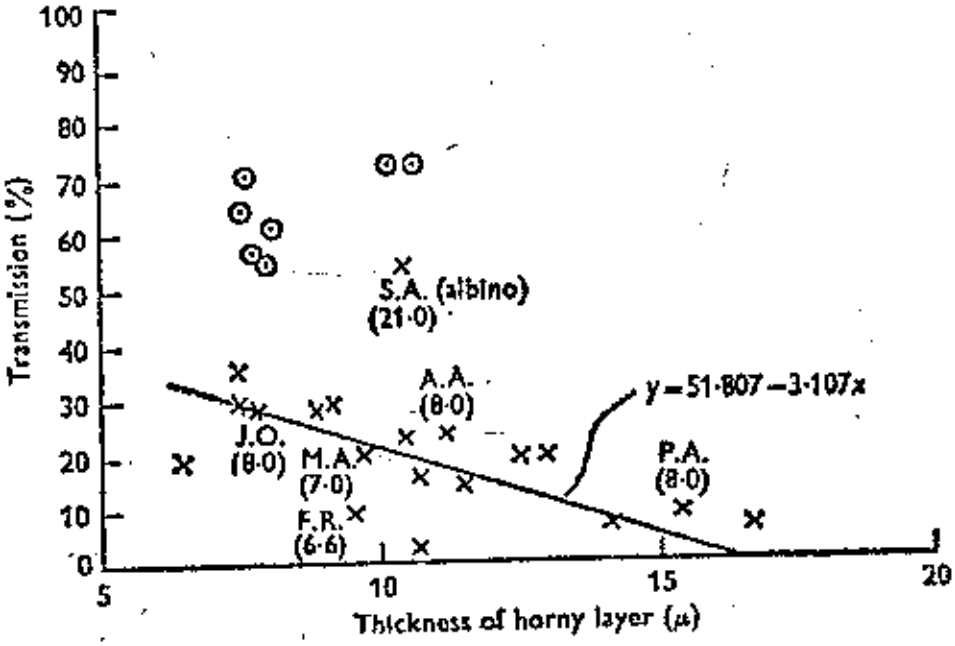

Fig. 3. Variation of transmission of solar ultraviolet light (3000 to 4000 angstroms) through the stratum corneum, plotted against thickness of this layer. ⊙, Europeans; ×, Africans. The numbers in parentheses after initials are percentages for reflectance of blue light on the forearm. [From M. L. Thomson (*6*)]

would cripple their ability to hunt game when they were adults. In this connection, Carleton Coon has written (*13*), "Up to the present century, if black skinned people were incorporated into any population living either north or south of the fortieth degree of latitude, their descendants would eventually have been selected for skin color on the basis of this vitamin factor alone." Howells agrees (*14*): "This variety of outer color has all the earmarks of an adaptation, of a trait responding to the force of sunlight by natural selection." The skin, he continues "admits limited amounts of ultraviolet, which is needed to form vitamin D, but presumably diminishes or diffuses dangerous doses by a screen of pigment granules."

Even in 1934 Murray clearly recognized the implications of these facts (*15*): "As primordial man proceeded northwards into less sunlit regions, a disease, rickets, accomplished the extinction of the darker, more pigmented elements of the population as parents and preserved the whiter, less pigmented to reproduce their kind and by progressive selection through prehistoric times, developed and established the white race in far northern Europe as it appears in historic times; its most extreme blond types inhabiting the interior of the northern-most Scandinavian peninsula."

It is a curious fact that Murray's thesis is almost unknown to the general public, including physiologists, biochemists, and physicians, and that it is not generally accepted by anthropologists, with the exception of Coon and Howells, quoted above, even though it fits the facts of Fig. 2. Both in Europe and China, skin pigmentation becomes lighter as one goes north, and it is lighter in young children; in almost all races the skin is lighter in the newborn infant (*16*) and gradually darkens as the individual matures, a change that parallels the declining need for vitamin D.

When Did European Hominids Begin To Turn White?

On the basis of the conclusion that white skin is an adaptation to northern latitudes because of the lowered availability of winter ultraviolet radiation, it appears probable that the early hominids inhabiting western Europe had lost much of their body hair and skin pigmentation even half a million years ago. Anthropological evidence indicates that early hominids such as the Heidelberg, Swanscomb, Steinheim, Fontechevade, and Neanderthal men lived north of the Mediterranean Sea—particularly during warm interglacial periods (*17*). It is important to recognize that the effect of latitude on the availability of ultraviolet light in winter is not related to climate but operates steadily and at all times, through glacial and interglacial periods alike.

Fig. 4. Distribution of early stone tools throughout the tropics of the Old World and in Europe as far north as 50°N. [From Brace and Montague, *Man's Evolution* (Macmillan, New York, 1965), p. 231]

Hand axes and other early stone tools have been found throughout the tropics of the Old World and also in Europe as far north as the 50th degree of latitude (Fig. 4). The presence of such stone tools as far north as England and France shows that some early hominids must already have adapted to the lowered level of ultraviolet radiation and consequent danger of rickets by partial loss of body hair and skin pigmentation, for without such adaptation they would have probably been unable to survive this far north.

England is at the same latitude as the Aleutian Islands, and no stone tools such as those found in southern England and France have ever been found in other areas at this latitude—for example, Mongolia and Manchuria. The unique combination of temperate climate and low levels of winter ultraviolet radiation in England and France is due to the powerful warming effect of the Gulf Stream on this particular northern area, which is unique in the world in this respect, for the Japan current in the Pacific is not as powerful as the Gulf Stream and warms only the Aleutian Islands, where no hominids existed until very recently.

Occupation of northern Europe and even Scandinavia up to the Arctic Circle seems to have taken place during the Upper Paleolithic, when presumably partially depigmented men already adapted to latitude 50°N lost nearly all their ability to synthesize melanin and so produced the blond-haired, blue-eyed, fair-skinned peoples who inhabit the interior of the northernmost part of the Scandinavian peninsula.

It has been held that the abundant appearance of stone scrapers in the Upper Paleolithic indicates that this far-northern extension of man's habitat followed his use of animal skins for clothing, a change that would select powerfully for infants with nearly transparent skin on their cheeks, who were thus still able to synthesize a minimum antirachitic dose of vitamin D even when fully clothed during the Scandinavian winter. Certainly the pink-to-red cheeks of northern European children are uniquely transparent; their color is due to the high visibility of the blood that circulates in the subepidermal region.

The one exception to the correlation between latitude and skin color in the Old World is the Eskimo; his skin is medium dark and yet he remains completely free of rickets (*18*) during the long dark arctic winters. Murray noted long ago that the Eskimo's diet of fish oil and meat contains several times the minimum preventive dose of vitamin D, concluding (*15*), "Because of his diet of antirachitic fats, it has been unnecessary for the Eskimo to evolve a white skin in the sunless frigid zone. He has not needed to have his skin bleached by centuries of evolution to admit more antirachitic sunlight. He probably has the same pigmented skin with which he arrived in the far north ages ago." Similar considerations would apply in the case of any coastal peoples of Europe and Asia, who would have been able to expand northward without depigmentation as long as they obtained sufficient vitamin D from a diet of fish; only when they ventured into the interior would antirachitic selection for blond types, as in Scandinavia, presumably have taken place.

Yellow, Brown, and Black Adaptation

Human skin has two adaptive mechanisms for resisting the penetration of solar ultraviolet: melanin-granule production in the Malpighian layer and keratohyaline-granule production in

the stratum granulosum. Melanin granules are black, whereas the keratohyaline granules produce keratin (from which nails, claws, horns, and hoofs are formed), which has a yellowish tinge. Particles of both types migrate toward the horny external layer, where they impart a black (melanin), yellow (keratin), or brown (melanin and keratin) tinge to the skin.

Thomson has shown (*6*) that, in Negroes, melanization of the stratum corneum plays the major role in filtering out excessive ultraviolet radiation, keratinization of the horny layer playing only a minor part. Mongoloids on the other hand have yellowish skin, since their stratum corneum is packed with disks of keratin (*13*) that allow them to live within 20 degrees of the equator even though their skin contains only small amounts of melanin (Fig. 2). On the equator itself, however, even Mongoloid-derived peoples acquire pigmentation—for example, the previously medium-light-skinned Mongoloids who entered the Americas over the Bering Straits at latitude 66°N as recently as 20,000 to 10,000 years ago (Fig. 2).

Even white-skinned peoples have to protect themselves against excessive doses of solar ultraviolet radiation in summer, for, as Blum has pointed out (*19*), on 21 June the solar ultraviolet is as intense in Newfoundland as it is at the equator, since at that time the two regions are at the same distance from the Tropic of Cancer (at 23°27′N). (At the equator, the solar ultraviolet is never less than on this date, while in Newfoundland it is never more.) In other words, adaptation to the variable intensities of solar ultraviolet in the north requires not only winter depigmentation but also the evolution of a reversible mechanism of summer repigmentation to keep the rate of vitamin-D synthesis constant throughout the year. It is significant that both the keratinization and melanization components of suntan are initiated by the same wavelengths which synthesize vitamin D, for it would be difficult to design a more perfect defense against excessive doses of vitamin D than this reversible response to ultraviolet light of these particular wavelengths—a pigmentation response that is further protected by the painful alarm bell of sunburn, which guarantees extreme caution against overexposure to solar ultraviolet in untanned individuals suddenly encountering a tropical sun.

Defenses against production of too much vitamin D therefore range from (i) reversible suntanning, as in Europeans, through (ii) constitutive keratinization, as in the Mongoloids of Asia and the Americas, to (iii) constitutive melanization, as in African and other truly equatorial peoples. The physiological superiority of melanization as a means of protection against ultraviolet was demonstrated by the ability, historically documented, of imported Nigerian slaves to outwork the recently adapted American Indians in the sun-drenched cane fields and plantations of the Caribbean and related tropical areas.

Additional evidence for the view that melanization of the stratum corneum is primarily a defense against the oversynthesis of vitamin D from solar ultraviolet is provided by the fact that the palms and soles of Negroes are as white as those of Europeans; *only* the palms and soles possess a thickly keratinized stratum lucidum (Fig. 5) under the external stratum corneum, which renders melanization of the latter unnecessary. The same reasoning explains the failure of the palms and soles of whites to sunburn during the summer.

Coon has written (*13*), "We cannot yet demonstrate why natural selection favors the prevalence of very dark skins among otherwise unrelated populations living in the wet tropics, but the answer may not be far away." Since overdoses of vitamin D administered orally are known to result in prompt and serious consequences, such as calcifications in the aorta and other soft tissues of the body, kidney stones, secondary renal disease, and death, it would appear that oversynthesis of vitamin D is sufficiently detrimental in young and old to favor the gradual selection for deeply pigmented skin near the equator, as seen, for example, in the repigmentation that has taken place among the equatorial American Indians during the last 10,000 years (Fig. 2).

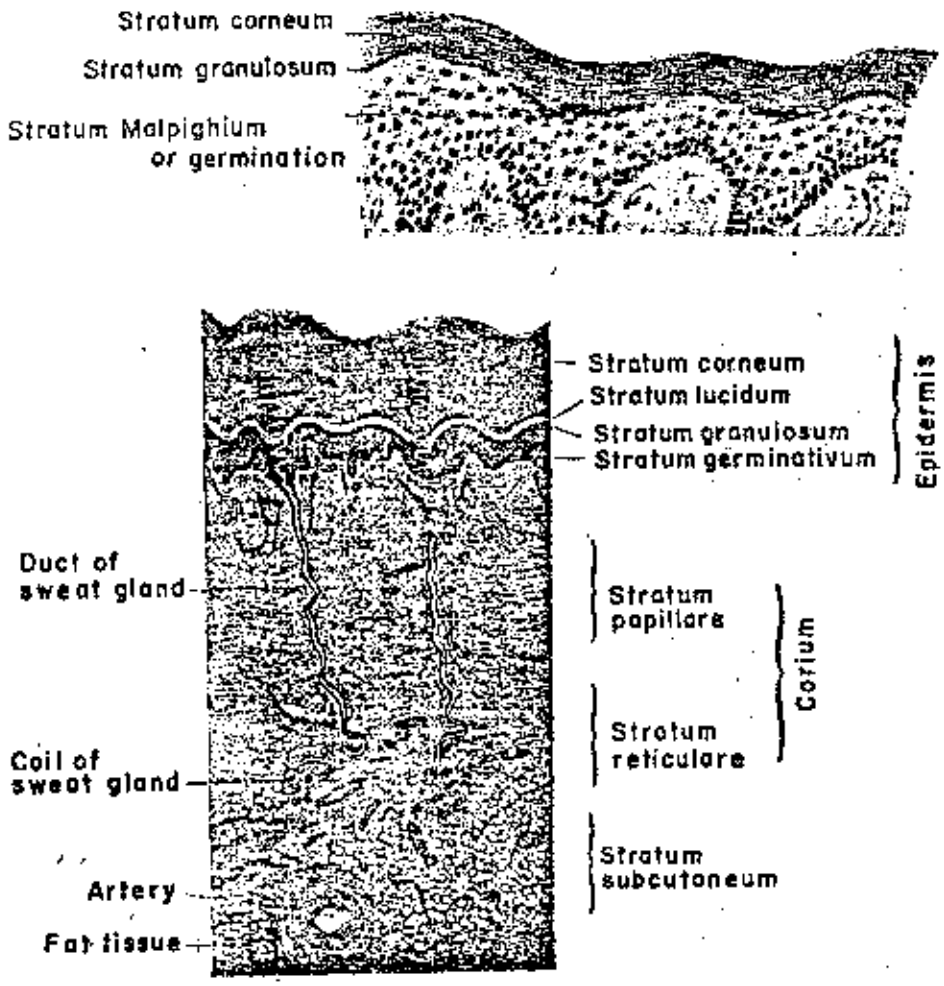

Fig. 5. Vertical section from (top) the shoulder and (bottom) the sole of the foot of a Caucasian adult. [From J. L. Bremer, *A Textbook of Histology* (Blakiston, Philadelphia, 1936)]

Secondary Results of Pigmentation and Depigmentation

It is known that black skin absorbs more heat than white skin; the studies of Weiner and his associates (*20*) show that black Yoruba skin reflects only 24 percent of incident light whereas untanned European skin reflects as much as 64 percent. Of themselves, these facts would lead one to expect that reflective white skin would be found near the equator while heat-absorbing black skin would be found in cold northern climate.

Since the exact opposite is true around the world, it seems clear that man has adapted his epidermis in response to varying levels of ultraviolet radiation despite the price he has had to pay in being badly adapted from the standpoint of heat absorbance and reflectance of visible and near-infrared wavelengths. Similar considerations naturally apply to summer pigmentation due to suntan; ultraviolet regulation rather than heat regulation explains why Caucasians are white in the winter but pigmented in the summer.

In addition to being badly adapted for maximum heat absorbance, white-skinned northern peoples are known to be particularly susceptible to skin cancer (*21*) and such skin diseases as psoriasis and acne. Therefore, only some powerful other advantage, such as relative freedom from rickets, would explain the worldwide correlation between high latitudes and white skin, for without some such factor it would seem that black or yellow skin would be the superior integument.

From this and other evidence, such as the fact that lion cubs and the young of other tropical animals develop rickets in northern zoos unless given cod-liver oil (*2*), it appears probable that depigmentation occurred north of latitude 40°N (a line marked by the Mediterranean Sea, the Great Wall of China, and the Mason-Dixon line) as an adaptation that allowed an increased penetration of winter ultraviolet radiation and consequent freedom from rickets. Certainly no other essential function of solar ultraviolet is known for man besides the synthesis of vitamin D.

Summary

The known correlation between the color of human skin and latitude (Fig. 2) is explainable in terms of two opposing positive adaptations to solar ultraviolet radiation, weak in northern latitudes in winter yet powerful the year around near the equator. In northern latitudes there is selection for white skins that allow maximum photoactivation of 7-dehydrocholesterol into vitamin D at low intensities of ultraviolet radiation. In southern latitudes, on the other hand, there is selection for black skins able to prevent up to 95 percent of the incident ultraviolet from reaching the deeper layers of the skin where vitamin D is synthesized. Selection against the twin dangers of rickets on the one hand and toxic doses of vitamin D on the other would thus explain the world-wide correlation observed between skin pigmentation and nearness to the equator.

Since intermediate degrees of pigmentation occur at intermediate latitudes, as well as seasonal fluctuation in pigmentation (through reversible suntanning), it appears that different skin colors in man are adaptations of the stratum corneum which regulate the transmission of solar ultraviolet to the underlying stratum granulosum, so that vitamin-D photosynthesis is maintained within physiological limits throughout the year at all latitudes.

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Vitamin D, Sunlight, and Natural Selection

Most readers will agree with Loomis (*1*) that there is generally a latitude influence on the distribution of human skin pigmentation, that melanin pigment absorbs light, and that the epidermal production of vitamin D requires ultraviolet radiation. I suspect, however, that only a few will agree, on the basis of the data given, with his thesis that the control of vitamin D synthesis is the primary role of human skin pigmentation.

First, the evolutionary implication does not support this view: the melanin pigments are widely distributed from fungi to man, and tyrosinase, the enzyme associated with melanin synthesis, occurs in bacteria as well. On the other hand, only mammals and birds are subject to rickets, and vitamin D is not found to occur regularly, or to play a physiological role, in forms more primitive than the bony fishes. In view of the wide distribution of the melanins, and the narrow distribution of vitamin D, it may be assumed that the former arose earlier in evolutionary development. It seems improbable, then, that a control system would evolve in advance of the thing to be controlled.

Second, the quantitative considerations which form the basis of Loomis's argument may be viewed somewhat differently from the way he views them. Starting with Bekemeier's data (*2*), one may prefer to take the average of the six experimental determinations, rather than the extreme value [particularly since Bekemeier reports (*3*) analytical variations of up to 300 percent from sample to sample]. One may also prefer not to assume that the total skin surface is maximally illuminated at a given instant (at least half the skin is on the side of the body *away* from the sun), or to assume that light incident at low angles to the skin is as effective as light incident normally. One may also wish to assume that people sit, walk, or stand during the day, exposing in each instance still less than half their total skin surface to the overhead sun. Finally, one may wish to assume that even primitive man did not dwell continually in direct sunlight, but sought the shade of the tropical rain forest for at least a part of the day. If a reader uses these assumptions in making the calculation for vitamin D synthesis, the daily yield of vitamin D (for either white or dark skin) turns out to be in the physiological range as defined by Loomis.

Third, the normal range of vitamin D intake is given in the argument as 400 to 100,000 international units, a range of variation of 1:250. Yet the hypothetical control system, if we use Loomis's data for the transmittances of the stratum corneum of dark and light skin, can control the input light over a range of variation of only 1:3. An excess or deficiency of light could lead to hyper- or hypovitaminosis only if the individual's daily synthesis of vitamin D were near one of the extremes of the physiological range, and even then could produce only one or the other, and not both, as proposed.

Fourth, if cutaneous pigmentation were of major importance in regulating vitamin D metabolism, one would expect to find hypervitaminosis D reported in albinos who had undergone exposure to the sun. I am unaware of any such reports.

The hypothesis that Loomis presents is quite interesting, and obviously skin pigmentation affects to some extent cutaneous synthesis of vitamin D; the thesis that this is the primary control mechanism is not proved by the data given.

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13 November 1967

In a recent article (*1*) and subsequent letter (*2*), W. Farnsworth Loomis cites a paper of mine (*3*) in support of his argument that the melanin pigment of Negro skin has evolved as a protection against excess vitamin D by modulating the effective amount of ultraviolet radiation in the skin. Loomis seems to have neglected a good part of the argument in my paper. I point out there that ultraviolet light has at least three effects which could conceivably exert selection pressure leading to genetic modification: sunburn, induction of skin cancer, and production of vitamin D. All three effects are elicited by wavelengths ranging from about 0.3 μ to the lower limit of sunlight at 0.29 μ; all may be expected to be greater in skins with little melanin, although we do not know how closely this generalization may hold. On the other hand, a high melanin content in the skin increases the absorption of total sunlight and so imposes a greater heat load upon the body. The selective pressure exerted by sunlight should thus be reckoned in terms of an algebraic summation of these four effects and probably others, and without much more information than is available the pressure of any one of them cannot be evaluated.

In his evolutionary argument Loomis invokes the distribution of ultraviolet light with latitude. This differs markedly from the distribution of total sunlight and, because of intense scattering back from the sky, different methods of measurement may give different results. A greater factor than latitude may be reflection from the terrain, most components of which absorb strongly, although snow is a good reflector. In addition, we have much to learn about the actual amount of absorption by melanin in different types of skin, the complicated optics of that organ making this very difficult to measure. All these things are discussed in my paper (*4*).

As regards Loomis's suggestion that darkly pigmented men moved from Africa to northern climates and there became lighter, it may be pointed out that the paleontological record gives us no evidence as to the time when the races of men were differentiated with respect to color, or as to where this occurred. Men moving from forested tropical Africa to northerly latitudes during one of the periods of glaciation might have received more ultraviolet radiation, because of reflection from snow, than they received in the tropics.

All such speculation seems to little purpose, however, in light of the complexity of the problem and our lack of knowledge. We should, in any event, be cautions about introducing teleology and undue determinism into our thinking about natural selection and the evolution of man.

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References and Notes

1. W. F. Loomis, *Science* 157, 501 (1967).
2. ———, *ibid.* 158, 579 (1967).
3. H. F. Blum, *Quart. Rev. Biol.* 36, 50 (1961).
4. For further information on the ultraviolet of sunlight, see P. Retter, *Strahlentherapie* 123, 6 (1964); R. Schulze, *Arch. Meteorol. Geophys. Bioklimatol.* 12, 185 (1963); P. T. Baker, *Headquarters Quartermaster Res. Eng. Command, U.S. Army, Tech. Rept. EP-75* (1958).

20 November 1967

I would like to answer Blois's interesting points first.

1) Phylogenetically older substances are sometimes used in phylogenetically newer control systems: (i) CO_2 is a metabolite found even in bacteria, yet it is used to control the rate of mammalian respiration; (ii) melanin is found even in fungi, as Blois points out, yet it is used to control the amount of light reaching the retina by rendering the mammalian iris opaque; (iii) protoporphyrin is present as cytochrome *c* in protista, yet it is used to control the oxygen- and CO_2-carrying power of the blood in the form of hemoglobin in animals that later evolved a circulatory system.

2) In my article I calculated the maximum daily amount of vitamin D [illegible] would be synthesized by an un[illegible]ed Caucasian exposed all day long [illegible] an equatorial sun without protection from clothing, trees, or shelters. I agree with Blois that, in fact, equatorial people could well be exposed to much less ultraviolet radiation; this lesser amount is probably still toxic in terms of hypervitaminosis D, as discussed in the next paragraph.

3) No general agreement has been reached on (i) the minimum toxic dose of vitamin D in short-term experiments or (ii) the least amount of the vitamin needed to produce chronic hypervitaminosis D, especially in children. Jeans and Stearns (*1*), for example, found that the linear growth of infants was retarded by the administration of as little as 1800 I.U. per day. Most estimates of the minimum toxic dose vary from 10,000 I.U. per day upward; a standard physiological textbook (*2*) reports that "the minimal toxic overdose does not appear to be far from the optimum curative dose." Wanting to be on the safe side, I stated in my arti[illegible] that daily ingestion of 100,000 I.U. per day could produce hypervitaminosis. This is undoubtedly true, but should not be interpreted as the actual toxicity threshold, which could easily be 1/10 this amount, or less. Thresholds for chronic toxicity must be lower than those for acute toxicity; yet any degree of toxicity could act overtime as a genetic selection factor for black skin in the tropics. Certainly more experimental work must be done to determine these thresholds with exactitude.

4) Blum is correct in stating that his paper provides no direct support for my theory; it does not even mention the disease hypervitaminosis D at all. I used his paper for indirect support as regards (i) the equatorial force of summer ultraviolet in the temperate zones and (ii) the difficulties in viewing either sunburn or skin cancer as effective genetic selection factors responsible for the black skins of equatorial primates. Since heat-load adaptation would lead one to expect Negroes in the north and white men in the south, it appears that adaptation to *ultraviolet penetration* was the major factor in the epidermal differentiation of man. As far as "teleology" is concerned, I look on the development of rickets-resistant strains of white men in northern latitudes and of hypervitaminosis D–resistant strains of black men in the tropics as no more teleological than the development of streptomycin-resistant strains in a culture of *Escherichia coli*.

5) I would like to mention here that Charles E. Bills of Johns Hopkins Hospital informs me that my figure of 3.8 percent provitamin D in the skin should have referred to this percentage of the total sterol in the skin; he adds, "Fortunately, this does not invalidate to any appreciable degree your thesis on the origin of blondes."

6) In a like vein, Peter Flesch of the University of Pennsylvania Hospital corrected the dermatological facts I abstracted from an anthropological textbook (*3*) by pointing out (*4*) that (i) "vitamin D synthesis does not take place solely in the stratum granulosum, but throughout the entire epidermis . . . and most probably also in the sebaceous glands or ducts"; (ii) keratohyaline is a hard keratinous structure without granular layers; and (iii) since keratin is essentially colorless, "the yellowish skin color of Mongoloids is caused by decomposed pigment 'excreted' with the horny layer."

In conclusion, I would like to mention two papers that have recently come to my attention. In the first (*5*), Pathak reports that over twice as much (290 to 320 mμ) ultraviolet passes through white stratum corneum as passes through Negro stratum corneum, this 2 : 1 difference in ultraviolet-light transmission being increased to 3 : 1 for whole epidermis. According to the second paper (*6*), significantly higher concentrations of serum calcium (10.9 as compared with 9.6 mg per 100 ml) and phosphorous (3.8 as compared with 3.1 mg per 100 ml), as well as higher levels of "serum antirachitic activity" (4.4 as compared with 2.6 units per milliliter), were found in comparable groups of 65 women living at latitude 18°N in Puerto Rico and 116 women living at latitude 43°N in Michigan. If the solubility product of these two minerals is viewed as a rough measure of calcification potential, then these two groups are already separated by a 25-percent difference (39.5 as against 29.8) in calcification. Further and probably toxic increases would be expected, from these findings, in people living on the equator at 0° latitude, especially if, unlike the Puerto Rican group studied, they wore little clothing, had outdoor occupations, and did not have partially pigmented skin.

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2. C. H. Best and N. B. Taylor, *The Physiological Basis of Medical Practice* (Williams and Wilkins, Baltimore, 1966), p. 1439.
3. C. S. Coon and E. E. Hunt, *The Living Races of Man* (Knopf, New York, 1965), p. 229.
4. Flesch cites the following: H. Bekemeier, *Intern. Z. Vitaminforsch. No. 10* (1966); J. I. Gaylor and F. M. Sault, *J. Lipid Res.* 5, 422 (1964).
5. M. A. Pathak, in *Advances in Biology of Skin*, vol. 8, *The Pigmentary System* (Pergamon, New York, 1967), chap. 25.
6. R. W. Smith, J. Rizek, B. Frame, *Amer. J. Clin. Nutr.* 14, 98 (1964).

26 December 1967

Although it is still widely regarded as a dietary-deficiency disease resulting from a lack of "vitamin D," it results in fact from a lack of sunlight. In smoky cities it was the first air-pollution disease

by W. F. Loomis

The discovery of the cause and cure of rickets is one of the great triumphs of biochemical medicine, and yet its history is little known. Indeed, it is so little known that even today most textbooks list rickets as a dietary-deficiency disease resulting from a lack of "vitamin D." In actual fact rickets was the earliest air-pollution disease. It was first described in England in about 1650, at the time of the introduction of soft coal, and it spread through Europe with the Industrial Revolution's pall of coal smoke and the increasing concentration of poor people in the narrow, sunless alleys of factory towns and big-city slums. This, we know now, was because rickets is caused not by a poor diet but by a deficiency of solar ultraviolet radiation, which is necessary for the synthesis of calciferol, the calcifying hormone released into the bloodstream by the skin. Without calciferol not enough calcium is laid down in growing bones, and the crippling deformities of rickets are the consequence. Either adequate sunlight or the ingestion of minute amounts of calciferol or one of its analogues therefore prevents and cures rickets, and so the disease has been eradicated.

That seems a clear enough story, and yet the textbooks speak of diet and vitamin D. How can that be? What happened is that the investigation of rickets proceeded along two quite independent lines. Intuitive folk medicine and then medical studies pointed in the direction of sunlight and calciferol. At the same time, however, common assumptions about poverty and poor nutrition and then studies by nutritionists pointed in the direction of diet and vitamin D. Now, with the inestimable advantage of hindsight, it is possible to trace these two chains of thought, to disentangle them and set the historical record straight.

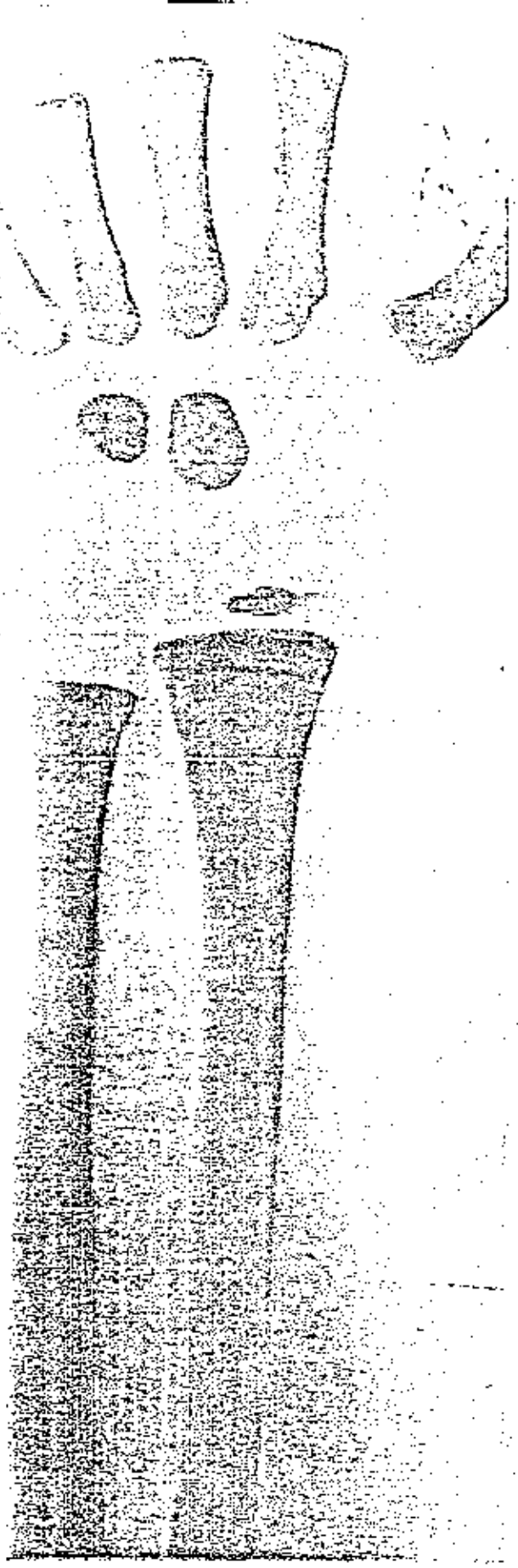
Now that one knows what to look for, the evidence of a climatic influence on rickets can be discerned quite early. In the early 19th century G. Wendelstadt published *The Endemic Diseases of Wezlar*, a German town of 8,000 population with exceptionally narrow streets and dark alleys. The town was infamous for rickets, he wrote, with entire streets where in house after house individuals crippled by rickets could be found. "The children must sit indoors . . . which ends in death or if they continue to live, they develop thick joints, cease to be able to walk or have deformed legs. The head becomes large and even the vertebral column bends. It comes to pass that such children sit often for many years without being able to move; at times they cease to grow and are merely a burden to those about them." This terrible picture of an entire town afflicted with severe rickets leads one to guess that many of William Hogarth's sketches of frightfully deformed men and women may have depicted the crippling effects of rickets in London in the 18th century.

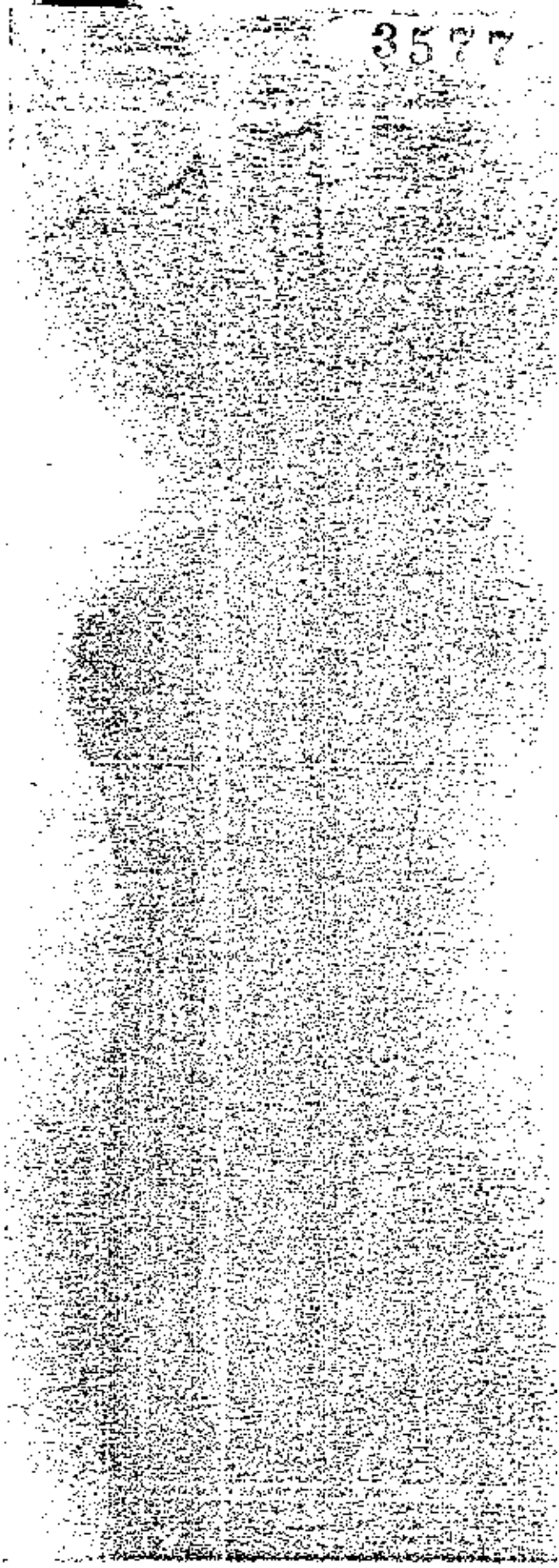
As early as 1888 the English physician Sir John Bland-Sutton found unmistakable evidence of rickets in animals in the London zoo—chimpanzees, lions, tigers, bears, deer, rabbits, lizards, ostriches, pigeons and many other species. He noted that "in spite of every care and keeping them in comfortable dens" lions in London developed rickets, whereas "in Dublin, Manchester, and some other British towns, lions can be reared successfully in captivity." It is clear in retrospect that the pall of coal smoke over London was the causative factor.

The geographical relation between rickets and cities was clearly noted in 1889 by the British Medical Association. After a survey of the incidence of the disease in the British Isles the association published maps [*see illustration on page 79*] that supported its major conclusion: There was widespread and severe rickets "in large towns and thickly peopled districts, especially where industrial pursuits are carried on," whereas rickets was almost totally absent in rural districts. Specifically, the report added, "almost the whole of London and the greater number of its outlying suburbs" reported severe rickets among rich and poor alike.

Solar ultraviolet may be blocked by many means, among them being the industrial smog in London and the sunless alleys of Wezlar, but beneath such specific industrial and urban conditions there is a major underlying factor: the far northern location of the entire European land mass. The area is made habitable by the benign influence of the Gulf Stream, yet its winter sun, hanging low in the sky, is almost without potency in effecting the crucial conversion of 7-dehydrocholesterol into calciferol. Elsewhere in the world, lands as northerly as Europe are largely uninhabited—the Aleutian Islands, for example, or Labrador or northern Siberia. The long, dark winters of Europe therefore powerfully predisposed European infants toward rickets during the winter months.

The seasonal variation was noted as early as 1884 by M. Kassowitz in Germany, who attributed it to the prolonged confinement of infants indoors during the winter. Then in 1906 D. Hansemann noted that nearly all German children who were born in the fall and died in the spring had rickets; those who were born in the spring and died in the fall were free of the disease. Noting the progressive rise of rickets during the winter months, he concluded that rickets was primarily a disease of "domestication,"





EFFECT OF RICKETS is deformation of bone for want of the calcifying hormone calciferol, which is synthesized on ultraviolet radiation. An X ray of normal arm and hand bones in an 18-month-old child (*left*) is compared with an X ray of the bones of a c of the same age with rickets (*right*). The disease can be prev or cured by sunlight or the ingestion of small amounts of calci-

live in complete freedom. Once caught, however, most of these formerly wild animals—and especially monkeys—show great disposition towards rickets. Hardly one young captured animal can avoid this danger. By observing rickets in people, who do not get this disease to such a degree as monkeys, one can also see that it is a sickness of *domestication*. We can say that in living locked indoors, with thick, heavy walls and windows facing brick walls in other houses, the natural habitat of a child is being disturbed—namely the outdoors." In 1909 G. Schmorl strongly documented this marked seasonal variation in the frequency of rickets with a series of 386 postmortem examinations carried out on children under four years old.

Perhaps the most brilliant investigation into the nature of rickets was made in 1890 by Theobald Palm, an English medical missionary who went to Japan and "was struck with the absence of rickets among the Japanese as compared with its lamentable frequency among the poor children of the large centres of population in England and Scotland." He wrote to other medical missionaries around the world, collated the results and was amazed to find that rickets was essentially confined to northern Europe and was almost totally absent from the rest of the world.

Dugald Christie wrote him from Mukden, for example, as follows: "I have met with not a single case of rickets during a residency of six years in Manchuria," and this in spite of the fact that there were "no sanitary conditions whatever" and the only articles of diet were millet, rice, pork and vegetables. C. P. Smith reported from Mongolia that he had not seen any rickets. "We have 10 months in the year of almost constant sunshine. In summer the children go practically naked, and even in winter, with the rivers frozen into a solid mass of ice, I have seen children running about almost naked, that is during the day while the sun is shining." From Java a Dr. Waitz reported what was a known fact there: European children suffering from rickets recovered from the malady within a few months of moving to Java and without any medical treatment.

From data such as this Palm deduced that rickets was caused by the absence of sunlight. "It is in the narrow alleys, the haunts and playgrounds of the children of the poor, that this exclusion of sunlight is at its worst, and it is there that

abundance. He proceeded to recommend "the systematic use of sun-baths as a preventive and therapeutic measure in rickets."

The first successful attempt to induce rickets experimentally in animals was 1908 by Leonard Findlay. He published conclusive pictures of puppies that had been confined in cages and developed rickets; unconfined animals did not become rachitic. His results convinced him that the cause of rickets was not any

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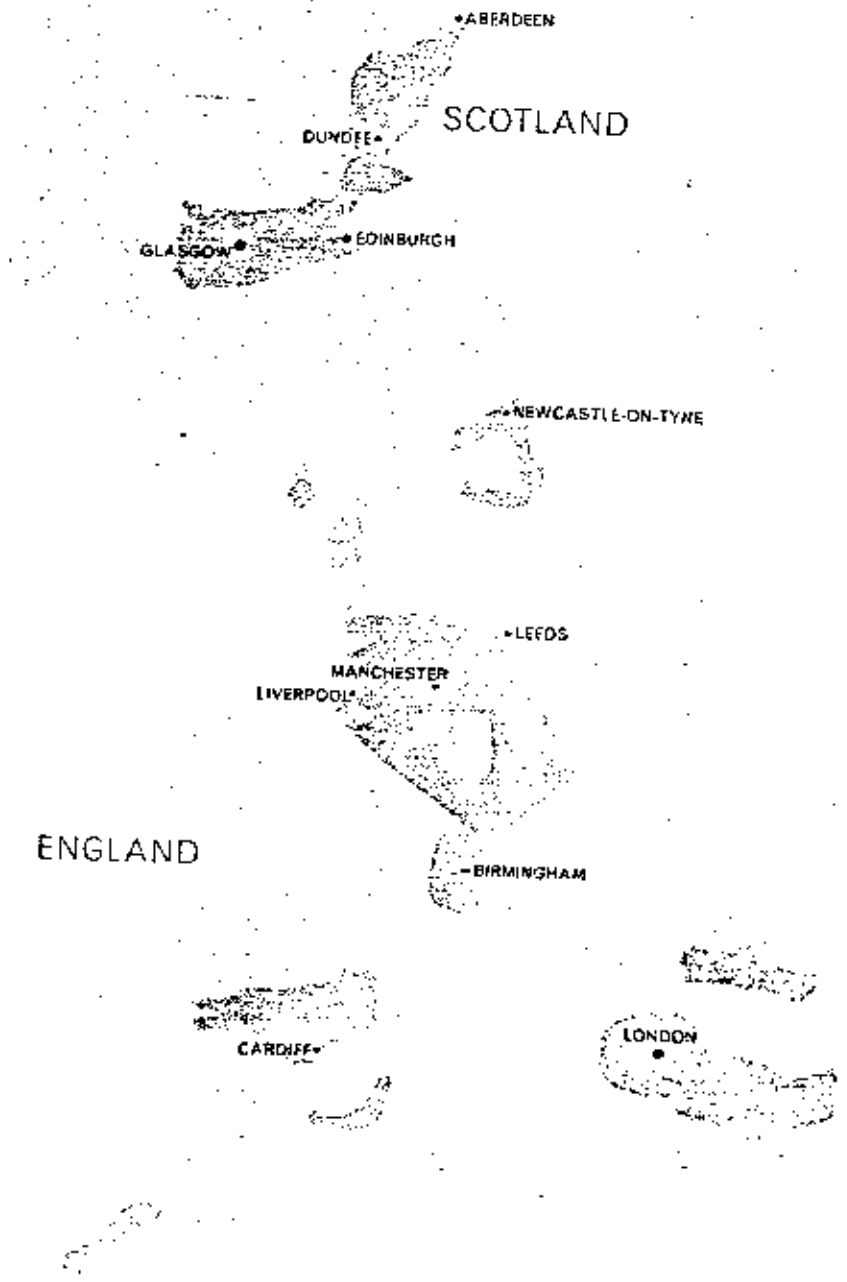


CORRELATION OF RICKETS with industrial areas and smoke from the burning of coal appeared in data assembled in 1889 by the British Medical Association. The map, which shows in gray the principal concentrations of rickets, is based on maps of England and Scotland prepared by the association. Since diets in these areas were in general better than those in poorer surrounding areas, the distribution of rickets is not what one would expect if the disease were of dietary origin. In actuality the cause was smoke that obscured sunlight.

to quite the right conclusion; he suggested that rickets was caused by "confinement, with consequent lack of exercise." More accurate was a brilliant experiment by Jan Raczynski of Paris in 1912. Raczynski pointed to lack of sunlight as the principal etiological factor in rickets. Two puppies, "newborn in the month of May from the same mother, were reared for six weeks, the first in sunlight from morning to evening, the second in deep shade in a large, well-ventilated cage. Both were fed in the same manner, that is exclusively on the milk of their mother." After six weeks the puppy kept out of the sunlight was markedly rachitic, a diagnosis confirmed by chemical analysis of its bones, which were found to contain 36 percent less calcium phosphate than the bones of the puppy that had been raised in the sun. Raczynski concluded that sunlight played a principal role in the etiology of the disease.

In 1918 Findlay returned to the problem with the assistance of Noel Paton. They did experiments with 17 collie puppies from two litters and reported that "all those kept in the laboratory showed signs of rickets to a greater or less degree. One which had been confined and had had butter was most markedly affected. It was unable to walk. Another of the confined animals which had had no butter was least affected." They concluded: "Pups kept in the country and freely exercised in the open air, although they had actually a smaller amount of milk fat than those kept in the laboratory, remained free of rickets, while the animals kept in the laboratory all became rickety." Findlay and Paton fed butter to some of their animals to check on the effect of diet, since the idea that rickets was a dietary-deficiency disease was already taking hold and milk fat was known as an important source of vitamin A. Their results argued against such a theory, of course. Moreover, it is known today that the adverse effect of butter they observed was due to the fact that "florid," or severe, rickets develops best in well-fed puppies; a poorly fed animal develops only mild rickets since the defect in calcification does not have as much effect in an animal whose bones are not growing. Rather than being a dietary-deficiency disease, therefore, florid rickets required a good diet, complete with vitamins A, B and C; only then could the puppy grow rapidly and hence develop incapacitating rickets.

Findlay's group had by now become known as the "Glasgow school," as op-

tionists. Their competing theories led to two important studies of human rickets, one in Scotland and one in India.

Margaret Ferguson studied 200 families living in Glasgow among whom marked rickets existed and decided that inadequate air and exercise appeared to be the most potent factors. "Over 40 percent of the rachitic children had not been taken out, while only 4 percent of the nonrachitic children had been confined indoors." It is clear now that being out of doors was the chief variable, for both sets of children were free to exercise at will.

The most clear-cut investigation was conducted by Harry S. Hutchinson in Bombay. He found no rickets at all among poor Hindus who subsisted on a pitifully inadequate diet but who worked outdoors all day "and while at work left their young infants at some nearby point in the open air." In contrast, he found that rickets was exceedingly common among the well-fed Moslems and upper-caste Hindus, whose women usually married at the age of 12 and entered purdah, where the ensuing infants usually remained with their mother for the first six months of life in a semidark room in the interior of the house. Hutchinson found that infants of both sexes kept in purdah suffered severely from rickets; the girls, who entered purdah when they were married, recontracted the disease then. He concluded that "the most important etiological factor in the production of rickets is lack of fresh air, sunlight, and exercise." He then proceeded to cure 10 such cases of purdah-induced rickets by taking the patients out into the open air, "showing that removal of the cause removes the effect. All other factors remained constant and no medicine was given."

Although it was becoming increasingly clear by 1919 to many physicians that sunlight had the power both to prevent and to cure rickets, no method of providing summer sunlight during European winters was available. Not only were such winters generally cloudy, with an ineffective sun less than 30 degrees from the horizon, but also the cold usually required exposure of children to the sun in glassed-in solariums whose windowpanes, it is now known, effectively filtered out the required ultraviolet rays. Folk custom had taught northern European mothers to put their infants out of doors even during January for "some fresh air and sunshine." The trouble was that in large cities with narrow

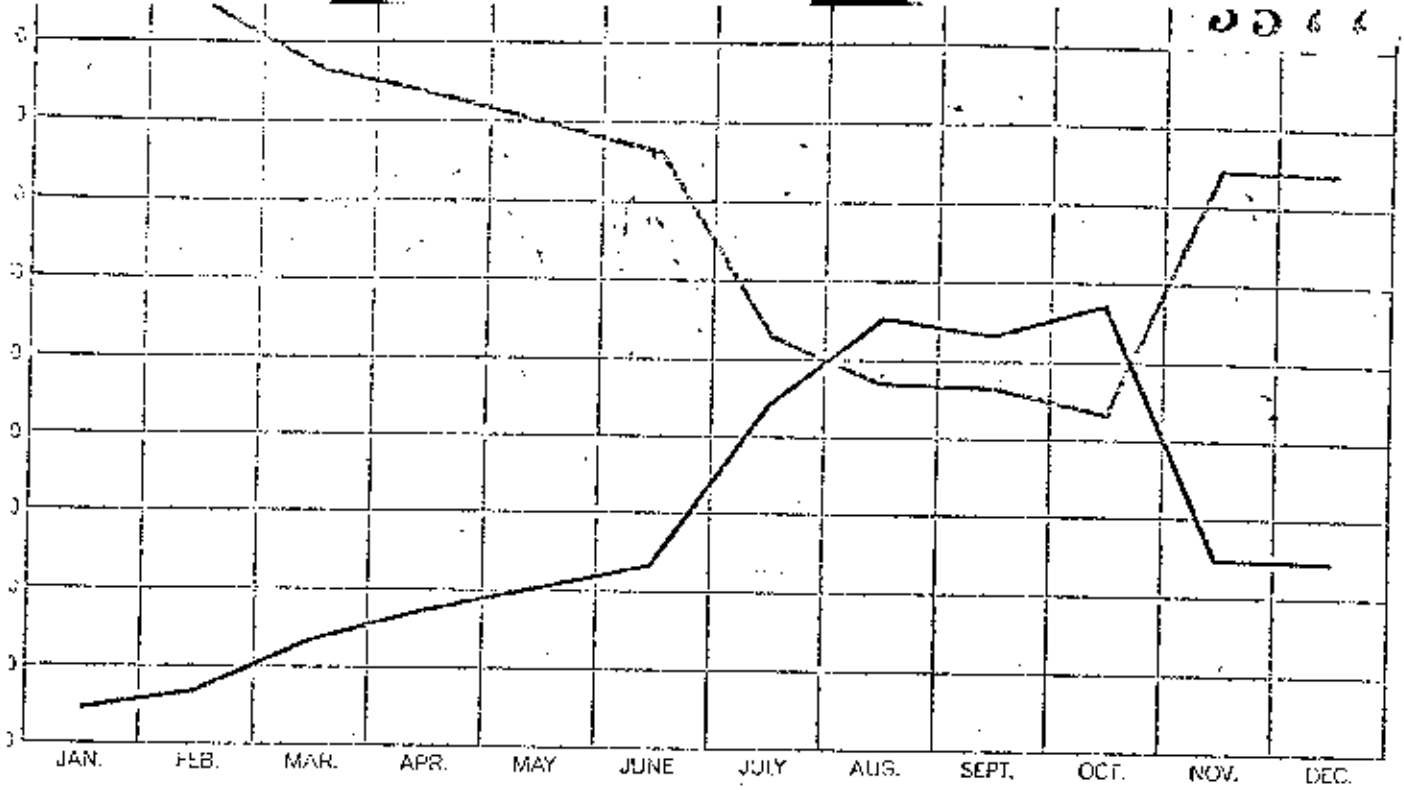
cause of the intervene[illegible] the pall of smoke.

With natural sunlight ineffective, [illegible] tors such as E. Buchholz in Germar[illegible] turned to artificial illumination such [illegible] that provided by the carbon-filame[illegible] electric bulb. Since the ultraviolet com[illegible] ponent of such light is very small, th[illegible] treatments did little good. Then in 191[illegible] a Berlin pediatrician, Kurt Huldschi[illegible] sky, tried the light from a mercury-vap[illegible] quartz lamp, which includes the ultr[illegible] violet wavelengths, on four cases of a[illegible] vanced rickets in children. He obtaine[illegible] complete cures within two months.

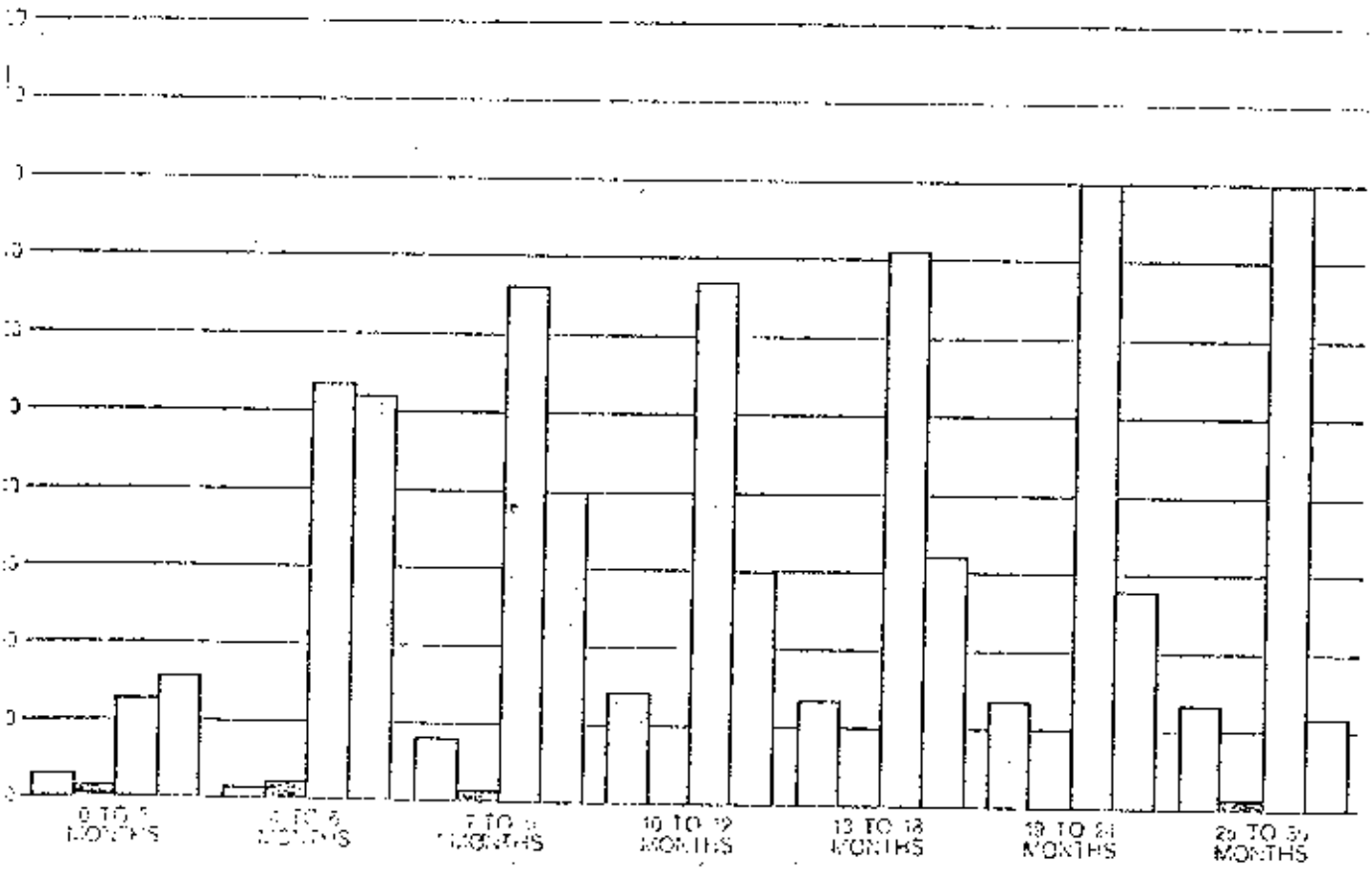
Huldschinsky's discovery of the subt[illegible] fact that it is the invisible portion of th[illegible] sun's rays that prevents rickets solve[illegible] the problem of this disease for all tim[illegible] In addition to providing a truly effecti[illegible] method of curing the disease, he pr[illegible] ceeded to show that an endocrine ho[illegible] mone must be involved. He irradiat[illegible] one arm of a rachitic child with ul[illegible] violet. Then he showed, with X-ray pi[illegible] tures, that calcium salts were deposit[illegible] not only in the irradiated arm but in t[illegible] other arm as well. This proved that [illegible] irradiation the skin released into t[illegible] bloodstream a chemical that had t[illegible] needed power to induce healing at a d[illegible] tance—in other words, a hormone.

After World War I, Huldschinsk[illegible] findings were extended by Alfred [illegible] Hess in New York. He showed that su[illegible] light alone had the power to cure rick[illegible] in children. He then showed that th[illegible] was true also of rats that had been ma[illegible] artificially rachitic by means of a lo[illegible] phosphate diet. In June, 1924, H[illegible] found that ultraviolet irradiation re[illegible] dered linseed or cottonseed capable [illegible] curing rickets. Similar results were [illegible] tained on whole rat rations later th[illegible] year by Harry Steenbock. Hess p[illegible] ceeded to show that a crude choleste[illegible] and plant sterols, as well as the skin, [illegible] quired the property of curing rick[illegible] when irradiated by ultraviolet light. [illegible] 1927 Otto Rosenheim and Thomas [illegible] Webster showed that the plant ste[illegible] ergosterol (derived from ergot, a fung[illegible] became enormously antirachitic wh[illegible] irradiated with ultraviolet light. This [illegible] the process that has now become ro[illegible] tine: Some .01 milligram per quart [illegible] ergocalciferol—or what is called "vi[illegible] min D_2"—is added to almost all the m[illegible] sold in the U.S. and most Europe[illegible] countries.

A description of the nature of the sk[illegible] hormone naturally released by irradiat[illegible] skin was finally provided in 1936 [illegible] Adolf Windaus of the University of G[illegible]



SONAL VARIATION in the frequency of rickets appeared in ies of 386 postmortem examinations of children with rickets lected by C. Schmorl in 1909. Children were classified accordingto whether they had an active case of rickets at the time of death (*color*) or a "healing" case (*black*). It was clear that the severity of the disease increased in the fall and decreased with spring.



'CT OF LATITUDE on the incidence of rickets among children of various ages in Puerto Rico (*gray*) and New Haven (*color*) demonstrated in a survey that was reported in 1933. The incidence is apparently related to the amount and strength of sunlight at 18 degrees of latitude and at 42 degrees. The light bars indicate clinical diagnosis of rickets, dark bars X-ray diagnosis.

CALCIFEROL IN FOOD is charted. The substance, which is often called vitamin D, is essentially absent from foodstuffs other than fish, particularly in winter. The numerals give the percentage of the minimum daily protective dose of calciferol in a gram of each food.

tingen. He demonstrated that 7-dehydrocholesterol is the natural prehormone that is found in the skin and showed how it becomes calciferol on ultraviolet irradiation.

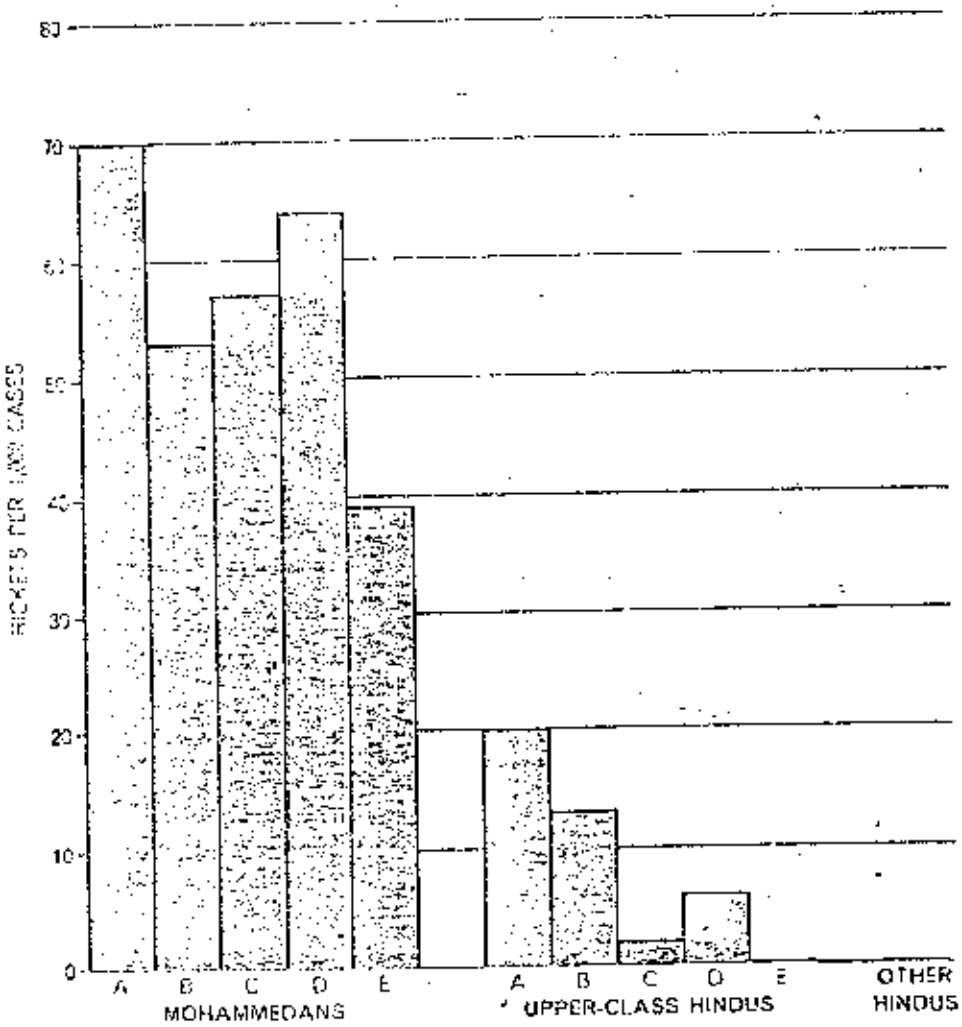
The hormonal nature of calciferol had been recognized to some degree as early as 1923 by such an authority as the American pediatrician Edwards A. Park, who wrote a careful summary of the history of rickets in *Physiological Reviews*. He summarized his view of the complex situation by saying that rickets is best compared to the endocrine-deficiency disease diabetes rather than to the genuine vitamin-deficiency diseases such as scurvy, pellagra, xerophthalmia and beriberi. Hess shared this view. In the first sentence of a 1929 monograph he stated that rickets "m[illegible] essentially a climatic d[illegible]

How, then, has the London school[illegible] view that rickets is due to a deficiency of "vitamin D" prevailed even up to the present day? Why is the error almost universally found in modern textbooks of endocrinology, physiology, biochemistry and medicine and further propagated by the words printed on every carton of milk sold in the U.S. and many other countries: "400 U.S.P. units vitamin D added per qt."? The remainder of this article will attempt to explain briefly the origin of the mistake.

Modern studies such as those of C. A. Blondin of Clark University support the long-suspected fact that fish, unlike birds and mammals, are able to synthesize calciferol enzymatically without ultraviolet light. Shielded by water, fish receive essentially no ultraviolet (290-to-320-millimicron) radiation, and yet the bluefin tuna has up to a milligram of calciferol per gram of liver oil—enough to provide a daily protective dose of calciferol for 100 children. Cod-liver oil contains less than 1 percent as much, enough to protect against rickets if it is consumed in amounts equal to four grams per day. It is an effective antirachitic medicine because of calciferol's unusual stability: an oil or fat containing the hormone preserves its efficacy for a long time.

In the north of Europe fish has always been a staple of diet, and so the normal diet tended to protect children against rickets. Slowly, over the years, the people of Scandinavia and the Baltic regions became aware of the specific therapeutic value of cod-liver oil as a preventive and even as a cure for rickets. By the end of the 19th century the therapy had come to the attention of physicians, but it was not generally accepted because a number of variables made the evaluation difficult: the advent of spring, chance exposure to sunlight or some unrelated retardation of growth that reduced the severity of the rickets could mask the effect of the cod-liver oil. It remained for Hess to make the unequivocal demonstration. In 1917 he conducted a controlled test with Negro children in New York City, among whom rickets was severe and almost universal, and proved the prophylactic value of routine administration of cod liver oil.

It was a significant finding but it helped to turn investigators away from sunlight and toward diet. In 1919, the very year in which Huldschinsky pointed directly to ultraviolet radiation as the crucial factor in preventing rickets, the British nutritionist Edward Mellanby re-



A UNDER 20
B 20 TO 24
C 25 TO 29
D 30 TO 34
E 35 AND OVER

INDOOR LIVING had a marked effect on rickets in India, according to a survey conducted by Harry S. Hutchinson in Bombay. He found a high incidence (*left*) among rich, well-fed Moslems, whose married women entered purdah and whose infants remained indoors; less among well-to-do Hindus (*middle*), whose children got outdoors more; none among poor Hindus (*right*), who had bad diets but who worked (and whose babies played) outdoors.

pall of industrial smoke, Mellanby found that puppies would rapidly develop florid rickets on a diet reinforced with yeast, milk and orange juice—a natural finding, considering that Bland-Sutton had shown that almost all the animals in the zoo in industrial London suffered from rickets. Mellanby's announcement of the "production" of rickets in dogs by means of a particular diet was in line with the new "vitamine" theories of Frederick Gowland Hopkins and Casimir Funk; Mellanby suggested that the efficacy of cod-liver oil was "most probably" due to vitamin A, which was presumably missing in his "rachitogenic" diet. The diet finding was greeted with enthusiasm—even though Findlay had produced rickets in dogs 11 years earlier by simply keeping them indoors.

Park has pointed out that the report of Mellanby's "first experiments was meagre and would probably have awakened little interest, had not the British Medical Research Committee endorsed the work and publicly committed itself to the view that rickets was a deficiency disease due to a lack in the diet of 'antirachitic factor.'" It is clear enough today that Mellanby's idea that the cod-liver oil factor was probably vitamin A was wrong, but it is not generally recognized that no specific medicine such as cod-liver oil can be called a dietary vitamin unless it is present in normal foods in significant amounts. (Orally administered thyroid, for example, cannot be regarded as a "vitamin T" even though patients with insufficient thyroid of their own are cured by thyroid extract. Both endocrine secretions require external factors, incidentally: ultraviolet radiation in the case of calciferol and iodine in the case of thyroxine.)

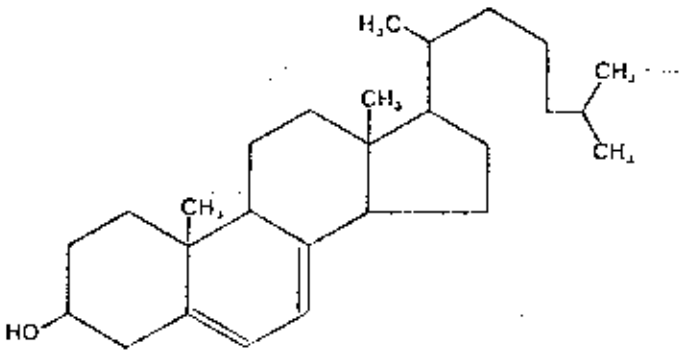
pashed the separation of fat-soluble vitamin A from water-soluble vitamin B, turned his attention to the rickets problem, putting his laboratory rats on Mellanby's "rachitogenic" diet. At first he could not produce rickets; being nocturnal animals, rats have become adapted to survival without direct sunlight and are resistant to rickets. Eventually Henry C. Sherman and Alwin M. Pappenheimer came on the trick of artificially giving rats a diet low in phosphate. Under this artificial stress the bones of young rats failed to calcify—unless they were placed in direct sunlight or were given cod-liver oil.

McCollum went on to establish the difference between the active factor in cod-liver oil and vitamin A in 1922 by showing that, after having been aerated and heated, cod-liver oil could still cure rickets but had lost its ability to cure xerophthalmia, which is due to lack of vitamin A. On this basis he called the cod-liver oil factor "vitamin D." Final recognition of the uniqueness of fish-liver oils came from the finding that animal fats such as butter and lard have essentially no calciferol, particularly in winter; the conclusion was clear that no nonfish diet of any kind could protect against rickets in a sunless environment. It was quite clear then that cod-liver oil was a medicine and not a food.

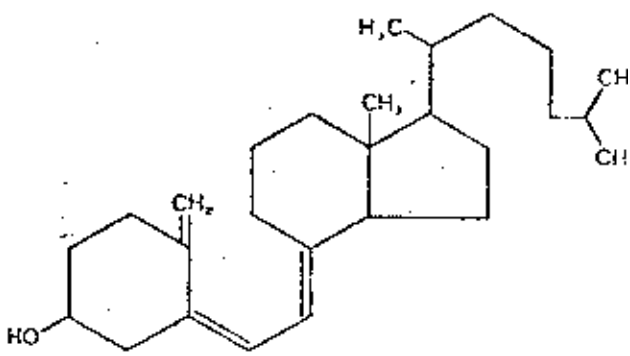
Nevertheless, McCollum had called it "vitamin D," and in the flush of enthusiasm for these new-found dietary factors the name acquired general acceptance. Semantic confusion now entered the picture in overwhelming force. Circular verbal proof made it evident that if "vitamin D" cured rickets, then rickets was a vitamin-deficiency disease! All the careful work demonstrating that rickets

vitamin. Chemists [illegible] about the task of deciphering its chemical structure. When Windaus received the Nobel prize in chemistry in 1928, was "for his researches into the constitution of the sterols and their connection with the vitamins"—a curious citation view of the fact that all biologically active sterols are manufactured by the body and are hormonal in character whereas none of the known vitamins have a steroid structure. Even the discovery that calciferol was produced naturally in the skin in the presence of ultraviolet did not wipe out its classification as a vitamin or the definition of rickets as a dietary problem. Meanwhile the addition of ergocalciferol to milk had essentially eradicated the disease in Europe and America. Ironically, its effectiveness tended to buttress the dietary concept of the disease.

It took time for the correct view to emerge. In 1927 the chairman of the American Medical Association's section on the diseases of children remarked that "cod-liver oil is our civilization's excellent, economical and practical substitute—at least during the colder and darker half of the year—for exposure to sunlight. Is it not strange that the established vitamin deficiencies such as xerophthalmia, beriberi and scurvy are so rare in infants fed human milk from mothers and that rickets is so common? The great primary importance of the actinic [chemically active] rays to normal growth is evidenced by the fact that rickets occurs most severely and most frequently at the end of winter, and especially in those infants whose skins are pigmented. These observations strongly suggest that in human infants vitamins do not play a primary role in the development of rickets.



7-DEHYDROCHOLESTEROL



CALCIFEROL

CHEMICAL STRUCTURES of calciferol (*right*) and its precursor, [illegible] in the skin, and ultraviolet radiation from the sun is crucial [illegible] 7-dehydrocholesterol (*left*), are closely related. The precursor is [illegible] effecting its conversion to calciferol, which then enters the blood

he fact that cod-liver oil, which contains the so-called "vitamin D," cures rickets does *not* prove that rickets observed in human infants primarily is a vitamin-efficiency disease."

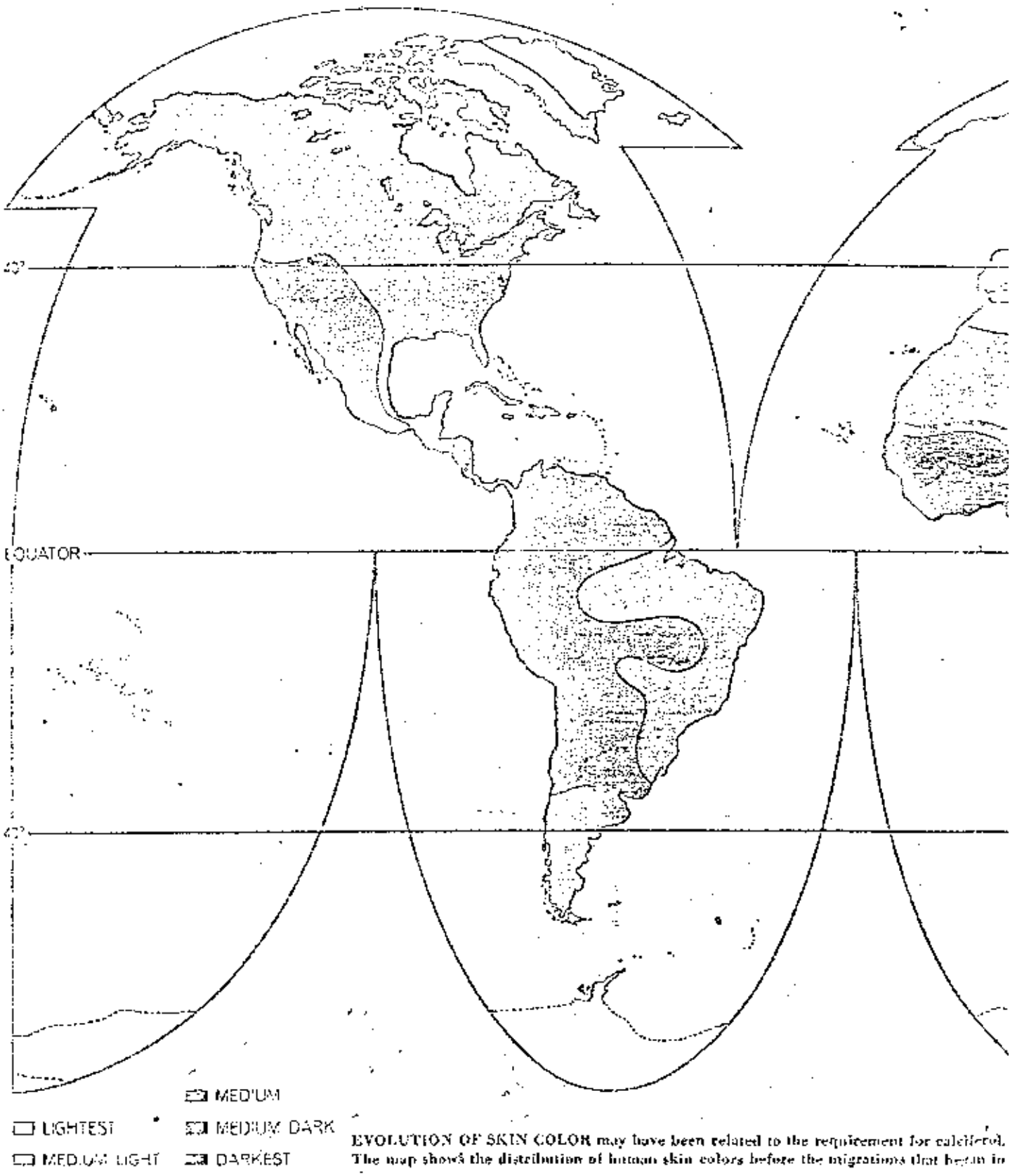
It is interesting to consider the essential difference between the methods of the Glasgow school and those of the London school. Whereas the Glasgow school studied rickets in humans as well as in animals, and from a medical point of view, the London nutritionists studied it only in animals, believing only the results of their experiments and essentially ignoring such brilliant medical studies as those of Palm and Hutchinson. The methodological differences between clinical medical research and beginning biochemistry are therefore behind the whole tangled story, and it is only today, 50 years later, that hindsight can explain the errors of those days.

A word should be said in answer to those who may ask what difference it makes whether calciferol is called a hormone or a vitamin. The answer lies in the point of view from which one approaches this vital calcifying factor needed for the healthy development of the skeleton. Calling calciferol "vitamin D" at least suggests that it forms the nucleus of some cellular coenzyme, as is the case with many vitamins. Calling calciferol a hormone, on the other hand, explains why three hormones, calciferol, hypocalcitonin and the parathyroid hormone are linked together in the delicate control of the level of calcium in the blood [see "Calcitonin," by Howard Rasmussen and Maurice M. Pechet; SCIENTIFIC AMERICAN, October]. Since no other cases of hormones and vitamins working together are known to medicine, it should not be surprising that calciferol turns out to be a steroid hormone whose production rate is under physiological control rather than being left to the vagaries of diet.

Other leads opened up by the hormonal view include the evolutionary development of the hormone. Fish synthesize it without ultraviolet light. Amphibians, reptiles, birds and mammals each apparently have some ultraviolet-receptive area of the body where the hormone is made, such as the ears of rabbits and the feet of birds. By and large, northern animals avoid rickets by living out of doors and by bearing their offspring in the spring, so that they are exposed to the summer sun during their growing period. Truly arctic animals, such as the polar bear and the seal, that live the year round in an area of deficient ultra-

violet obtain their calciferol mainly from their staple diet of fish. (The same was true of the E-kimos, who were entirely free of rickets until they were placed on a European diet by missionaries—when they too rapidly developed the disease.)

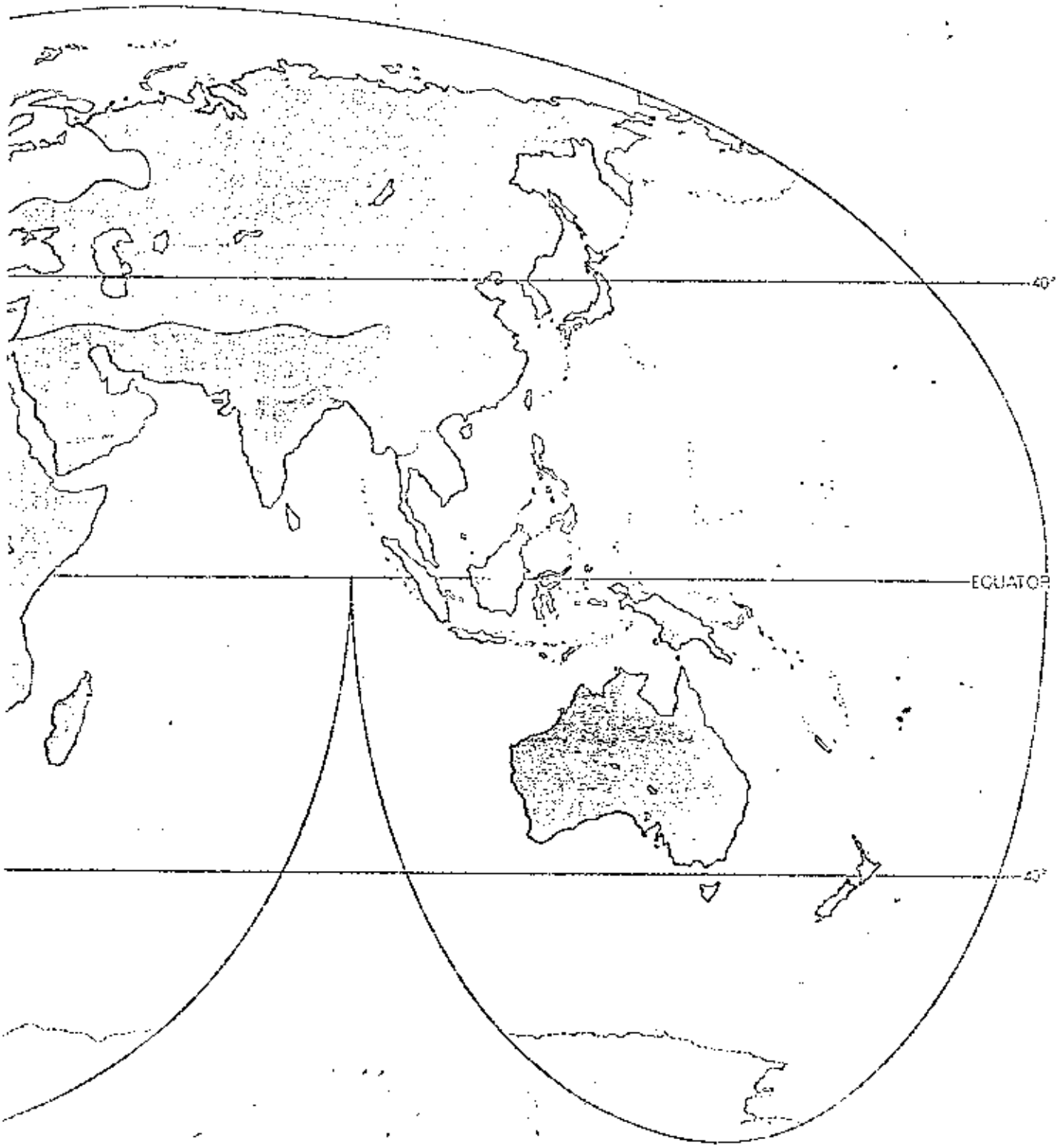
The recognition of calciferol as an ultraviolet dependent hormone gives fresh meaning to a number of seemingly unrelated physiological and cultural adaptations. Tropical man probably avoids the dangers of too much calciferol production by virtue of his dark skin; the melanin granules in the outer layers protect the lower layers of the skin. European man, on the other hand, needed to use all the scanty ultraviolet light available, and consequently was gradually selected for an unpigmented skin such as is present in extreme degree in the blond-haired, blue-eyed, fair-skinned and rosy-cheeked infants of the English, north German and Scandinavian peoples. Indeed, the [illegible] idea of female beauty [illegible] girl with trim ankles, straight legs, fair skin and rosy cheeks must never have suffered from rickets and hence would probably bear strong sons and daughters if chosen as a mate. The very phrase "a fair young damsel" implies that a girl who is beautiful in the eyes of northern beholders is the possessor of an unpig-



EVOLUTION OF SKIN COLOR may have been related to the requirement for calciferol. The map shows the distribution of human skin colors before the migrations that began in

dented skin. Delicate wrists are further proof of the absence of a history of rickets, as is a free-swinging walk, which is only possible in the absence of the pelvic deformities of rickets that would later endanger the process of childbirth.

June weddings tend to bring the first baby in the spring; an infant born in the fall was almost certain to have rickets by the time he was six months old. The fish-on-Friday tradition was as adaptive as the scurvy-preventing eating of an apple a day. Taking the baby out of doors even in the middle of winter for "some fresh air and sunshine" became a northern folk custom. Pink cheeks are visible evidence of the thinness of the unpigmented skin in the one area left uncovered in babies wrapped up warmly and placed out of doors in winter. The ability of outdoor-living Europeans to become deeply bronzed by the sun prevents the synthesis of too much calciferol. [illegible] significant that this [illegible] tion is induced by the ultraviolet 290 to 320 millimicron radiation that produces calciferol. Clearly northern European man, bronzed in summer but [illegible] white in winter, has an epidermis well adapted to the seasonal variation in ultraviolet radiation. Only with the advent of industrial smog did rickets appear in England and northern Europe.



the 16th century. Dark skin presumably protected against overproduction of calciferol. In Europe, much of which is at very high latitudes, man needed all the ultraviolet he could get, particularly for winter, and was presumably selected for unpigmented skin.

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THE EFFECT OF A LOW CALCIUM AND VITAMIN D FREE DIET ON THE SKELETON AND TEETH OF ADULT RATS[1]

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Several investigations have shown that the histological structure and the chemical composition of the continually growing incisor tooth of the rat may be altered by deficient diets (1, 2, 4, 6, 7, 13). Toverud (14) investigated the influence of a calcium low diet and pregnancy on the calcification of bone and teeth of rats. The ash content of the femur diaphysis was greatly decreased, while that of the incisors and molars was 3.5 per cent and 2.0 per cent, respectively, below the average for the normal control rats. Schour (12) has concluded, from a review of his work and that of others, that the mineral phase of mature teeth of limited growth is not susceptible of alteration. However, it does not appear that the effect of a low calcium and vitamin D free diet on the composition of the bones and of the enamel and dentin of the separately examined incisor and molar teeth of adult rats has been studied.

EXPERIMENTAL

Twenty male albino rats were used in the present investigation. Their age of 11 months and their average initial and final weights of 332 and 343 gms. indicate that their molar teeth were fully developed at the beginning of the experimental period. All animals were given, for 5 weeks, the adequate or control diet (Table I) to adjust them to the experimental conditions. They were then divided into 2 equal groups. One group was continued on the control diet for 220 days and will be referred to as the control group, while the other group was given the experimental diet for an equal time. The 2 diets (Table I) were so constructed as to include equal amounts of all ingredients and elements except calcium and vitamin D. The calcium and phosphorus contents of the prepared diets, as determined by chemical analysis, is given in Table II. The animals were fed at a level of 10 grams of these prepared diets per day and the food consumption was determined daily. Distilled water was supplied *ad libitum*.

After the 5 week preliminary period the calcium and phosphorus ingested and the calcium and phosphorus in the combined excreta were determined for each ten-day period. To the excreta for each rat, collected in an 800 cc. Kjeldahl flask were added 100 cc. of concentrated hydrochloric acid, and the contents heated until a homogeneous mixture was obtained. This mixture was cooled to

[1] This work was supported by a grant from the Carnegie Corporation while one of us (A. P. L.) held a Carnegie Fellowship in Densistry. Read at the 20th General Meeting of the International Association for Dental Research, New York, March 14 and 15, 1942 (J. D. Res., 21: 335, 1942). Received for publication July 20, 1942.

room temperature and made up to a known volume. Aliquots of this solution were wet ashed according to the method of Neumann (9). Aliquots of the wet

TABLE I

Composition of the diets

	CONTROL DIET	EXPERIMENTAL DIET
	per cent	*per cent*
Egg white*	12.0	12.0
Salt mixture	4.4	2.773
Embo†	10.0	10.0
Cerelose	56.6	58.227
Corn oil	5.0	5.0
Crisco	12.0	12.0
Beta carotine	1.6 gm./kgm. of food	1.6 gm./kgm. of food
Viosterol	0.2 gm./kgm. of food	1.6 gm./kgm. of food

Salt mixture for the control diet‡ (10)

Ca Citrate·$4H_2O$	154.83 gms.	A
Ca $(H_2PO_4)_2 \cdot H_2O$	56.62	A
K_2HPO_4 (anhyd.)	109.86	A
KCl	62.64	A
NaCl	38.70	A
$CaCO_3$	34.48	A
$MgCO_3$	16.71	A
$MgSO_4$	19.25	A
Ferric Citrate·$1\frac{1}{2}H_2O$	94.18 gms.	B
NaF	3.68	B
$MnSO_4$	1.17	B
$K_2Al_2(SO_4)_4 \cdot 24H_2O$	0.67	B
KI	0.30	B

493.06 gms. of A mixed with 6.90 gms. of B constitutes the salt mixture used in the control diet.

Salt mixture for the experimental diet‡

KH_2PO_4 (anhyd.)	44.50 gms.	A_1
$NaH_2PO_4 \cdot H_2O$	16.25	A_1
K_2HPO_4 (anhyd.)	109.86	A_1
KCl	62.64	A_1
NaCl	38.70	A_1
$MgCO_3$	16.71	A_1
$MgSO_4$ (anhyd.)	19.25	A_1

367.01 gms. of A_1 mixed with 6.90 gms. of B, constitutes the salt mixture used in the experimental diet.

* Commercial egg white. Detoxified according to the method of Parsons (11).

† A General Mills Product rich in thiamine, riboflavin, and vitamin E.

‡ Reagent grade chemicals were used in preparing the salt mixtures.

ashed material were used for the determination of calcium according to standard methods and phosphorus according to the method of Fiske and Subbarow (3)

as adapted for the use of the Evelyn photoelectric colorimeter. The sum of the data obtained for each group per 10 day period was divided by the number of animals, thus giving average value for calcium and phosphorus excreted per rat per 10 day period. From these data, and from the data of food consumption, the mean intake and excretion of calcium and phosphorus for an average rat for an average 10 day period during the 220 days of observation employing the different diets was calculated and the standard deviations (Sx) computed. The data with regard to mineral balance are shown in Table III.

When the animals were sacrificed, the carcasses were boiled in water and the soft tissues removed by dissection. The humeri, femurs, molar and incisor teeth were cleaned and preserved in alcohol.

TABLE II

Calcium and phosphorus in the experimental and control diets

DIET	Ca	P	PER CENT Ca : PER CENT P
	per cent	*per cent*	
Experimental diet	0.007	0.451	0.015
Control diet	0.462	0.458	0.009

TABLE III

Average calcium and phosphorus ingested and excreted per rat for an average of 20 10-day periods together with the standard deviation of the means

Results in milligrams

GROUP	Ca Sx	Ca Sx	P Sx	P Sx
	Ingested	Excreted	Ingested	Excreted
Experimental	6.8 ± 0.58	33.7 ±10.53	467.8 ±38.68	423.5 ±81.44
Control	455.2 ±33.88	422.9 ±42.45	405.0 ±83.00	433.2 ±65.58

During the removal of the teeth from the alveolar bone the following observations were made: In the experimental group the alveolar bone was very soft and friable. The alveolar crest was resorbed and the teeth were loose. The molar teeth of the members of the experimental group showed more wear of the occlusal surfaces than those of the control group.[2] In the control group, on the other hand, the alveolar crest displayed no resorption, and the teeth were firm in their sockets. The incisor teeth appeared to be as well developed in the experimental group as in the control group. The mean weight of the cleaned, fat extracted and dried incisor teeth of single animals was 0.4258 ± 0.0200 (Sx) and 0.4210 ± 0.0346 (Sx) gms. for the experimental and control groups respectively.

The long bones were rendered fat free by continuous extraction for 24 hours using a Soxhlet extractor with alcohol and ether as the solvent. They were

[2] This excessive attrition may have been due to occlusal trauma caused by the looseness of the teeth rather than to softness of the teeth.

dried in an oven at 110° for 12 hours, cooled and weighed. The left humerus was used to determine percentage of calcium and phosphorus, and calcium and phosphorus per cubic centimeter of bone. In order to determine the volume of the bone, a special pycnometer was designed, wherein mercury was used as a submersion medium. The determinations were made at a definite temperature in order that the weight of pycnometer filled with mercury would be a constant value, and that the volume of the mercury displaced by the bone could be calculated without applying corrections. Volume and density of the bones were calculated as follows:

$$\text{Vol. of bone} = \{[\text{Wt. of pycnometer full of Hg}] - [(\text{Wt. of pycnometer} + \text{Hg} + \text{bone}) - (\text{Wt. of bone}^3)]\} \text{ (times)}$$
$$\text{Vol. of 1 gm. of Hg at experimental temperature.}$$
$$\text{Density of bone} = \text{Vol. of bone/Wt. of bone before immersion in Hg.}$$

To test the accuracy of the method 5 determinations of the volumes of 3 humeri from different rats were carried out. The results in terms of cubic centimeters were as follows:

Bone	*Average volume ± Standard deviation*
1	0.122 ±0.0021
2	0.140 ±0.0005
3	0.139 ±0.0012

The average volume in cubic centimeters and the average density together with the standard deviation were determined for the left humeri for both the experimental and control groups. The results were as follows:

GROUP	AVE. VOL. ± Sx	AVE. DENSITY ± Sx
Experimental	0.198 ±0.0200	0.994 ±0.0646
Control	0.194 ±0.0220	1.111 ±0.0600

Each left humerus was dissolved in normal hydrochloric acid and made up to volume. Aliquots of this solution were used for the determination of calcium and phosphorus by the methods mentioned above. The percentages of calcium and phosphorus on the weight per cent basis and in terms of unit volume of bone were calculated. The average results, are given in Table IV.

The left femur from each animal, prepared for analysis as described above, was used for ash determination. The average results are as follows: Experimental group 65.21 ± 2.74 per cent, control group 63.83 ± 1.78 per cent.

The incisor and molar teeth were separately crushed, fat extracted and dried as described above and crushed in a diamond mortar until all particles passed through a 100-mesh sieve. The molar teeth from the experimental animals crushed easily, but vigorous pounding on the pestle with a hammer was neces-

[3] The weight of the bone after submersion in mercury was subtracted in the above equation rather than the true weight since a small but variable quantity of mercury may enter the porous bones.

sary to crush the molar teeth from the control animals. The friability of the incisor teeth of the 2 groups was not noticeably different. The enamel and dentin was separated into 5 fractions according to the method of Manly and

TABLE IV

Average contents of calcium and phosphorus in the humeri together with the standard deviations

GROUP	Ca	P	MGM. Ca/CC. BONE	MGM. P/CC. BONE
	per cent	*per cent*		
Experimental	25.3 ±0.88	11.74 ±1.07	251.0 ±17.35	115.6 ±13.18
Control	26.17 ±0.49	12.58 ±0.31	301.9 ±37.30	139.5 ±15.50

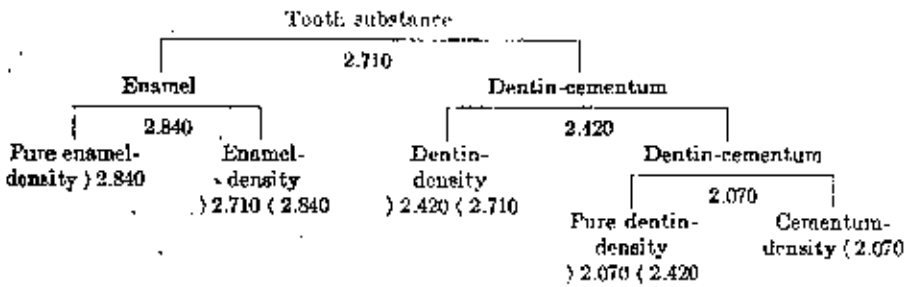

FIG. 1. Separation of powdered teeth into fractions according to density. Numbers enclosed within symbols) and (refer to the density limits of each fraction. Numbers not so enclosed refer to density of liquid employed at indicated place for separation of various fractions.

TABLE V

Average composition of fractions of incisor and molar teeth

DENSITY	EXPERIMENTAL ANIMALS		CONTROL ANIMALS	
	Ca	P	Ca	P
	per cent	*per cent*	*per cent*	*per cent*
Incisor teeth				
>2.093 <2.414	25.51 ±0.84	15.18 ±0.27	26.35 ±1.08	14.84 ±0.61
>2.414 <2.701	33.84 ±1.09	16.69 ±0.40	33.91 ±0.68	16.75 ±0.30
>2.701 <2.84	37.73 ±1.83	17.97 ±0.90	37.51 ±0.12	17.98 ±0.65
>2.840	38.41 ±0.16	18.29 ±0.88	38.46 ±0.75	18.24 ±0.15
Molar teeth				
>2.037 <2.425	30.23 ±0.93	13.79 ±0.33	30.24 ±0.60	13.79 ±0.79
>2.425 <2.711	35.40 ±1.44	16.53 ±0.45	35.42 ±1.42	16.82 ±0.63
>2.711 <2.841	36.18 ±0.80	17.07 ±0.39	36.53 ±1.09	16.90 ±0.40
>2.840	36.86 ±0.92	17.07 ±0.56	36.73 ±2.73	17.65 ±1.18

Hodge (8). A diagrammatic description of the course of the separations and the density limits of each fraction is shown in *fig. 1*.

These fraction of tooth substance were dried in the oven at 110° for 1 hour, and samples were weighed on a semi-micro balance, dissolved in normal hydro-

chloric acid and made up to volume. Aliquots of these solutions were used in determining calcium and phosphorus according to the methods listed above. The percentage of calcium and phosphorus was calculated together with the average values and standard deviation of the means for each group of animals. The results are shown in Table V. The indicated density limits of the fractions shown in Table V are the determined densities of the fluids used in the isolation of the fractions.

SUMMARY AND CONCLUSIONS

The influence of a diet very low in calcium and essentially vitamin D free, but otherwise adequate, on the calcium and phosphorus balance and on the composition of the skeleton and of the enamel and dentin of adult rats has been studied.

The animals on the experimental diet were in a negative calcium balance during the experimental period.

The molar teeth from the experimental rats were friable, but chemical analysis does not reveal any decalcification. The alveolar bone of these animals, however, was decalcified, the alveolar crest resorbed and the teeth loose in their sockets. The incisor teeth from the experimental rats grew and developed normally in weight and chemical composition.

The average ash content of the femurs of the experimental rats was not significantly lower than that of the control group.

The average density of the humeri from the experimental animals was decreased, but their volume was not altered. The percentage of calcium and phosphorus per unit weight of bone does not distinguish osteoporotic bones from normal bones, but calculations of weight of calcium or phosphorus or ash per unit volume of bone may serve to indicate the degree of osteoporosis.

The data indicate, under the conditions which produced osteoporosis of this experiment, that bone substance is lost from the interior of the bone without loss from the surface and that the mineral and organic phases are proportionally decreased in the osteoporotic bone.

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BIOLOGICALLY ACTIVE METABOLITES OF VITAMIN D

(Order No. 66-13,809)

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The University of Wisconsin, 1966

Supervisor: Professor Hector F. DeLuca

Chemical and biological investigations were made on the antirachitic metabolites of vitamin D. Radiochemically pure vitamin D (general H^3 vitamin D_3, 1,2-H^3 vitamin D_3, 7-H^3 vitamin D_3, 22,23-H^3 vitamin D_4, or 4-C^{14} vitamin D_3) was administered to vitamin D-deficient rats either orally or intravenously. The tissues were extracted by a methanol-chloroform-water procedure, and the chloroform layers were chromatographed on silicic acid columns. At least three chloroform-soluble metabolites have been found in addition to the parent vitamin, all of which are partially or fully biologically active. These have been found in varying amounts in kidney, intestine, blood, liver, and bone. In bone, an important target of vitamin D action, 49% of the chloroform-soluble radioactivity was found as a polar metabolite (peak IV) which was as biopotent as standard vitamin D in curing rickets in rats. The least polar, partially bipotent metabolite (peak I) was never formed in large amounts under the conditions studied. It has been tentatively identified as vitamin D ester(s) because it yields vitamin D upon saponification. Peak II is eluted from the columns with solvents slightly less polar than those required for elution of vitamin D and it also has not been found in large quantities. Its identity is unknown, but it is not pre-vitamin D and is probably not the isomer, 5,6-trans vitamin D. Peak III was tentatively identified as unaltered vitamin D because of its chromatographic behavior in several systems and its biopotency. The amounts of metabolites formed at various times (from two to forty-eight hours) after the oral administration of vitamin D were determined in rat liver, blood, and carcass. In all three tissues, the relatively constant amount of peak IV from four to forty-eight hours was the striking feature, being much more constant than the amount of vitamin D itself. It was also found that the relative amount of peak IV, but not of the other metabolites, increased as the dose of vitamin D decreased, so that it was the major form of the vitamin present at dosage levels approaching those required to prevent deficiency symptoms. Biologically active peak IV was also found in human blood serum, comprising 18-32% of the chloroform-soluble radioactivity. Peak IV given in vivo stimulated calcium transport in rat everted intestinal sacs, as did vitamin D. With ten unit (10 I.U.) doses, vitamin D caused a response in sixteen to twenty hours, whereas a response to peak IV occurred in eight hours, considerably earlier. On the basis of these observations, it has been suggested that the peak IV metabolite could be the metabolically active form of vitamin D.

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Formation of Vitamin D Esters *in Vivo*[1]

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A nonpolar metabolite of vitamin D appears in the liver and carcass within 2 hours after ^{3}H-vitamin D_3 administration to vitamin D-deficient rats. Its concentration in liver and carcass remains low regardless of the amount of vitamin D administered or time after dosage. It has not been detected in extracts of blood. This metabolite migrates on silicic acid columns identically with vitamin D palmitate and yields almost entirely vitamin D_3 on saponification. The vitamin D_3 thus produced was identified by co-chromatography with crystalline vitamin D_3 and by bioassay. Thus the metabolite has been identified as an ester of vitamin D and a long chain fatty acid.

The metabolic fate of the vitamin D molecule is at present largely unknown. This can be attributed mostly to technical difficulties arising from the small amounts required or tolerated by an animal. It has only recently been possible to carry out definitive experiments on the metabolism of vitamin D with the preparation of radioactive vitamin D of moderate to high specific activity (1-3). Prior to that, Kodicek and co-workers were able to detect "breakdown" products of vitamin D using a low specific activity vitamin D preparation (4). These metabolic products are as yet unidentified.

Norman, Lund, and DeLuca reported for the first time three metabolites of vitamin D which possess at least partial ability to cure rickets (5). In further work, Lund and DeLuca (6) have shown one of these metabolites (their peak 4) to be present in large amounts in bone, blood, and liver; to be highly biologically active; and to be found in large proportions in rats given amounts of vitamin D approaching a minimum daily dose (6). These findings suggest that this metabolite may be the metabolically active form of the vitamin, but it also still remains unidentified.

Another one of the biologically active metabolites of vitamin D described in this laboratory (peak 1, in references 5 and 6) is eluted with 5-10% diethyl ether in Skelly B (a distilled petroleum (65-67°) fraction) from silicic acid columns. Its elution position suggested that it might be an esterified form of vitamin D. It is the purpose of this report to demonstrate that the peak 1 described previously is an ester of vitamin D which is biologically effective, and is formed rapidly in the liver, but which is found only in small amounts regardless of the dose of the vitamin D given. During the course of this work, Fraser and Kodicek have also reported the formation of esters of vitamin D *in vivo* (7).

METHODS

Radiochemically pure, randomly labeled ^{3}H-vitamin D_3 or 1,2-^{3}H-vitamin D_3, prepared in this laboratory, were used in all experiments (1, 2). Each preparation used was purified prior to use by silicic acid column chromatography as described previously (1).

The palmitate ester of vitamin D was prepared essentially as described by Pasatis and Bell (8). The ester had an ϵ of 18,200 at 261 mμ and was able to cure rickets in rats as revealed by the line test assay method described below.

Animals. Weanling male albino rats were obtained from the Holtzman Co. of Madison, Wisconsin, and were given food and water *ad libitum*. They were housed in individual hanging wire cages

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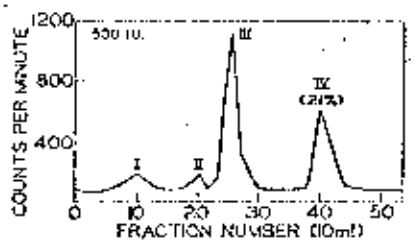

FIG. 1. Silicic acid chromatogram of an extract from the livers of vitamin D-deficient rats given 500 IU of ^{3}H-vitamin D_3 24 hours before sacrifice. The livers were extracted by a methanol chloroform procedure. The extract was dissolved in Skelly B and chromatographed on silicic acid columns by a gradient elution procedure as described earlier (6).

and were fed a purified diet described previously which contained 0.47% Ca and 0.3% P (9). After 3-4 weeks on this diet, they became vitamin D-deficient as judged by a reduced serum calcium concentration and reduced growth (10). The animals were then used for the experiments described.

Extraction and chromatography. The ^{3}H-vitamin D preparations were administered orally to deficient rats in 0.1 ml cottonseed oil (Wesson) by means of a syringe fitted with a blunt needle. At either of the times indicated or 24 hours after dosage, the rats were killed by decapitation and their blood was collected. The livers were removed and the entire gastrointestinal tract was excised and discarded. The carcass was skinned, ground in a meat grinder, and extracted with methanol and chloroform as described previously (6). The blood and liver were also extracted as previously described. The chloroform phase from each extract was taken to dryness by means of a flash evaporator and the residue was redissolved in a distilled petroleum (65-67°) fraction. This material was chromatographed on silicic acid columns. Aliquots of each fraction were removed, taken to dryness under a stream of air, and dissolved in a toluene liquid scintillation counting solution which contains 2 gm 2,5-diphenyloxazole (PPO) and 100 mg 1,4 - bis - 2 - (4 - methyl - 5 - phenyloxazolyl)benzene (POPOP) per liter. In cases where optical density readings at 264 mμ were desired, another aliquot of each fraction was dried under a stream of N_2, redissolved in ethanol, and read in a Beckman DU spectrophotometer. The vitamin D position in the chromatogram was finally established by ultraviolet absorption spectra using a Cary 11 recording spectrophotometer.

Co-chromatography of vitamin D palmitate and radioactive peak I fraction. The synthetic palmitate of vitamin D (6 mg) was mixed with radioactive peak I and placed on a silicic acid column as already described. Gradient elution was carried out with a 250 ml Erlenmeyer mixing chamber and a 300-ml holding chamber to give a convex gradient (1, 5, 6). The gradients were from Skelly B to 1% diethyl ether in Skelly B; from 1% diethyl ether in Skelly B to 5% ether in Skelly B; 5% ether in Skelly B to 50% ether in Skelly B; and finally 50% ether in Skelly B to 100% methanol. Ten-ml fractions were collected and an aliquot was taken for measurement of O.D. at 264 mμ, while another aliquot was used for determination of radioactivity.

Saponification. Peak I fraction was dissolved in 95% ethanol containing 10% KOH (w/v). The solution was flushed with N_2 and 0.5 mg α-tocopherol was added. The mixture was refluxed under an atmosphere of N_2 for 30 minutes and cooled on ice, and an equal volume of water was added. It was then extracted three times with diethyl ether. The ether phase was successively washed with 3.6% HCl (v/v) and three times with water. It was dried over anhydrous $CuSO_4$ overnight. The ether was finally removed by means of a flash evaporator and replaced with Skelly B in preparation for chromatography.

Bioassay. The hydrolysis product shown in Fig. 3 was believed to be vitamin D_3 and was assayed biologically by the line test assay method. This assay was performed essentially as described in the U.S. Pharmacopeia (11).

The doses for the metabolites were prepared assuming that they had a specific activity equal to that of the starting ^{3}H-vitamin D_3. For example, if the parent ^{3}H-vitamin D_3 had a specific activity of 800 dpm per 0.025 μg, then it was assumed that 800 dpm of a metabolite would be 0.025 μg. It was on this basis that biopotency was estimated. The metabolite to be tested was dissolved in 0.1 ml cottonseed oil and administered orally as previously described.

RESULTS

Figure 1 demonstrates a chromatogram of a chloroform extract of liver from rats killed 24 hours after receiving 500 IU of ^{3}H-vitamin D_3. As previously described, at least four components could be detected. Peak III has already been identified as the unaltered vitamin (5, 6) while peaks I, II, and IV are as yet unidentified. It has already been shown that the present chromatographic system is free of artifacts and that the above peaks really do represent metabolites of vitamin D_3 (6).

As the dose of vitamin D is varied, the

TABLE I

ACCUMULATION OF PEAK I METABOLITE FRACTION AS A FUNCTION OF ^{3}H-VITAMIN D_3 DOSAGE

Vitamin D-deficient rats maintained on a diet containing 0.47% Ca and 0.3% P were given the indicated dose of 1,2-^{3}H-vitamin D_3 orally in Wesson oil and were killed 24 hours later. Tissue extracts were prepared and chromatographed as described in Fig. 1.

Tissue	Dose(IU)	% of dose as Peak I
Liver	10	0.23
	100	0.30
	500	0.42
	2500	0.47
	10,000	0.43
Blood	16–10,000	None detected
Carcass	10	0.04
	100	0.1
	500	0.1
	2500	0.09
	10,000	1.00

TABLE II

TIME COURSE APPEARANCE OF PEAK I METABOLITE IN LIVER AFTER 1,2-^{3}H-VITAMIN D_3 ADMINISTRATION

Vitamin D-deficient rats were given 500 IU of 1,2-^{3}H-vitamin D_3 orally in Wesson oil and were killed at the times indicated. Tissue extracts were chromatographed as described in Fig. 1.

Time after dose (hours)	% radioactivity administered	Units
0.25	0	0
2	0.02	0.27
4	0.22	4.6
8	0.52	10.8
10	0.28	5.7
24	0.42	9.3
48	0.34	6.0

percentage of the dose appearing in the peak I fraction of liver or carcass does not change appreciably (Table I). Thus it seems unlikely that this metabolite holds a significant position either as a storage compound or as a possible metabolically active form.

The results presented in Table II show that the peak I metabolite appears in significant concentration even 2 hours after vitamin D dosage. Thus this metabolite of vitamin D is formed in a reasonably short

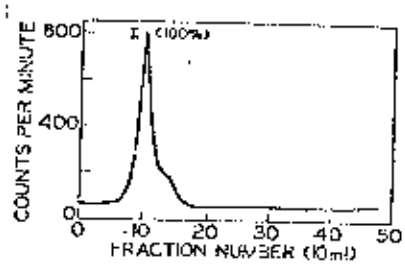

FIG. 2. Chromatography of combined fractions from liver extract which chromatographed as peak I. The peak I fractions obtained from the extracts described in Tables I and II were combined and chromatographed on silicic acid columns as described in Fig. 1.

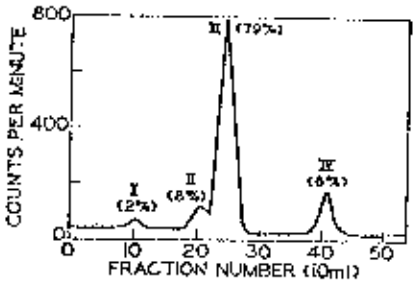

FIG. 3. Chromatography of peak I material after saponification. The peak I fraction from Fig. 2 was subjected to saponification as described in the text and rechromatographed on silicic acid columns as in Fig. 1.

time after administration. In experiments not shown, co-chromatography of peak I with vitamin D_3 showed it to be a distinct metabolite and its chromatographic behavior to be unaffected by the presence of the vitamin. The combined peak I fractions from Tables I and II were rechromatographed on silicic acid columns and showed only one peak (Fig. 2).

When peak I thus isolated was subjected to alkaline hydrolysis and the products chromatographed, the elution pattern shown in Fig. 3 resulted. Most of the radioactivity migrated in a position usually occupied by vitamin D_3. In fact, co-chromatography of the hydrolyzed peak I with crystalline vitamin D_3 shows that the major material is identical with vitamin D_3 (Fig. 4). As little as 2% of the original peak I material chromatographed in its original position after alkaline treatment. It is interesting that some radioactivity of the hydrolyzate appeared in the

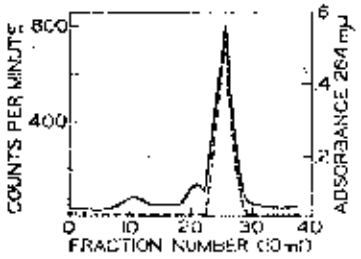

FIG. 4. Co-chromatography of saponified peak I with crystalline vitamin D_3. Saponified peak I from Fig. 2 was mixed with 3 mg crystalline vitamin D_3 and chromatographed on silicic acid columns as described in Fig. 1. Optical density was recorded at 264 mμ (---) on all fractions as was the radioactivity in cpm. (—)

TABLE III

BIOASSAY OF PEAK III (FIG. 3) RELEASED BY THE SAPONIFICATION OF PEAK I (FIG. 1)

Each of four rachitic rats fed the Steenbock and Black diet 2965 for 3 weeks was fed the indicated dose in 0.1 ml cottonseed oil. Seven days later they were killed, and their radii were dissected out, sectioned, and stained with silver nitrate. The stained radii were scored according to the procedure described in the U.S. Pharmacopeia (11).

Dose	Calcification score
0	0
4 IU standard D_3	4.0
3200 dpm (4.0 IU/rat) peak III	3.0
3200 dpm (4.0 IU/rat) peak III	5.0
1800 dpm (2.0 IU/rat) peak III	1.0

regions of peak II and peak IV, suggesting that peak I may contain small amounts of these metabolites in esterified form. As additional proof that vitamin D is released by the hydrolysis of peak I, the peak III recovered from the saponified peak I was assayed for its ability to cure rickets by the line test assay method. The results show (Table III) that the peak III so recovered did in fact assay equivalent to standard vitamin D.

That peak I is in fact an ester of vitamin D and a long chain fatty acid is shown in Fig. 5. These results show that the radioactivity of peak I corresponds exactly with the 264 mμ absorption induced by vitamin D_3 palmitate on silicic acid column chromatography.

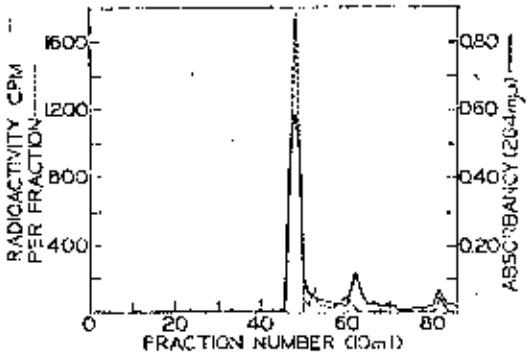

FIG. 5. Co chromatography of vitamin D_3 palmitate and peak I metabolite. The exact gradient elution is described in the text. (- - -) OD 264 mμ; (———) CPM radioactivity, dpm.

DISCUSSION

There is now adequate evidence that vitamin D is esterified *in vivo*. The present report shows conclusively that vitamin D is generated by saponification of the peak I metabolite derived from the administration of ^{3}H-vitamin D_3 to rats. The radioactive vitamin D_3 thus generated was identified by chromatographic position, by its exact co-chromatography with crystalline vitamin D_3, and finally by its biological activity. Synthesis of vitamin D palmitate and co-chromatography on silicic acid columns also shows it to chromatograph identically with peak I (Fig. 5). Thus there is little doubt that esters of vitamin D are formed *in vivo*.

Fraser and Kodicek have also found esters of vitamin D in liver and kidneys of rats given ^{3}H-vitamin D_3 (7). They have compared migration of synthetic esters of vitamin D_3 with the esters found in liver extracts and have concluded that palmitate, linoleate, stearate, and oleate esters comprise 39, 16, 22, and 13% of the esterified vitamin D found, respectively.

It is of interest that no peak I metabolite appears in the blood serum of rats. A complete absence of this metabolite has also been observed in the case of human serum despite the appearance of peak II and peak IV metabolites (DeLuca, Lund, Lobeck, and Rosenbloom, submitted for publication). This is in contrast to retinol, where the esters represent an important transport and storage

form and is found in significant amounts in blood.

The purpose of the esterified form of vitamin D in the animal is unknown at the present time. It has never been found in large amounts under any level of vitamin D dosage or at any time after dosage. Thus there is no evidence to support the idea that this form of the vitamin functions in a storage capacity. Because their concentrations are low at low dosages, it seems unlikely that they would function as a metabolically active form. It seems likely that the esters of vitamin D may be only a metabolic curiosity to which little or no functional importance can be ascribed at the present time.

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Parathyroid Activity and Vitamin D

Observations on Rats, Chickens and Guinea Pigs

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Introduction

The absence of a hypercalcemic effect of a parathyroid extract on the calcemia of a rachitic dog was observed starting from 1930 by Morgan and Garrison (16) in contrast to the hypercalcemia observed in a dog receiving irradiated ergosterol or cod liver oil. This idea of synergism between the effects of the parathyroid hormone and vitamin D on the calcium metabolism has been espoused during the last few years. Harrison and Harrison (1964 (7), Raoul and Marnay, 1965 (24) have discussed the observations which led to this idea.

In vitro, the stimulating effect of the parathyroid hormone (PTH) on the liberation of Ca was observed only in the liver mitochondria of rats receiving vitamin D (De Luca, Sallis, 1965 (4). Bone reabsorption under the influence of parathyroid hormone in an embryonal radius section of the rat is decreased if the milieu contains the serum of vitamin D deficient rats (Raisz [23]).

However, with suboptimal amounts of parathyroid hormone (PTH), the addition of small quantities of vitamin D increased reabsorption in the calvaria of mice (Goldhaber [6]).

The existence of small quantities of vitamin D in certain preparations of parathyroid hormone (Marnay, Gerrillot-Vinet and Raoul [13]), shows that the interaction between these two factors of calcium metabolism is possible without the knowledge of the investigator.

In vivo, the hypercalcemic response to the injection of parathyroid hormone is weak or non-existent in the case of vitamin D deficiency. The amount sufficient to restore this response is very small, 0.05 IU X 6 (0.0075 µg) of calciferol for the rat (Rasmussen, De Luca, Arnaud, Hawker and Stedinck, 1963 [26]). This will explain the contradictions found in the literature concerning the rat (Toverud [30]), the mouse (Nichols, Schartum and Vaes [19]) or the dog (Ney, Au, Kelly, Radde and Bartter [18]). The slight hypercalcemia observed under the influence of PTH in different animals deficient in vitamin D would be due to the persistance of traces of this vitamin in their organisms.

However, the hypophosphoremic activity of the parathyroid in vivo or the stimulation of the captation of phosphate by the mitochondria in vitro appear to be independent of the presence of vitamin D (26).

In this work, we have tried to specify the role of vitamin D on the activity of the parathyroid hormone in vivo (usual or purified preparation) by varying the tests, the animal species and the regimes. We have used the classic tests: prevention of hypocalcemia, hyperphosphoremia and phosphaturia after parathyroidectomy in D deficient or non-deficient rats. Further, we studied the variations in calcemia of chickens, more susceptible to rickets than rats, and also in guinea pigs deficient in vitamin C, after administration of parathyroid hormone.

Experimental Conditions

1. Animals

We used male Wistar rats, tricolored male guinea pigs and male Hubbard chicks.

2. Regimes

The diet given to rats was that of Harrison, Harrison and Park (8), replacing the beer leaven by a vitamin mixture (18); vitamin D_3 administered following the details indicated in the text in amounts of 2.5 µg per week, per rat. The distilled water and the diets are given in ad libitum.

The diet for the guinea pigs was that of Th. Terroine (29) with the saline mixture of Sober, Mannering, Cannon, Elvehjem and Hart (28). The guinea pigs received this diet with a few greens (carrots, salad) two to three days after their arrival in the laboratory, then we took away this supplement. Vitamins C or D_3 were administered to the groups in amounts of 60 mg vitamin C and 100 IU of vitamin D_3 two times per week.

The newly hatched chicks went a day without food (water, as desired) and then received the rachitogenic diet of the A.O.A.C. (20) for three weeks, then supplemented by 7.5 p. 100 of the saline mixture used for the rats' diet (Wesson [33]). They were kept in the shelter of daylight and warmed with a carbon filament lamp.

3. Samplings, Operating Techniques

The blood was sampled by cardiac puncture under light ether-chloroform anesthesia (9 v-1-v) in the rats and guinea pigs. The sample from the heart was taken without anesthesia in the chicks following the technique described by Lepine, Cadillon and Chaumont (10).

Urine excretion 6 hours after parathyroidectomy was measured in groups of 2 rats placed in glass metabolism cages enabling the separation of feces and urine.

The parathyroidectomy was performed in the rat by extensive cauterization (affecting about 1/4 of each thyroid lobe (Hirsh, Gauthier, Munson, 1963 [9]). In the guinea pig, we performed surgical thyroparathyroidectomy by ligating the thyroid arteries. These operations were done under ether-chloroform anesthesia (9 v-1 v).

4. Amounts of Ca and P

The quantitative determinations of calcium were performed on 0.2 ml of serum (blood collected without anti-coagulants) after calcination by an electric furnace (+ <600°), then recovered in 5 ml of a 8.82 p. 100 solution of lanthane chloride; the amount of calcium is determined by atomic absorption (Perkin Elmer 303 apparatus) following the procedure described by Zettner and Selligson (34). Serum phosphorus or urine phosphorus was determined by the colorimetric method of Briggs, modified according to Raoul and Vinet (25).

5. Parathyroid Hormonal Preparations

The commercial preparation used was Paratyrone BYLA titrated to about 100 USP per ml. A purified preparation was made from frozen bovid glands following the method of Rasmussen, Sze and Young (27) or that of Aurbach and Potts (1). The separations on the Sephadex G 100 were done at +4°. The optic density measured at 265 mμ served to identify the peptides. The amount of proteins was determined according to the method of Lowry, Rosebrough, Farr and Randall, 1950 (11). The fractions thus separated were lyophiled and stored at -20° C. Figure 1 represents a curve of the elution with designation of the peptides.

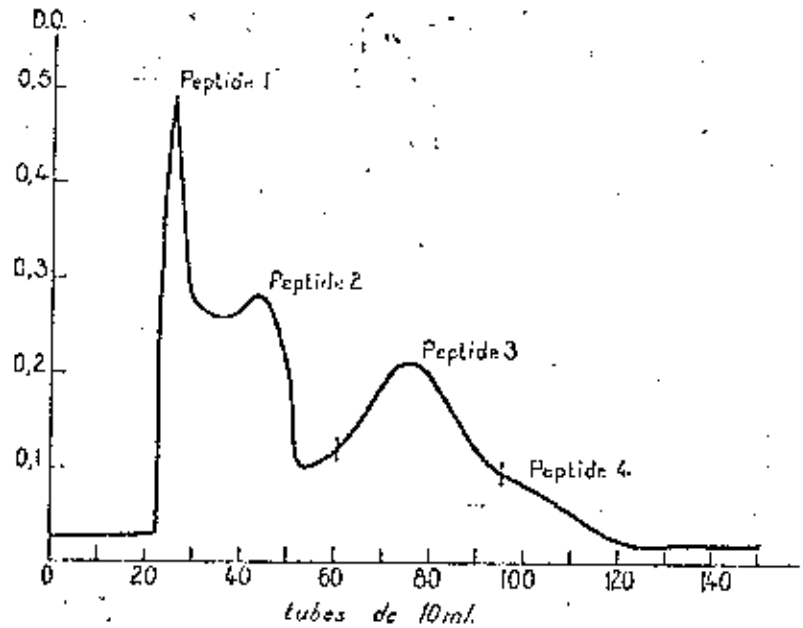

Figure 1. Separation on Sephadex G 100 of the parathyroid hormone according to Rasmussen, Sze, Young, 1964 (27)

Injections were given by the subcutaneous route, the product being dissolved in distilled water. The control animals were subjected to the same samplings and received an injection of distilled water.

Results

1. On the parathyroidectomized rat

The peptides separated on the Sephadex had been assayed by Munson's test (17) separately on parathyroidectomized rats either deficient or non-deficient in vitamin D (Table 1).

Peptides 1, 2 and 3 caused hypercalcemia in rats receiving vitamin D, but had practically no effects on the deficient animals.

Study of the variations of phosphorus metabolism following the administration of the same fractions showed a certain degree of dissociation between the activity on the calcium and on the phosphorus; with vitamin D deficiency,

Table I.

Activity of Parathyroid Hormones Separated on Sephadex on the Serum Calcium of Rats 6 Hours After Parathyroidectomy

	Without vitamin D	+ vitamin D_3	P
Calcemia of the parathyroidectomized controls (mg Ca/1)	70	80	<0.1
Variation after:			
Peptide 1 (1.4 mg)	+ 9	+32	<0.05
Peptide 2 (3.6 mg)	- 1	+15	<0.05
Peptide 3 (0.52 mg)	+ 4.5	+15	<0.1
Peptide 4 (0.06 mg)	- 6.5	-12	>0.1

peptides 1 and 3 caused significant lowering of serum P after parathyroidectomy accompanied by an increase of urinary excretion of this element (Table II).

2. On the chick

The A.O.A.C. diet included 0.6 p. 100 Ca and 0.68 p. 100 P, and under these conditions, the chickens receiving 0.5 µg of vitamin D_3 per day had a normal calcemia but the injection of 100 USP of parathyroid hormone had no hypercalcemic effect (Marnay, Aldbais, Raoul [12]). (Table III).

The sufficient addition of calcium to the diet to reach the level of 1.86 p. 100 enabled us to observe clear hypercalcemic activity, but only in the presence of vitamin D_3. The simultaneous addition of phosphate did not modify this response; on the other hand, the elevation of the elevation of the level of phosphate without modifying that of calcium (diet IV) brought very divergent responses, from 0 to 28.7 in the presence of vitamin D_3 while without vitamin D, the chickens died before the end of the experiment.

Table II

Activity of Parathyroid Peptides Separated on the Sephadex on the Urinary and Serum Phosphorus of the Rat 6 Hours After Parathyroidectomy

	Without vitamin D		+ vitamin D	
	Serum P (mg/l)	Urinary P in 6 hours (mg)	Serum P (mg/l)	Urinary P in 6 hours (mg)
Parathyroidectomized controls	150	1	131	4.9
Variation after:				
Peptide 1 (1.4 mg)	- 19(*)	+1.8	- 25(*)	+7
Peptide 2 (3.6 mg)	+ 4(**)	-0.03	- 28(**)	+4
Peptide 3 (0.52 mg)	- 47(***)	+2.3	- 14(***)	-1.4
Peptide 4 (0.06 mg)	- 10(****)	+1	- 7(****)	-4.2

(*) P>0.1 N.S.
(**) P<0.05
(***) P<0.1
(****) P<0.1 N.S.

Table III

Effect of 100 USP of Commercial Parathyroid Hormone (PTH) on the Calcemia of the Chicken as a Function of the Levels of Ca, P and Vitamin D in the Diet

Diets	Composition of diets p. 100 Ca	p. 100 P	D3/j	Inital Calcemia mg l	3 hours after PTH	P
I	0.60	0.68	0	72.3±3.3	- 6.9±2.5	
	0.60	0.68	0.5 µg	95.8±2.7	- 1.1±2.7	<0.10
II	1.86	0.68	0	75.9±3	- 5.1±1	
	1.86	0.68	0.5 µg	106.9±1.1	+28.6±0.9	<0.005
III	1.86	1.31	0	74.6±1.8	+ 2.8±1.8	
	1.86	1.31	0.5 µg	97.7±1	+23.2±1.8	<0.005
IV	0.60	1.31	0.5 µg	105.7	+12.2	NS

The second fraction separated on the Sephadex comparatively tried on the vitamin D deficient or non-deficient chickens as a function of time with diet III (Ca 1.86 p. 100, P 131 p. 100) allowed the same conclusion (Table IV).

Table IV

Kinetics of the Activity of the Fraction of Parathyroid Hormone Purified on Sephadex G 100 on the Calcemia of the Vitamin D Deficient or Non-deficient Chicken (mg/l)

	1 h 30	3 h	4 h 30	6 h	P
With vitamin D_3	+24 (3)(*)	+25 (6)	+21 (3)	+25 (5)	<0.05
Without vitamin D	- 5 (6)	- 1 (5)	- 1 (3)		

*Number of experiments

3. On the guinea pig

With the semi-synthetic diet, without the addition of hay and without vitamin D, the guinea pigs showed a certain degree of hypocalcemia in 4 to 6 weeks (Marnay, Nghiem, [14]) and the injection of 200 USP of parathyroid hormone was practically without effect whether the animal was thyroparathyroidectomized or not. By contrast, in the animals receiving, besides this diet, 200 IU of vitamin D_3 per week, hypercalcemia was clear 24 hours after injection of the same dose of parathyroid hormone (Table V).

Discussion

1. The importance of vitamin D in the information on the hypercalcemic effect of the parathyroid hormone has elicited a number of works which have been analyzed almost simultaneously by Harrison and Harrison, 1964 (7) and Raoul and Marnay (24). At that time, the hypercalcemic effects observed

Table V

Effect of 200 USP of Parathyroid Hormone on the Calcemia of Vitamin D Deficient or Non-deficient, Normal or Thyroparathyroidectomized (Thpx) Guinea Pigs

	Initial calcemia	24 hours after 200 USP of parathyroid hormone	P
Unoperated upon without D	93.4	+ 3.4	
Unoperated upon + D_3	101	+17.6	<0.05
Thpx without D	86.5	+ 6.8	
Thpx + D_3	91	+18.4	<0.05

on animals deficient in vitamin D were linked to the weakness of the necessary amount of vitamin D (Rasmussen, et al., 1963, loc. cit.), and the difficulty of obtaining totally deficient animals. The results which we just described completely confirm this idea. Actually, on the rat and the guinea pig, which are difficult to make totally deficient in vitamin D, we observed a residual hypercalcemic activity after injection of parathyroid hormone purified on the Sephadex (Table 1, peptides 1 and 3) or of commercial parathyroid hormone (Table V). However, with the chicken which is more susceptible to vitamin D deficiency the two types of commercial preparations or the purified one (Table IV) were completely without effect. Let us emphasize that contrary to most authors, we have always compared the responses of long-term stabilized animals, deficient or not, thus allowing for comparisons less subject to rapid kinetic variations.

The lack of comparable controls has actually often affected the results presented as contrary to the preceding conclusions. Such is the case with hypercalcemic activity observed in rachitic dogs by Ney, Au, Kelly, Radde and Bartter (18), or the comparison with the dogs receiving

the same diet supplemented with vitamin D not having been made, it is impossible to make clear conclusions. The activity observed may here be explained as much by an insufficient deficiency of vitamin D so that the calcemia is slightly lowered by the diet used and by the fact that thyroparathyroidectomy causes a perceptible lowering of calcemia, indicating a significant functional activity on the part of the animal parathyroid.

2. The hypophosphoremic activity seemed by contrast to be relatively independent of vitamin D, although the results of various authors are not in agreement; for example, Harrison and Harrison (7) observed no parathyroid hormone effect on the serum phosphorus of vitamin D deficient rats while Rasmussen, De Luca, Arnaud, Hawker and von Stedingk (26) observed with this hormone a fall in hyperphosphoremia of vitamin D deficient parathyroidectomized rats. In agreement with these observations, we obtained a dissociation between the hypercalcemic (absent without D) and hyperphosphoremic activity in parathyroidectomized rats, in particular with peptides 1 and 3 (Table II).

3. The existence of a principal active fraction under the best conditions of preparation (Aurbach and Potts [1]) does not exclude the distribution of hormonal activity among the various peptides separated on the Sephadex by Rasmussen, Sze and Young (27) or ourselves. The same distribution was observed with hypophysic peptides (Upton, Lerner and Lande, 1966 [31]) and may be related to the different forms of composition rather than to clearly different compounds. In this same vein, Rasmussen, Sze and Young (loc. cit.) had obtained peptides which didn't have the same relative activity on the mobilization of Ca and P in parathyroidectomized rats, nor in isolated mitochondria *in vitro*. However, in spite of the differences

in the activity of the hormone on calcium and phosphorus discussed above, the existence of two distinct hormonal fractions is not established and underlines the importance of vitamin D as an element of differentiation.

4. The test of mitochondrial effect used by some authors may, however, be prone to criticism as to its value for the evaluation of the actual activity of the parathyroid hormone. Pastan (22) insists on the lack of specificity of this test; for example, protamine acts like the parathyroid hormone on the capture of phosphate, on respiration, and on calcium liberation at the mitochondrial level. Protamine does not act on the calcemia of parathyroidectomized rats; a more specific test of the parathyroid hormonal effect.

5. Another result merits being emphasized: the existence of a hypocalcemic-causing fraction in a preparation of parathyroid hormone purified on Sephadex (peptide 4, Table I) revealed by its effect on the calcemia of parathyroidectomized rats receiving vitamin D.

We had observed with certain commercial preparations of PTH, a hypocalcemia-causing effect on the dog or the rabbit (Marnay, Prelot [15]). Copp (3) also pointed out the presence, in certain parathyroid preparations of a hypocalcemia-causing hormone which he named "Calcitonin". Carr, Aurbach and Potts (2) made the same observation. The presence of a hypocalcemia-causing hormone in the thyroid: thyrocalcitonin (Foster, Baghdiantz, Kumar, Slack, Soliman, MacIntyre, 1964 [5]) causes consideration of a risk of contamination by parts of the thyroid on the frozen parathyroids, commercially extracted which was the primary material of our preparations. Nevertheless, the simultaneous existence of a hypocalcemia-causing hormone and hypercalcemia-causing hormone in the parathyroid cannot be definitely excluded.

Conclusion

The activity of purified parathyroid hormone on calcemia is linked to the necessary presence in the animal diet of small quantities of vitamin D; the demonstration of this was done on parathyroidectomized rats and confirmed on guinea pigs and young chicks. By contrast, the activity of purified fractions on the blood or urinary phosphorus does not always seem to be linked to the presence of vitamin D.

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ACTIVITÉ PARATHYROIDIENNE ET VITAMINE D
OBSERVATIONS SUR LE RAT, LE POULET ET LE COBAYE

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INTRODUCTION

L'absence d'action hypercalcémiante d'un extrait parathyroïdien sur la calcémie du chien rachitique a été observée dès 1930 par MORGAN et GARRISON (16) par opposition à l'hypercalcémie observée sur le chien recevant de l'ergostérol irradié ou de l'huile de foie de morue. Cette notion de synergie d'action entre l'hormone parathyroïdienne et la vitamine D sur le métabolisme calcique s'est imposée au cours de ces dernières années. HARRISON et HARRISON (1964 (7), RAISZ et MARNAY, 1965 (24) ont discuté les observations qui ont conduit à cette notion.

In vitro, l'action stimulante de la parathormone (PTH) sur la libération du Ca ne s'observe que sur les mitochondries de foie de rat recevant de la vitamine D (DE LUCA, SALLIS, 1965 (4). La résorption osseuse sous l'influence de la parathormone dans les coupes de radius embryonnaire de rats est diminuée si le milieu contient du sérum de rat carencé en vitamine D (RAISZ [23]).

Par ailleurs, aux doses suboptimales de parathormone (PTH), l'addition de petites quantités de vitamine D renforce la résorption des calvaria de souris (GOLDHABER [6]).

L'existence de petites quantités de vitamine D dans certaines préparations de parathormone (MARNAY, GUERILLOT-VINET et RAOUL [13]), montre que l'interaction entre ces deux facteurs du métabolisme calcique est possible à l'insu de l'expérimentateur.

In vivo, la réponse hypercalcémiante à l'injection de parathormone est faible ou nulle en cas de carence en vitamine D. La dose suffisante pour restaurer cette réponse est très faible 0,05 UI = 6 (0,0075 µg) de calciférol pour le rat (RASMUSSEN, DE LUCA, ARNAUD, HAWKER et STEDINCK, 1963 [26]). Ceci expliquerait les contradictions relevées dans la littérature concernant le rat (TÖVERUD [30]), la souris (NICHOLS, SCHARTUM et VAES [19]), ou le chien (NEY, AC. KELLY, RADDE et BARTTER [18]). La faible hypercalcémie observée sous l'influence de PTH sur ces différents animaux privés de vitamine D serait due à la persistance de traces de cette vitamine dans leur organisme.

Cependant l'activité hypophosphorémiante de la parathormone *in vivo* ou la stimulation de la captation du phosphate par les mitochondries *in vitro* semblent indépendantes de la présence de vitamine D (26).

Dans ce travail, nous avons tenté de préciser le rôle de la vitamine D sur l'activité *in vivo* de la parathormone (de préparation courante ou purifiée) en variant les tests, les espèces animales et les régimes. Nous avons utilisé les tests classiques : prévention de l'hypocalcémie, de l'hyperphosphorémie et de la phosphaturie après parathyroïdectomie chez le rat carencé ou non en vitamine D. En outre, nous avons étudié les variations de la calcémie du poulet plus sensible au rachitisme que le rat et également celles du cobaye carencé de vitamine C, après administration de parathormone.

CONDITIONS EXPÉRIMENTALES

1. Animaux

Nous avons utilisé des rats mâles Wistar, des cobayes mâles de race tricolore et des poussins mâles de souche Hubbard.

2. Régimes

Le régime donné aux rats est celui de HARRISON, HARRISON et PARK (8) en remplaçant la levure de bière par un mélange vitaminique (18); la vitamine D3 est administrée suivant les précisions indiquées dans le texte à la dose de 2,5 µg par semaine et par rat. L'eau distillée et le régime sont donnés *ad libitum*.

Le régime du cobaye est celui de Th. TERROINE (29) avec le mélange salin de SOBER, MANNERING, CANNON, ELVEHJEM et HART (28). Les cobayes reçoivent ce régime avec un peu de verdure (carottes, salade) deux à trois jours après leur arrivée au laboratoire, puis on leur retire ce supplément. Les vitamines C ou D3 sont administrées suivant les groupes à la dose de 60 mg de vitamine C et 100 UI de vitamine D3, deux fois par semaine.

Les poussins juste éclos restent une journée sans nourriture (eau à volonté) et reçoivent ensuite le régime rachitigène de l'A.O.A.C. (20) pendant trois semaines, puis supplémenté par 7,5 p. 100 du mélange salin utilisé pour le régime des rats (WESSON [33]). Ils sont gardés à l'abri de la lumière du jour et chauffés avec une lampe à filament de carbone.

3. Prélèvements, technique opératoire

Le sang est prélevé par ponction cardiaque sous anesthésie légère à l'éther-chloroforme (9 v-1 v) chez le rat et le cobaye. Le prélèvement au cœur est fait sans anesthésie sur le poulet suivant la technique décrite par LÉPINE, CADILLON et CHAUMONT (10).

L'excrétion urinaire 6 heures après parathyroïdectomie est mesurée sur des groupes de 2 rats placés dans des cages à métabolisme en verre permettant la séparation des fécès et de l'urine.

La parathyroïdectomie est faite chez le rat par cautérisation large (atteignant ainsi environ le 1/4 de chaque lobe thyroïdien (HIRSCH, GAUTHIER, MUNSON, 1963 [9]). Chez le cobaye, on pratique une thyroparathyroïdectomie chirurgicale en ligaturant les artères thyroïdiennes. Ces interventions sont effectuées sous anesthésie à l'éther-chloroforme (9v-1v).

4. Dosages du Ca et du P

Les dosages de calcium sont faits sur 0,2 ml de sérum (sang recueilli sans anti-coagulant) après calcination au four électrique ($t < 600°$), puis reprise dans 5 ml d'une solution de chlorure de lanthane à 8,82 p. 100; la teneur en calcium est déterminée par absorption atomique (appareil Perkin Elmer 303) suivant le procédé décrit par ZETTNER et SELLIGSON, (34). Le phosphore sérique ou le phosphore urinaire sont déterminés par la méthode colorimétrique de BRIGGS adaptée suivant RAOUL et VINET (23).

5. Préparations hormonales parathyroïdiennes

La préparation commerciale utilisée est la Paratyrone BYLA titrant environ 100 USP par ml. Une préparation purifiée a été faite à partir de glandes congelées de bovidés suivant la méthode de RASMUSSEN, SZE et YOUNG, (27)

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ou celle d'AURBACH et POTTS, (1). Les séparations sur Sephadex G 100 ont été effectuées à + 4°. La densité optique mesurée à 265 mμ sert à identifier les peptides. La teneur en protéines est déterminée suivant la méthode de LOWRY, ROSEBROUGH, FARR et RANDALL, 1950 (11). Les fractions ainsi séparées sont lyophilisées et conservées à - 20°C. La figure 1 représente une courbe d'élution avec la désignation des peptides.

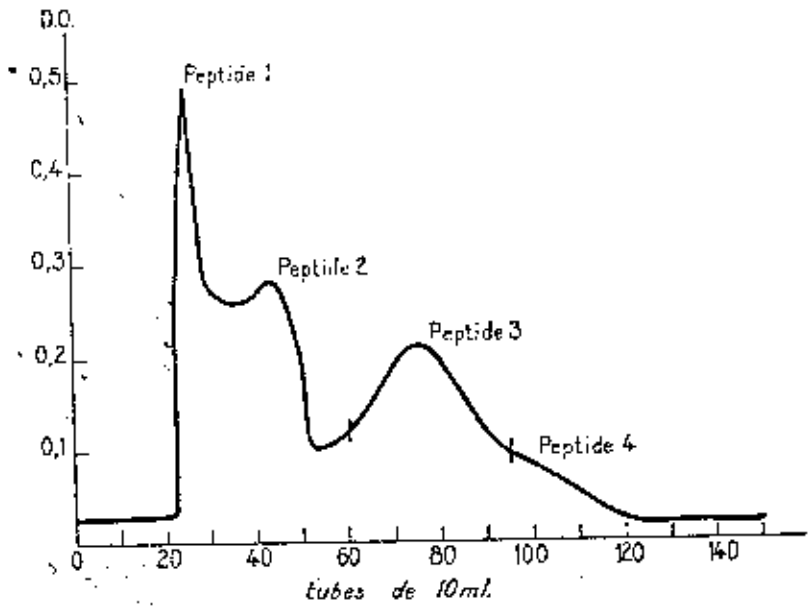

FIG. 1

Séparation sur Sephadex G 100 de la parathormone préparée selon RASMUSSEN, SZE, YOUNG, 1964 (27)

Les injections sont faites par voie sous-cutanée, le produit étant dissous dans l'eau distillée. Les animaux témoins subissent les mêmes prélèvements et reçoivent une injection d'eau distillée.

RÉSULTATS

1. Sur le rat parathyroïdectomisé

Les peptides séparés sur Séphadex ont été essayés par le test de MUNSON (17) séparément sur le rat parathyroïdectomisé carencé ou non en vitamine D (tableau I).

TABLEAU I

Activité des peptides parathyroïdiens séparés sur Sephadex sur le calcium sérique du rat 6 heures après parathyroïdectomie.

	Sans vitamine D	+ vitamine D3	P
Calcémie des témoins parathyroïdectomisés (mg Ca/l)	70	80	0,1
Variation après :			
Peptide 1 (1,4 mg)	- 9	- 32	0,05
Peptide 2 (3,6 mg)	1	+ 15	0,05
Peptide 3 (0,52 mg)	+ 4,5	15	0,1
Peptide 4 (0,06 mg)	- 6,5	- 12	0,1

Les peptides 1, 2 et 3 sont hypercalcémiants sur les rats recevant de la vitamine D mais pratiquement inactifs sur les animaux carencés.

L'étude des variations du métabolisme phosphoré suivant l'administration des mêmes fractions montre un certain degré de dissociation entre l'activité sur le calcium et sur le phosphore : en carence vitaminique D les peptides 1 et 3 provoquent une baisse importante du P sérique après parathyroïdectomie accompagnée d'une augmentation de l'excrétion urinaire de cet élément (tableau II).

TABLEAU II

Activité des peptides parathyroïdiens séparés sur Sephadex sur le phosphore urinaire et sérique du rat 6 heures après parathyroïdectomie.

	Sans vitamine D		+ vitamine D	
	P sérique	P urinaire en 6 heures	P sérique	P urinaire en 6 heures
	(mg/l)	(mg)	(mg/l)	(mg)
Témoins parathyroïdectomisés	150	1	131	4,9
Variation après :				
Peptide 1 (1,4 mg)	- 19 (*)	+ 1,8	- 25 (*)	+ 7
Peptide 2 (3,6 mg)	+ 4 (**)	- 0,03	- 28 (**)	+ 4
Peptide 3 (0,52 mg)	- 47 (***)	+ 2,3	- 14 (***)	- 1,4
Peptide 4 (0,06 mg)	- 10 (****)	+ 1	- 7 (****)	- 4,2

(*) P > 0,1 N.S.
(**) P < 0,05.
(***) P < 0,1.
(****) P > 0,1 N.S.

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2. Sur le poulet

Le régime de l'A.O.A.C. apporte 0,6 p. 100 de Ca et 0,68 p. 100 de P et dans ces conditions les poulets recevant 0,5 µg de vitamine D3 par jour ont une calcémie normale mais l'injection de 100 USP d'hormone parathyroïdienne n'a pas d'action hypercalcémiante (MARNAY, ALONAIS, RAOUL [12]), (tableau III).

TABLEAU III

Action de 100 USP de parathormone commerciale (PTH) sur la calcémie du poulet en fonction des taux de Ca, P et vitamine D du régime.

Régimes	Composition des régimes : p. 100 Ca	p. 100 P	UI	Calcémie initiale (mg/l)	3 heures après PTH	P
I	0,60	0,68	0	72,3 ± 3,3	− 6,9 ± 2,5	< 0,10
	0,60	0,68	0,5 µg	95,8 ± 2,7	1,1 ± 2,7	
II	1,86	0,68	0	75,9 ± 3	5,1 ± 1	< 0,005
	1,86	0,68	0,5 µg	106,9 ± 1,1	− 28,6 ± 0,9	
III	1,86	1,31	0	74,6 ± 1,8	+ 2,8 ± 1,8	< 0,005
	1,86	1,31	0,5 µg	97,7 ± 1	+ 23,2 ± 1,8	
IV	0,60	1,31	0,5 µg	105,7	− 12,2	NS

L'addition suffisante de calcium au régime pour atteindre le taux de 1,86 p. 100 permet d'observer une activité hypercalcémiante nette, mais seulement en présence de vitamine D3. L'addition simultanée de phosphate ne modifie pas cette réponse; par contre l'élévation du taux de phosphate sans modification de celui du calcium (régime IV) entraîne des réponses très dispersées, de 0 à + 28,7, en présence de vitamine D3, tandis que sans vitamine D les poulets meurent avant la fin de l'expérience.

La fraction nº 2 séparée sur Sephadex essayée comparativement sur les poulets carencés ou non en vitamine D en fonction du temps avec le régime III, (Ca 1,86 p. 100, P 1,31 p. 100) permet la même conclusion. (Tableau IV).

TABLEAU IV

Cinétique de l'activité de la fraction purifiée sur Sephadex G 100 de parathormone sur la calcémie du poulet carencé ou non en vitamine D (mg/l).

	1 h 30	3 h	4 h 30	6 h	P
Avec vitamine D3	+ 24 (3) (*)	+ 25 (6)	+ 21 (3)	+ 25 (5)	< 0,005
Sans vitamine D	− 5 (6)	− 1 (5)	− 1 (3)		

(*) Nombre d'essais.

3. Sur le cobaye

Avec le régime semi-synthétique, sans addition de foin et sans vitamine D, les cobayes présentent un certain degré d'hypocalcémie en 4 à 6 semaines (MARNAY, NOHIEM. (14) et l'injection de 200 USP d'hormone parathyroïdienne est pratiquement sans effet que l'animal soit thyroparathyroïdectomisé ou non. Par contre, chez les animaux recevant en plus de ce régime 200 UI de vitamine D3 par semaine, l'hypercalcémie est nette 24 h après injection de la même dose de parathormone. (Tableau V).

TABLEAU V

Action de 200 USP de parathormone sur la calcémie du cobaye carencé ou non en vitamine D, normal ou thyroparathyroïdectomisé (Thpx).

	Calcémie initiale	24 heures après 200 USP de parathormone	P
Non opéré sans D	93,4	− 3,4	< 0,05
Non opéré + D3	101	+ 17,6	
Thpx sans D	86,5	+ 6,8	< 0,05
Thpx + D3	91	+ 18,4	

DISCUSSION

1. L'importance de la vitamine D dans la mise en évidence de l'action hypercalcémiante de la parathormone a suscité de nombreux travaux qui ont été analysés presque simultanément par HARRISON et HARRISON, 1964 (7) et RAOUL et MARNAY (24). A cette époque les activités hypercalcémiantes observées sur des animaux privés de vitamine D ont été rapportées à la faiblesse de la dose de vitamine D nécessaire (RASMUSSEN et coll., 1963, loc. cit.) et à la difficulté d'obtenir des animaux totalement carencés. Les résultats que nous venons d'exposer confirment tout à fait cette notion. En effet, sur le rat et le cobaye, difficiles à carencer totalement en vitamine D, nous observons une activité hypercalcémiante résiduelle après injection de parathormone purifiée sur Sephadex (Tableau I, peptides 1 et 3) ou de parathormone com-

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merciale (Tableau V). Par contre, sur le poulet plus sensible à la carence en vitamine D, les deux types de préparation commerciale (Tableau III) ou purifiée (Tableau IV) sont tout à fait inactives. Soulignons qu'à la différence de la plupart des auteurs, nous avons toujours comparé les réponses d'animaux longuement stabilisés, carencés ou non, permettant ainsi des comparaisons moins sujettes à des variations cinétiques rapides.

Le manque de témoins comparables a en effet souvent affecté des résultats présentés comme contraires aux conclusions précédentes. Tel est le cas de l'activité hypercalcémiante observée sur le chien rachitique par NEY, AU, KELLY, RADDE et BARTTER (18) où la comparaison avec des chiens recevant le même régime supplémenté en vitamine D n'ayant pas été faite, il est impossible de conclure avec netteté. L'activité observée peut ici s'expliquer d'autant mieux par une carence insuffisante en vitamine D que la calcémie est peu abaissée par le régime employé et que la thyroparathyroïdectomie provoque une baisse sensible de la calcémie, indiquant une activité fonctionnelle importante des parathyroïdes de l'animal.

2. L'activité hypophosphorémiante semble par contre être relativement indépendante de la vitamine D quoique les résultats des divers auteurs ne soient pas concordants : par exemple HARRISON et HARRISON (7) n'observent pas d'action de la parathormone sur le phosphore sérique des rats carencés en vitamine D tandis que RASMUSSEN, DE LUCA, ARNAUD, HAWKER et VON STEDINCK (26) observent avec cette hormone une chute de l'hyperphosphorémie du rat parathyroïdectomisé carencé en vitamine D. En accord avec ces observations, nous avons obtenu une dissociation entre l'activité hypercalcémiante (annulée sans D) et hypophosphorémiante sur le rat parathyroïdectomisé, en particulier avec les peptides 1 et 3 (Tableau II).

3. L'existence d'une fraction principale active dans les meilleures conditions de préparation (AURBACH et POTTS) [1], n'exclut pas la dispersion de l'activité hormonale entre divers peptides séparés sur Sephadex par RASMUSSEN, SZE et YOUNG (27) ou nous-mêmes. La même dispersion s'observe avec les peptides hypophysaires (UPTON, LERNER et LANDE, 1966 [31]) et peut être rapportée à divers états d'agrégation plutôt qu'à des composés nettement différents. Dans cet ordre d'idées, RASMUSSEN, SZE et YOUNG (loc. cit.) avaient obtenu des peptides qui n'avaient pas la même activité relative sur la mobilisation du Ca et du P du rat parathyroïdectomisé, ni sur les mitochondries isolées *in vitro*. Cependant, malgré les différences d'activité de l'hormone sur le calcium et le phosphore discutées plus haut, l'existence de deux fractions hormonales distinctes n'est pas acquise et souligne donc l'importance de la vitamine D comme élément de différenciation.

4. Le test de l'action mitochondriale utilisé par certains auteurs peut d'ailleurs prêter à la critique quant à sa valeur pour l'évaluation de l'activité réelle de l'hormone parathyroïdienne. PASTAN (22) insiste sur le manque de spécificité de ce test : la protamine, par exemple agit comme la parathormone sur la captation du phosphate, la respiration, la libération calcique au niveau

mitochondrial. La protamine n'agit pas sur la calcémie du rat parathyroïdectomisé : test plus spécifique de l'action hormonale parathyroïdienne.

5. Un autre résultat mérite d'être souligné : l'existence d'une fraction hypocalcémiante dans une préparation de parathormone purifiée sur Sephadex (peptide 4, tableau I) décelée par son action sur la calcémie du rat parathyroïdectomisé recevant de la vitamine D.

Nous avions observé avec certaines préparations commerciales de PTH, une activité hypocalcémiante sur le chien ou le lapin (MARNAY, PRELOT [15]). COPP (3) signale aussi la présence, dans certaines préparations parathyroïdiennes, d'une hormone hypocalcémiante qu'il nomme « calcitonine ». CARR, AURBACH et POTTS (2) ont fait la même observation. La présence d'une hormone hypocalcémiante dans la thyroïde : la thyrocalcitonine (FOSTER, BAGHDIANTZ, KUMAR, SLACK, SOLIMAN, MAC INTYRE, 1964 [5]) fait peser un risque de contamination par des fragments de thyroïde des parathyroïdes de bovidés prélevées industriellement, matière première de nos préparations. Néanmoins, l'existence simultanée d'une hormone hypocalcémiante et d'une hormone hypercalcémiante dans la parathyroïde ne peut être encore définitivement exclue.

CONCLUSION

L'activité de la parathormone purifiée sur la calcémie est liée à la présence nécessaire dans le régime de l'animal de petites quantités de vitamine D; cette démonstration a été faite sur le rat parathyroïdectomisé et confirmée sur le cobaye et le jeune poulet. Par contre l'activité de ces fractions purifiées sur le phosphore sanguin ou urinaire ne paraît pas toujours liée à la présence de vitamine D.

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A Chick Serum Fraction that Stimulates Alkaline Phosphatase Activity in Embryonic Chick Femora in Culture

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A specific response of alkaline phosphatase activity of 6- to 7-day embryonic chick femora in organ culture to an active principle in chick serum has been demonstrated. The active principle appears to be a nonprotein molecule which is probably negatively charged, heat stable, base stable, acid labile, slowly dialyzable, charcoal adsorbable, and not hydrolyzed by *E. coli* phosphomonoesterase or by a proteinase of low substrate specificity.

Studies of the type of effect of the active principle on the enzyme activity indicate it is not a response to overall improved growth of the rudiment. The suggestion is made that the active principle may act as an inducer.

Studies of the effect of additions to or omissions from the control medium showed no alkaline phosphatase response to the absence of L-cysteine, the addition of proline, OH-proline, hexosamine, the amino acids nonessential for chick rudiment culture, inorganic pyrophosphate and ascorbic acid.

Studies reviewed by Biggers (1) and Kiemy (2) of the requirements of embryonic chick cartilage rudiments in culture have resulted in chemically defined media which makes it possible to use these organs for the determination of exogenous factors affecting their growth and differentiation. Although chemical media can be shown to supply the requirements for increase in length and DNA synthesis in cultured chick rudiments (3), the osteogenesis observed in mammalian limbs cultured on a completely-defined chemical medium (4, 5) cannot be observed in chick limbs cultured in media without a biologic supplement (6, 7). The appearance of the enzyme, alkaline phosphatase, precedes or is associated with osteogenesis in the course of normal development of limb cartilage into bone tissue (8). When the rudiments are removed from an embryo at the cartilage stage and are cultured on a completely natural medium or on a biologically-supplemented medium, alkaline phosphatase activity appears during the cultivation period (9) or increases in activity if present in the organ at the time of explantation (10). When the rudiments are cultured on a chemically defined medium, they fail to show the increased alkaline phosphatase activity exhibited by the supplemented rudiments although they resemble the supplemented cultures in outward appearance. This paper reports a study of the factor or factors in the biological supplement, chick serum, which stimulates the activity and/or development of alkaline phosphatase in cultured chick cartilage rudiments.

MATERIALS AND METHODS

Explants. The femora from 6- to 7-day chick embryos were isolated under a dissecting microscope in a few drops of Hank's solution. The excess soft tissue was removed by rolling them on a damp filter paper.

Culture technique. The rudiments were cultivated for 6 days by Chen's method for liquid media (11). They were incubated at 37° in humidified 5% carbon dioxide in air and the medium was changed every other day. The alkaline phosphatase response of the rudiments on a chemically defined medium and a biologically-supplemented medium were compared. The chemically defined medium was BGJb (12) without the antibiotics

and with 5 μg $ZnSO_4$ per 100 ml of medium added in some of the experiments. The biologically-supplemented medium was BGJb supplemented to a concentration of 25% of total medium volume with a fraction prepared as described below from dried reconstituted chick serum (Difco Laboratories, Detroit, Michigan). There were six rudiments per test group. The rudiments were distributed among the experimental groups such that size difference would be minimized and no rudiment from the same chick appeared in the same experimental group.

Measurement. At the end of the culture period each set of six rudiments was removed from culture, washed with Hank's solution, rolled on damp filter paper to remove excess tissue, and homogenized in an ice chilled Potter-Elvehjem glass homogenizer (13) in 1 ml of 0.001 M glycyl glycine buffer (pH 7.5). After homogenizing, 0.6 ml of the homogenate was transferred into 0.4 ml perchloric acid or trichloroacetic acid for DNA determination and 0.3 ml homogenate was used to determine alkaline phosphatase activity.

Alkaline phosphatase activity of the homogenates was determined by following the release of phenol from phenylphosphate by a modified King and Armstrong method (14). The assay consisted of incubating 0.3 ml homogenate together with 2.5 μmoles disodium phenylphosphate (Fischer Scientific Co., New Jersey), 25 μmoles glycine buffer (pH 9.6) and 5 μmoles $MgCl_2$ in a reaction volume of 0.7 ml for 30 min. The reaction was initiated by the addition of the reaction mixture to the homogenate after a 5 min preincubation of the homogenate and the reaction mix. The reaction was stopped by the addition of 5.0 ml 10% Na_2CO_3. One milliliter Folin-Ciocalteau reagent was added to each reaction tube. The color was read at 620 mμ after 10 min in a Bausch and Lomb Spectronic 20 colorimeter. The alkaline phosphatase activity was expressed as μmoles phenol released per μg DNA-P.

Lactic acid dehydrogenase was assayed by following the formation of DPNH from DPN and calcium lactate in a 1.0-cm silica cuvette for 15 min at room temperature (15). The reaction mixture consisted of 10 μmoles Tris(hydroxymethyl)-aminomethane buffer (pH 8.5), 20 μmoles calcium lactate (Cal. Biochem., Los Angeles, California), 0.5 μmoles DPN (Pabst Laboratories, Milwaukee, Wisconsin), and 0.4 ml homogenate. The enzyme activity was expressed as μmoles DPNH per μg DNA-P.

The DNA was extracted from 0.6 ml homogenate by heating in 5% trichloroacetic acid for 15 min at 90° as described by Schneider (16), or it was extracted with 10% perchloric acid at 70° for 20 min as described by Ogur (17). After extraction, the DNA was determined by the method of Ceriotti (18).

Serum fractionation. The chick serum was separated into 2 fractions on a 42 × 2.5-cm Sephadex G-25 (medium) column. Desiccated chick serum was reconstituted with deionized water. Five milliliters of the reconstituted serum was placed on the column and eluted with deionized water. The protein fraction was collected in 16 ml after passage of the void volume. The next 40 ml was collected as the small molecule fraction (SMF). The fractions were checked for 280 and 260 mμ absorption on the Beckman DU spectrophotometer and fractions were collected on this basis. The fractions were also checked for chloride ion to be sure that all the inorganic ions had passed through the column. The protein fraction was placed in a dialysis bag and the bag buried in Aquaride (Cal. Biochem.) overnight in the cold to remove the water. The protein was redissolved in 5 ml deionized water. The small molecule fraction was desiccated at room temperature in the vacuum, after which it was redissolved in 5 ml deionized water. The recombined fraction was prepared by mixing together 2.5 ml each of the redissolved protein and small molecule fractions, desiccating under vacuum and redissolving in 2.5 ml deionized water. The fractions were sterilized by passage through a Millipore filter with a pore size of 0.45 μ. The Millipore filter was extracted with hot deionized water and rinsed with cold water before use to remove the detergent from the filter (19).

For the sake of easier reference, the media will be abbreviated as follows through the paper. BGJb will mean unsupplemented BGJb. All supplemented BGJb will be referred to by its supplement. For example, BGJb supplemented with SMF will be referred to simply as SMF.

RESULTS AND DISCUSSION

Specific Effect of Chick Serum on Alkaline Phosphatase Activity

Figure 1 shows the alkaline phosphatase activity of cartilage rudiments cultured for six days on BGJb supplemented to a concentration of 25% of total medium volume with bovine serum albumin (General Biochemicals, Chagrin Falls, Ohio), bovine serum (Difco Laboratories), or chick serum. The bovine serum albumin solution was prepared to contain the same protein concentration as an equal amount of chick serum. Chick serum has at least two times the effect on alkaline phosphatase activity as does

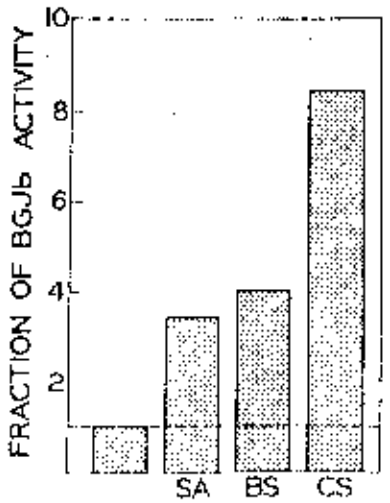

FIG. 1. The effect of serum albumin, bovine serum and chick serum supplements to BGJb on alkaline phosphatase activity of 6- to 7-day chick embryo femora grown in culture. Each bar represents a single determination of a homogenate of six femora. The specific alkaline phosphatase activity of the rudiments cultured on the various media is expressed as the fraction of the activity on the control BGJb. SA (serum albumin); BS (bovine serum); CS (chick serum).

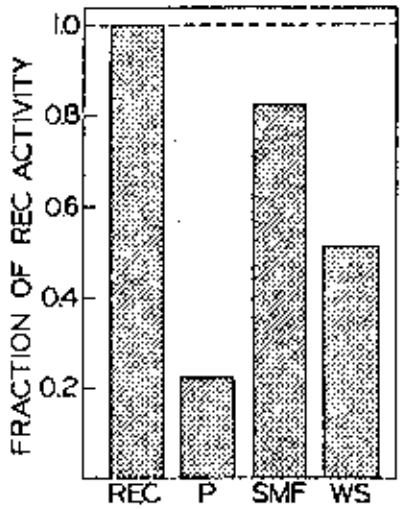

FIG. 2. The effect of Sephadex G-25 chick serum fractions on the alkaline phosphatase activity of 6- to 7-day chick embryo femora cultivated *in vitro*. Each bar represents the mean of single determinations of two separate homogenates of 6 femora. The specific alkaline phosphatase activity of the rudiments cultured on the Sephadex fractions is expressed as the fraction of the activity on the recombined-fraction medium. REC (recombined serum fractions); P (protein fraction); SMF (small molecule fraction); WS (whole serum).

bovine serum or serum albumin, while BGJb alone resulted in very little enzyme activity.

Determination of Sephadex G-25 Fraction Affecting Alkaline Phosphatase Activity

The serum was separated into two fractions on a Sephadex G-25 column as described in the methods section. Equal amounts of these fractions and the recombined fraction were assayed for their effect on the alkaline phosphatase activity of the rudiments. The results (Fig. 2) show that the small molecule fraction (SMF) has the greatest effect on the alkaline phosphatase activity. The SMF is not significantly different in effect from the activity of the recombined fraction but is more effective than the whole serum. This does not appear to be due to enzymatic degradation in the whole serum of the active principle necessary for the alkaline phosphatase activity, because when the whole serum is incubated aseptically at 37° for as long as 24 hr before fractionation there is only a slight decrease in the alkaline phosphatase response to the SMF.

Studies of the Alkaline Phosphatase Response to the Small Molecule Fraction

If the rudiments are exposed to the small molecule fraction for a short period of time, rinsed thoroughly then transferred to BGJb for the remainder of the culture period, there is no response to the SMF after 2 or 24 hrs exposure (Fig. 3). However if the rudiments are exposed to the SMF for 4 days then cultured on BGJb for either 2 or 4 days more, the alkaline phosphatase activity is the same as that of the rudiments cultured continually in the presence of the SMF.

The rudiments which were exposed to the SMF for 24 hrs or 4 days had a DNA content equal to that of the rudiments cultured in the presence of SMF for the entire culture period (Table I). Since the alkaline phosphatase

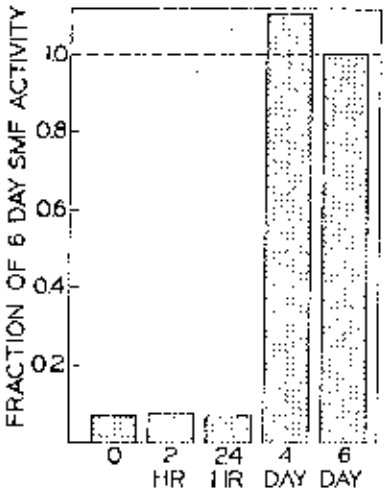

FIG. 3. Effect of short time exposure of rudiments to SMF on the alkaline phosphatase response of 6- to 7-day chick embryo femora *in vitro*. Each bar represents the mean of single determinations of two separate homogenates of six femora. The specific alkaline phosphatase activity of the rudiments exposed to SMF for shorter lengths of time is expressed as the fraction of the activity of the rudiments exposed to SMF for the entire culture period. Length of time of exposure to SMF is given beneath the graph.

TABLE

DNA CONTENT OF CHICK EMBRYO FEMORA AFTER SHORT TIME EXPOSURE TO SMF[a]

Length of SMF exposure	DNA (μg DNA-P)
24 hr	2.8
	3.0
4 day	2.6
	2.8
6 day	2.3
	2.2
	2.9

[a] DNA was determined on 0.6 ml of a homogenate of six femora as described in the Methods section.

response did not appear in the 24-hr exposure to the SMF but did in the 1 day period, this tends to dissociate the response from a growth effect. Another factor which supports this dissociation is the fact that the rudiments continue to show the response 2–4 days after the removal of the SMF, although it appears from the lack of response to short-term exposure that the rudiment cannot store the active principle. Also another enzyme, lactic acid dehydrogenase, was assayed in the rudiment homogenate prepared from rudiments cultured in the presence or absence of SMF. The response of this enzyme to the SMF was about one twelfth that of the alkaline phosphatase response (Fig. 4) indicating a more specific response of the alkaline phosphatase rather than an overall growth response to the SMF.

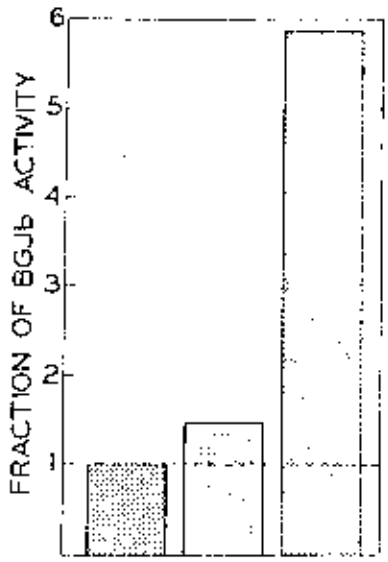

FIG. 4. The effect of SMF on lactic acid dehydrogenase activity of 6- to 7-day chick embryo femora cultured *in vitro*. The first two bars represent the mean of single determinations of two to three separate homogenates, the third bar, one homogenate of six femora. The specific alkaline phosphatase activity of the rudiments cultured on SMF is expressed as the fraction of the activity of the rudiments cultured on BGJb. BGJb (hatched bar); lactic dehydrogenase (stippled bar); alkaline phosphatase (striped bar).

The lack of response to very short time exposure to SMF and the fact that the greatest response occurs after 4 days in culture (Fig. 5) indicate that the alkaline phosphatase activity is not the result of a direct enzyme activation but that it may be due to an indirect action of the SMF. The time course of alkaline phosphatase appearance (Fig. 5) indicates a continued increase in ac-

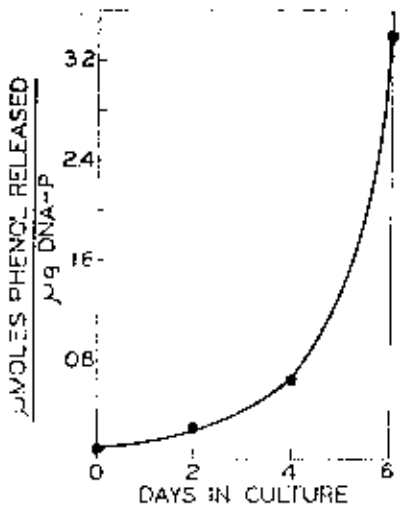

FIG. 5. The time course of appearance of alkaline phosphatase activity of 6- to 7-day chick embryo femora grown on SMF medium for six days. Each point represents a single determination of one homogenate of six femora.

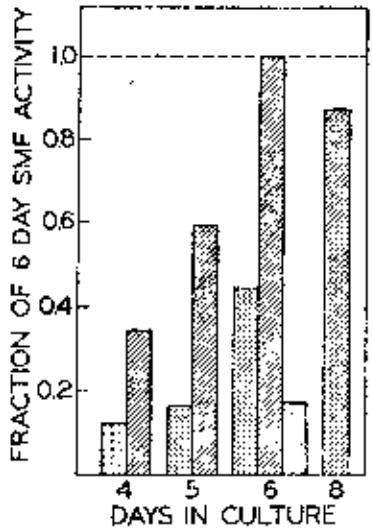

FIG. 6. The effect of culturing rudiments on BGJb for 4 days, then supplementing with SMF for 2-4 days more, on the alkaline phosphatase response of 6- to 7-day chick embryo femora *in vitro*. Each bar represents the mean of a single determination of two separate homogenates or the determination of one homogenate of six femora. The specific alkaline phosphatase activity of the rudiments cultured on BGJb or SMF for the duration of the culture period or cultured on BGJb for 4 days then transferred to SMF, is expressed as the fraction of the activity of the rudiments cultured on SMF for 6 days. BGJb (stippled bar); SMF (striped bar); BGJb 4-day then transferred to SMF (hatched bars).

tivity over the culture period with the greatest increase between the fourth and sixth day in culture. This time course of activity could suggest that the SMF is causing an acceleration of cartilage maturation. Histological examination of the rudiments shows the BGJb-cultured rudiments consisting of a greater number of immature cartilage cells while the SMF-supplemented rudiments show increased hypertrophy of the cartilage cells. A similar effect of serum on cartilage differentiation has been shown by H. Ende (20) who concluded on the basis of histological studies of 9 day chick embryo femora cultured on a medium consisting of embryo extract, horse serum and Gey's saline, that serum favored the proliferation and hypertrophy of cartilage cells. It is an open question whether or not maturation of cartilage is functionally associated with alkaline phosphatase or whether the two events independently occur at the same time in the course of differentiation or if one precedes the other. Whatever the case, the two events are associated and induction of alkaline phosphatase with a resulting acceleration of cartilage maturation cannot be eliminated.

The lack of response to short term SMF exposure suggests the possibility that the rudiments may have to reach a certain developmental state, after which they may show an immediate response to SMF similar in size to that seen between 4 and 6 days on SMF. In view of the large increase in enzyme activity after day 4, the rudiments were cultured on BGJb for 4 days before the addition of the SMF then washed and transferred to a medium containing SMF. Figure 6 shows that the pattern of alkaline phosphatase response was the same in the rudiments cultured for 4 days on BGJb before the addition of SMF as it was for the rudiments cultured on SMF from the day of explantation. There is no response of the rudiments cultured for 4 days on BGJb followed by 24 hr on BGJ plus SMF. However, the alkaline

phosphatase activity increases on the second day after SMF addition and increases more rapidly than in the rudiments cultured only on SMF-supplemented BGJb. This indicates that the response is dependent on the stage of development of the rudiment but that it cannot be made an immediate (24-hr) response to SMF even when the rudiments are at an older culture age at the time the SMF is added. It appears that if this is an induced response, the inducer must be present for at least 4 days of *in vitro* growth in order to see the full response.

Effect of Omissions from or Additions to BGJb on the Alkaline Phosphatase Activity

In pursuit of the answer to the question about possible nutrition effects, compounds related to cellular metabolism were added to BGJb and assayed for effect on alkaline phosphatase activity. A possible inhibitor and a possible stimulator of alkaline phosphatase activity were also examined. The concentrations were chosen so as to approximate the amount which would be contained in the SMF.

Since L-cysteine was reported by Cox to repress alkaline phosphatase in human cell cultures (21), the L-cysteine was omitted from BGJb. There was no difference in alkaline phosphatase activity in the rudiments when cultured in the presence or absence of L-cysteine.

The nonessential amino acids omitted from BGJb (aspartic acid, serine, proline, OH-proline, citrulline, glycine, alanine) were added back in a group at concentrations of each amino acid of 8, 16, and 24 mg/100 ml of medium. There was no significant increase in alkaline phosphatase response at any of the three concentrations. However, the balance between the amino acids is important so it cannot be unequivocally stated, on the basis of such an incomplete study, that none of these amino acids are involved in the alkaline phosphatase response, although these amino acids have been shown to be synthesized from glucose by embryonic chick tibiotarsi (22) and should not have to be supplied by the medium.

Because of the relationship of proline, OH-proline and hexosamine to collagenous bone matrix, these compounds were added individually to the medium. Proline (12.5 μg/ml of medium), OH-proline (15 μg/ml of medium) and galactosamine (10 μg/ml of medium) showed no effect beyond that of control BGJb.

It has been shown that inorganic pyrophosphate and a long chain polyphosphate slightly activated mineralization of 9-day chick femora at low concentrations but were inhibitors at higher concentrations (23). This suggested that alkaline phosphatase could act to remove the inhibitory effect by hydrolyzing the polyphosphate. Perhaps then, a concentration of polyphosphate above a certain level could cause an increase in the activity of alkaline phosphatase. With this in mind, sodium pyrophosphate (0.2 and 10 μg/ml of medium) was added to BGJb. No effect on alkaline phosphatase was observed at either concentration.

Ascorbic acid has been shown by Reynolds to stimulate the synthesis of collagen in embryonic chick bone rudiments (24, 25). BGJb with 17 μg/ml ascorbic acid added showed no increase in alkaline phosphatase activity of the cultured rudiments. The concentration of ascorbic acid used is lower than that used by Reynolds but it is nearer that concentration which would be added to the medium in the SMF.

Studies of the Characteristics of the Small Molecule Fraction

The stability of the active principle to heating at 90°F for 15 or 30 min or to boiling for 2 min is shown in Fig. 7. The active principle appears to be quite stable to heating as there is no significant difference between the alkaline phosphatase activity of the rudiments cultured on the heat-treated SMF and the untreated SMF.

Figure 8 shows the average results of SMF exposure at room temperature to acid (pH 1 or 2) or to base (pH 8, 12). The SMF was titrated to the given pH with HCl or ammonium or sodium hydroxide. The HCl and ammonia were removed by vacuum desiccation. Exposure time was 1 hr or more. When

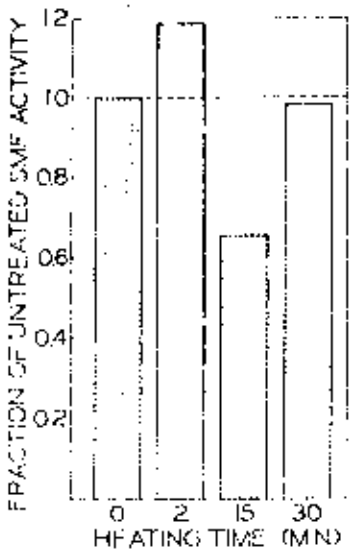

FIG. 7. The effect of heating the small molecule fraction on the alkaline phosphatase response of 6- to 7-day chick embryo femora cultivated *in vitro*. Each bar represents the mean of single determinations of two separate homogenates of six femora. The specific alkaline phosphatase activity of the rudiments cultured on the heat-treated SMF is expressed as the fraction of the activity of the rudiments cultured on untreated SMF. The fraction of the alkaline phosphatase activity of the rudiments cultured on BGJb to that of the rudiments cultured on SMF was 0.00. No heat treatment (hatched bars); boiled (stippled bars); heat at 60° (striped bars).

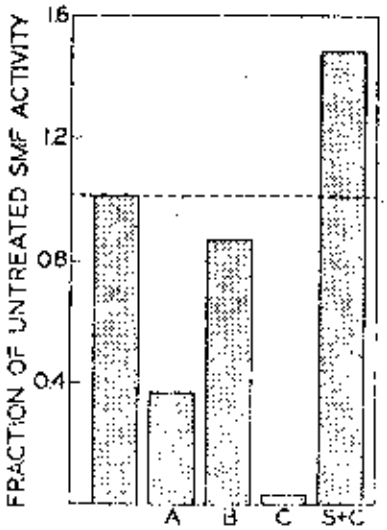

FIG. 8. The effect of acidification, alkalinization and combustion of the SMF on the alkaline phosphatase response of 6- to 7-day chick embryo femora *in vitro*. Each bar represents the mean of single determinations of three to five separate homogenates of six femora. The specific alkaline phosphatase activity of the rudiments cultured on acid-treated, base-treated or combusted SMF is expressed as the fraction of the activity of the rudiments cultured on untreated SMF. The fraction of the alkaline phosphatase activity of the rudiments cultured on BGJb to that of the rudiments cultured on SMF was 0.04. Acid treated (A); base treated (B); combusted (C); SMF plus combustion ash (S + C).

the NaOH was used, the SMF was back-titrated to neutral pH after 6 hr. For the same length of time in acid or base, the active principle was consistently labile to acid but not significantly affected by base.

The SMF was passed through a DEAE cellulose column and a carboxymethyl cellulose column in an attempt to determine its ionic characteristics. After eluting the columns with water, it was found that the active principle was adsorbed on the anion exchange column but not on the cation exchanger. Two groups of cultures showed the cation exchange column effluent to be four times as effective in stimulating alkaline phosphatase activity as the anion exchange column effluent and to be equally as effective as the untreated SMF. The DEAE column effluent was similar in effect to that of the control BGJb. This indicates that the active principle is probably negatively charged.

Three milliliters of the SMF was ashed in a platinum crucible for 10 min at the minimum temperature required for combustion of the organic compounds. The completeness of combustion of organic compounds was determined by Johnson's method for measuring organic matter (26). The resulting ash was redissolved in 3 ml deionized water, sterilized, and added to the culture medium. Figure 8 shows the destruction of the effectiveness of the SMF on alkaline phosphatase activity as a result of ashing. Addition of the SMF ash to untreated SMF shows the ash to be noninhibitory. These results indicate that the active principle is either an organic or an inorganic compound which is volatilized under combustion conditions.

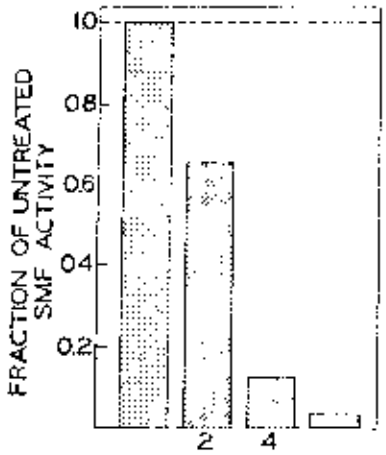

Fig. 9. The effect of dialysis of the SMF or its passage through highly activated charcoal on the alkaline phosphatase response of 6- to 7-day chick embryo femora *in vitro*. Each bar represents the mean of single determinations of two to three separate homogenates of six femora. The specific alkaline phosphatase activity of the rudiments cultured on the dialyzed or charcoal-treated SMF is expressed as the fraction of the activity of the rudiments cultured on untreated SMF. The untreated SMF of the charcoal experiment contained the water effluent of a blank charcoal column. The fraction of the alkaline phosphatase activity of the rudiments cultured on BGJb to that of the rudiments cultured on SMF was 0.12. Untreated SMF (hatched bar); dialyzed SMF (striped bars); charcoal column treated SMF (stippled bars). Time (hours) of dialysis is given below the graph.

A small inorganic molecule would pass through a dialysis bag quite readily compared to a larger organic molecule. Dialysis of 4 ml of the SMF against 400 ml distilled water for 2 hr in the cold resulted in about 35% reduction of the effect on alkaline phosphatase activity (Fig. 9). Further dialysis to a total of 4 hr resulted in complete loss of the alkaline phosphatase response. A previous dialysis study in which 280 mμ absorption of the SMF was observed before and after 2 hr dialysis showed an 86% decrease in OD as a result of dialysis. Ninhydrin-positive compounds passed through the dialysis bag within 1 hr showing that the amino acids and probably the other small molecules had been removed. The 280-mμ data indicate the loss of 86% of the aromatic containing compounds such as small peptides or other large ring containing compounds in about 2 hr. The length of time required for complete loss of effectiveness of the SMF would seem to indicate a large molecule. The Sephadex column filtration does not eliminate the possibility of the SMF containing large peptides, because basic amino acids and aromatic amino acids are known to be adsorbed to the Sephadex when eluting with water. In fact, a 260-, 280-mμ profile of the SMF show an adsorption peak about 15–20 ml beyond the protein fraction where the inorganic ions are already being eluted. If a peptide had a predominant number of the aromatic or basic amino acids, or both, it would come off the column in the SMF and account for the slow dialysis pattern.

Another effort to elucidate the nature of the active principle of the SMF was to process the SMF through a highly activated charcoal column. Upon passing 3 or 4 ml of SMF through a 1 × 1 cm Nuclear column and assaying the 12 ml water effluent, no effect on alkaline phosphatase activity was observed in four experiments (Fig. 9). Furthermore, elution of the column with 25 ml of 0.25, 1, or 10% ammonia failed to remove the active principle from the column. Addition of the ammonia effluent to the untreated SMF did not inhibit the SMF activity, indicating that no compounds deleterious to the alkaline phosphatase activity were being washed from the column. Highly activated charcoal has been reported to adsorb nonpolar compounds such as ring compounds, but larger straight-chain molecules are also adsorbed with increasing adsorption per length of carbon chain (27). These data indicate the active principle to be a carbon containing compound, possibly long-chain or a ring-containing compound, either of which would be adsorbed on nonpolar charcoal.

The probable negative charge of the active principle, its acid lability, organic nature, and the possibility that it would be a substrate for a phosphatase, suggested that it may be a phosphate ester. To test this, the stability of the active principle to *E. coli* phosphomonesterase was investigated. There was no significant difference between the effect of heat inactivated enzyme controls or

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active enzyme on the active principle. Similarly, a proteinase (*Streptomyces griseus*) of low substrate specificity was used to determine if a protein was involved in the SMF effect on alkaline phosphatase. No significant effect of proteolysis of the SMF was observed.

The characterization data suggests a non-protein, organic molecule. Its slow dialysis property indicates a comparatively large molecule and its adsorption to charcoal suggests a ring containing or long-chain carbon compound. Secondary or tertiary structure can probably be eliminated on the basis of its heat stability. It is base stable, acid labile and probably negatively charged. Elimination of more than one active component of the SMF is not possible with the present data.

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VITAMIN D AND MAGNESIUM ABSORPTION[1]

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The effect of vitamin D on the absorption of Mg is of more than passing interest, for while Day and McCollum ('39) found that 85% of ingested Mg was absorbed by rats on a low P ration, it has been assumed generally that it is poorly absorbed. (Mendel and Benedict, '09; Schmidt and Greenberg, '35; Tibbetts and Aub, '37; Leichsenring et al., '51.) This conclusion was based on results obtained with other animals than the rat and with other than purified diets. The role of vitamin D cannot be assessed from the published data. In the experiments of Day and McCollum vitamin D was fed to all of the rats used in their experiments. Outside of the data obtained in metabolic trials the published data on the Mg content of tissues also are not helpful in determining the effect of vitamin D. While the Mg content of rat bones has been found to be increased in rickets (Gassmann, '10; von Euler and Rydbom, '31) the amount in the blood serum apparently is reduced (Bomskov and Kruger, '31).

Our interest in the effect of vitamin D on Mg absorption originated through our concern over the possible modifying effect of changes in Mg absorption on Ca and P balances when vitamin D was given. An ionic antagonistic effect of Ca and Mg is well recognized and as some of our observed effects of vitamin D were not those ordinarily attributed to this vitamin

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we have sought to ascertain if the Mg content of the diet could be a factor. On the supposition that differences in the published reports of the availability of Mg might have been arrived at with rations differing in P content or in the nature of the P in the rations some experiments were carried out with semi-synthetic rations in which P was added as inorganic phosphate or as phytic acid under acidic as well as basic conditions.

EXPERIMENTAL METHODS

Ninety-gram rats of the Sprague-Dawley strain were placed in metabolism cages for a 12-day period, during the last 9 days of which urine and feces were collected. Daily, for three or more half-hour periods, the rats were transferred for feeding to similar cages provided with sub-bottoms of filter paper to absorb any urine voided and to catch feces. At the end of the balance period the feces excreted in these cages were added to those collected in the metabolism cages and the filter papers, after brushing off any spilled food, were eluted with hot normal hydrochloric acid and rinsed twice with hot water. These elutions and rinses were added to the urine collections.

The food consumption was limited to 20 gm per rat for each three-day period in such a manner that this allotment lasted throughout the period and yet was consumed completely. This amount supported only very limited growth but this equalization of food intake eliminated difficulties due to variations in appetite and in the resultant mineral intake.

The basal ration was a modification of the semi-synthetic diet of Steenbock et al. ('51). It was a low P (0.016%), low Ca (0.012%), low Mg (0.02%) ration consisting of Cerelose (D-glucose monohydrate) 67, cooked egg white 18, roughage[2] 3, cottonseed oil[3] 10, and Ca-P-Mg-free salts 2. In this ration the level of magnesium has been reported to supply 4 times

[2] Ruffex.
[3] Wesson.

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the minimum needs of the rat for growth, reproduction and lactation (Schmidt and Greenberg, '35).

Crystalline vitamins were added to the ration in a mixture with glucose. One gram of the mixture per kilogram of ration provided the following number of milligrams of the respective vitamins; thiamine hydrochloride, 4; riboflavin, 5; pyridoxine, 5; calcium pantothenate, 28; nicotinamide, 10; p-aminobenzoic acid, 200; and inositol, 200. Choline chloride was added to the ration at a level of 500 mg per kilogram of ration as an alcoholic solution sprayed on the Cerelose before the ration was mixed.

A supplement of 70 μg of β-carotene, 105 μg of 2-methyl-1,4-naphthoquinone, and 875 μg of α-tocopherol in cottonseed oil was supplied each week in three doses. Vitamin D, when given, was administered orally at the rate of 75 I.U. per three-day period as crystalline calciferol dissolved in cottonseed oil.

The salt mixture used by Steenbock et al., ('51) was modified by the omission of the Mg ingredient. Mineral additions to the basal diet were made at the expense of the Cerelose. Magnesium phosphate of the desired Mg/P ratio was prepared by dissolving $MgCO_3$ in standardized phosphoric acid, drying and grinding. The Mg phytate was prepared from a technical calcium phytate preparation[4] by removal of the Ca and precipitation with $MgCO_3$. Two preparations of varying Mg/P ratio were made by precipitating them respectively at a pH of 5 and 8. The former, later referred to as acidic Mg phytate, contained 8.3% Mg and 12.7% P while the latter, referred to as basic Mg phytate, contained 15.2% Mg and 8.0% P. The basic preparation contained an excess of $MgCO_3$ over that which could react with the phytic acid in the solution. The amount of Mg salts which could be fed was limited by their cathartic effect. Since a level of 0.24% Mg was the maximum which could be fed, the amount included in the diet was limited to 0.12% to avoid all danger of the incidence of diarrhea. Water was supplied for ad libitum consumption.

[4] Staley.

The urines after concentration and the feces as obtained were wet-ashed with nitric acid followed by perchloric acid. Inorganic P was determined essentially according to the method of Fiske and Subba Row ('25) while phytic acid was determined by the method of Pringle and Moran ('42). Mg was determined by an analysis for P in the magnesium ammonium phosphate precipitated after the removal of Ca as the oxalate.

RESULTS

The data in table 1 reveal that vitamin D increased Mg absorption. While the increase is small, never exceeding 19% of the intake which perforce had to be limited to 7 to 8 mgs daily, it was obtained consistently in 12 separate trials entailing the use of 170 rats. There is no evidence that this increase was the result of an increase in Mg requirements due to a stimulation of growth, as growth was increased inconsistently and, when it did occur, the increase was small, undoubtedly because of the limitation in food intake. Assessed on the basis of normal Mg requirements (Schmidt and Greenberg, '35) the intake of our animals exceeded this by a multiple of 20. Furthermore, as shown in table 2, the decrease in fecal excretion was more than compensated for by an increase in the urine so that the retention of Mg was actually decreased when vitamin D was given.

It is apparent from our data that Mg was absorbed readily even when given as the phosphate or phytate or under very basic conditions such as were provided by additions of 1.0% $CaCO_3$ and 2.6% $NaHCO_3$. Obviously the retention was limited with the carbonate as the phosphorus content of the basal ration was too low to meet the requirements of tissue growth but when P was provided either as Mg phosphate or as Mg phytate retention became possible. The greatest retention occurred at the highest level of P intake, namely, at 0.34% of the ration when Mg was furnished as the phosphate.

TABLE 1

The effect of vitamin D on magnesium absorption

ADDITIONS TO THE BASAL RATION [1]	INCREASE IN WEIGHT OF RATS IN 9 DAYS		Mg ABSORBED [2]		DIFFERENCE
	− D	+ D	− D	+ D	
	gm	gm	%	%	%
None	13	19	67.6	69.7	2.1
$MgCO_3$	19	15	60.2 (± 2.4)	68.3 (± 2.6)	8.1
$MgCO_3$ + 1.0% $CaCO_3$ + 2.6% $NaHCO_3$	9	11	71.3 (± 3.2)	76.7 (± 2.9)	5.4
Mg phosphate	7	7	58.5 (± 4.0)	66.5 (± 3.6)	8.0
Acidic Mg phytate	7	8	52.9 (± 3.4)	62.4 (± 2.9)	9.5
Acidic Mg phytate + 1.0% $CaCO_3$	11	8	58.0 (± 9.5)	61.2 (± 1.7)	3.2
Acidic Mg phytate + 0.42% $MgCO_3$	7	12	63.4 (± 2.1)	66.4 (± 2.9)	3.0
Basic Mg phytate	12	11	59.3 (± 2.8)	68.1 (± 2.3)	8.8

[1] Basal ration was low in P (0.016%), Ca (0.012%), and Mg (0.02%). Magnesium additions were all made at a level equivalent to 0.42% $MgCO_3$.

[2] Figures in parentheses give standard error of the mean calculated as follows: $\frac{\sqrt{\frac{\Sigma d^2}{n}}}{\sqrt{n}}$ where d is the deviation from the mean and n is the number of observations. Each group consisted of 6 rats except those fed the basic Mg phytate ration which [illegible] 12.

TABLE 2

The effect of inorganic and phytic acid phosphorus on the absorption and excretion of magnesium

ADDITIONS TO BASAL RATION	ADDITION OF VITAMIN D	NO. OF RATS	DAILY INTAKE	DAILY EXCRETION [1]			DAILY BALANCE
				Feces	Urine	Total	
	25 i.u./day		mg	mg	mg	mg	mg
$MgCO_3$ (0.016% P)	No	3	7.58	2.92	4.27	7.19	+ 0.39
	Yes	3	7.72	1.98	5.81	7.79	− 0.07
Mg phosphate (0.34% P)	No	3	7.32	2.46	1.36	3.82	+ 3.50
	Yes	3	7.41	2.09	2.30	4.39	+ 3.02
Mg phosphate (0.14% P)	No	6	7.08	2.94 (± 0.23)	3.07 (± 0.23)	6.01 (± 0.37)	+ 1.07
	Yes	6	7.20	2.41 (± 0.25)	4.62 (± 0.15)	7.03 (± 0.07)	+ 0.17
Mg phytate (0.14% P)	No	6	8.13	3.83 (± 0.16)	3.59 (± 0.16)	7.42 (± 0.11)	+ 0.71
	Yes	6	8.20	3.08 (± 0.14)	5.02 (± 0.10)	8.10 (± 0.07)	+ 0.10
Mg phytate + 1.0% $CaCO_3$ (0.[illegible]% P)	No	6	8.42	3.64 (± 0.57)	3.96 (± 0.51)	7.60 (± 0.44)	+ 0.82
	Yes	6	8.06	3.13 (± 0.15)	4.68 (± 0.12)	7.81 (± 0.26)	+ 0.25

[1] The standard error of the mean is given in parentheses except where pooled samples were used.

TABLE 3

The effect of magnesium, calcium, and phytic acid on phosphorus balances

ADDITIONS TO BASAL RATION	ADDITION OF VITAMIN D	DAILY INTAKE	DAILY EXCRETION [1]			DAILY BALANCE
			Feces	Urine	Total	
	25 i.u./day	*mg*	*mg*	*mg*	*mg*	*mg*
Mg phosphate	No	9.05	2.82 (± 0.16)	2.96 (± 0.40)	5.78 (± 0.65)	+ 3.27
(0.12% Mg, 0.14% P)	Yes	9.20	1.86 (± 0.14)	4.36 (± 0.33)	6.22 (± 0.24)	+ 2.98
Mg phytate	No	9.44	4.65 (± 0.10)	2.00 (± 0.30)	6.65 (± 0.35)	+ 2.79
(0.12% Mg, 0.14% P)	Yes	9.53	3.03 (± 0.21)	3.05 (± 0.33)	6.08 (± 0.38)	+ 3.45
Mg phytate + 1.0% $CaCO_3$	No	9.60	7.41 (± 1.35)	0.09 (± 0.02)	7.50 (± 1.33)	+ 2.10
(0.12% Mg, 0.14% P)	Yes	9.19	5.06 (± 0.26)	0.20 (± 0.06)	5.26 (± 0.21)	+ 3.93
Mg phytate	No	6.58	3.64 (± 0.35)	1.11 (± 0.27)	4.75 (± 0.12)	+ 1.83
(0.12% Mg, 0.11% P)	Yes	6.89	1.47 (± 0.38)	0.59 (± 0.20)	2.06 (± 0.59)	+ 4.83
Mg phytate + $MgCO_3$	No	6.70	3.89 (± 0.27)	0.79 (± 0.23)	4.68 (± 0.17)	+ 2.02
(0.20% Mg, 0.11% P)	Yes	7.22	1.88 (± 0.42)	1.26 (± 0.27)	3.14 (± 0.34)	+ 4.08

[1] The standard error of the mean is given in parentheses. All groups consisted of 6 rats.

TABLE 4

The effect of magnesium and calcium on the hydrolysis of phytic acid

ADDITIONS TO BASAL RATION	ADDITION OF VITAMIN D	DAILY PHYTATE P INTAKE	DAILY FECAL EXCRETION [1]			PHYTATE HYDROLYSIS
			Phytate	Inorganic	Other	
	25 i.u./day	*mg*	*mg*	*mg*	*mg*	%
Mg phytate	No	9.44	1.25	2.50	0.51	86.7
(0.12% Mg, 0.14% P)	Yes	9.53	0.59	1.32	0.52	90.7
Mg phytate + 1.0% $CaCO_3$	No	9.60	4.82	2.39	0.57	49.8
(0.12% Mg, 0.14% P)	Yes	9.19	2.28	1.66	0.72	75.2
Mg phytate	No	6.58	0.93 (± 0.12)	1.40 (± 0.15)	1.08 (± 0.31)	85.9
(0.12% Mg, 0.11% P)	Yes	6.89	0.21 (± 0.08)	0.69 (± 0.16)	0.60 (± 0.26)	96.9
Mg phytate + $MgCO_3$	No	6.70	0.89 (± 0.11)	1.77 (± 0.14)	1.35 (± 0.34)	86.7
(0.20% Mg, 0.11% P)	Yes	7.23	0.37 (± 0.12)	0.91 (± 0.15)	0.68 (± 0.23)	94.9

[1] The standard error of the mean is given in parentheses except where pooled samples were used. All groups consisted of 6 rats.

The phosphate balances in 4 out of 5 experiments (table 3) reveal a very definite increase in P retention when vitamin D was given, the main import of which, together with the evidence on phytic acid hydrolysis (table 4) is: that phytic acid given as Mg phytate was available as a source of P in the absence of vitamin D even when its hydrolysis was depressed to 49.8% of the total intake by the addition of 1% $CaCO_3$. When fed without $CaCO_3$ the hydrolysis of added phytate was almost as great in the absence of vitamin D as when it was given. The retention of P also was essentially unchanged.

We have no explanation either for the mechanism of the increase in Mg absorption or for the decrease in its retention when vitamin D is given. Specifically the former may be attributed to an increase in the acidity of the tract or to an as yet unidentified improvement in metabolic activity in the intestinal tract as well as in other tissues. The decrease in the retention of Mg may be due to the correction of the increase in Mg content of tissue when Mg is substituted for Ca in a diet free from vitamin D. This would be in harmony with the observations of Gassmann ('10) and von Euler and Rydbom ('31) who found that the Mg content of bone is increased in rickets.

SUMMARY

A series of experiments with 90-gm rats revealed that Mg was absorbed equally well when added at a level of 0.12% of the ration as the carbonate, phosphate, or phytate to a low-P, low-Ca semi-synthetic diet. The amount absorbed in the absence of vitamin D ranged from 50 to 71% of that ingested to a range of 53 to 77% when vitamin D was given. This increase was obtained consistently with all rations and in the absence of a large need for the Mg available since most of that ingested appeared in the urine. Dietary changes, such as the addition of calcium carbonate and sodium bicarbonate, or inorganic phosphate did not affect this absorption but the addition of phytic acid reduced it slightly. Similarly, Mg

limited to the levels which could be fed without inducing catharsis was without effect on the absorption of P, or on the hydrolysis of phytic acid.

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Arch Biochem. 20: 325, 1949

Interaction of Calcium, Phosphorus, and Vitamin D.[1] III. Study of Mode of Action of Vitamin D using Ca^{45}

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Introduction

The mode and site of action of vitamin D still remains a controversial problem. Evidence exists to substantiate the theory that the vitamin influences the absorption of calcium or phosphorus or both (3,5). Expeciments with Ca^{45} by Greenberg (2) led to the conclusion that viatmin D has a dual action, that of promoting absorption of calcium and of exerting a direct effect on the mineralization of bone.

Another possible theory of vitamin D action, which explains the observations made on chicks by Migicovsky and Emslie (4), is that the vitamin acts on the newly formed bone, by resisting the solution effect of an adverse mineral equilibrium which exists in a rachitic animal. The promotion of calcium absorption by vitamin D, according to this theory, would be considered a secondary effect.

The feeding experiments of Migicovsky and Emslie (4) have been followed by the present investigation on the mode of action of vitamin D, in which radioactive calcium is used, with excretion of calcium and phosphorus as the criterion of vitamin D activity. The results lead to the conclusion that the initial action of vitamin D is to prevent the solution of bone calcium.

Experimental

One-day old leghorn chicks were fed Assoc. Official Agri. Chemists rachitogenic diet (1) without vitamin D until placed on experiment. After a specified time, chicks were dosed twice with Vitamin D or corn oil, followed by a removal of feed the next day and a repetition of the doses. On the third day the chicks were given another dose of vitamin D and the excreta were collected for 48 hr. Another dose was given during the collection period, on the fourth day.

[1] Technical Contribution No. 156, Division of Chemistry, Science Service, Dept. of Agriculture, Ottawa, Canada.

With all the experiments, except No. 140, the treatments were given to quadruplicate groups of 10 chicks per group; experiment 140 was carried out in triplicate only.

In experiments where the effect of calcium or phosphate was being studied the mineral dose was given the day the excreta collection began.

The excreta were collected in either two consecutive 24 hr. samples or one 48 hr. sample. The collection was made in galvanized metal pans, washed into beakers and analyzed for calcium and phosphorus, using perchloric acid digestion followed by permanganate titration in the case of calcium, and in the case of phosphorus by titration of the molybdate. It should be pointed out that the term "excreta" refers to the combined urinary and fecal excrement.

The experiments with radioactive calcium were carried out as described above, except that several doses of radioactive calcium as the chloride were given one week prior to the start of the experiment. In this way chicks were prepared that had a deposit of radioactive mineral in the tissues. The dose of radioactive calcium was approx. 0.2 μc/chick/day for 3 days. These experiments were also conducted in quadruplicate with 5 chicks per group.

Measurement of radioactivity was made with a nucleometer. The calcium was precipitated on a filter paper pad as the oxalate, the thickness of the precipitate being less than 1.0 mg. Ca/cm.[2] In this manner, absorption corrections were avoided.

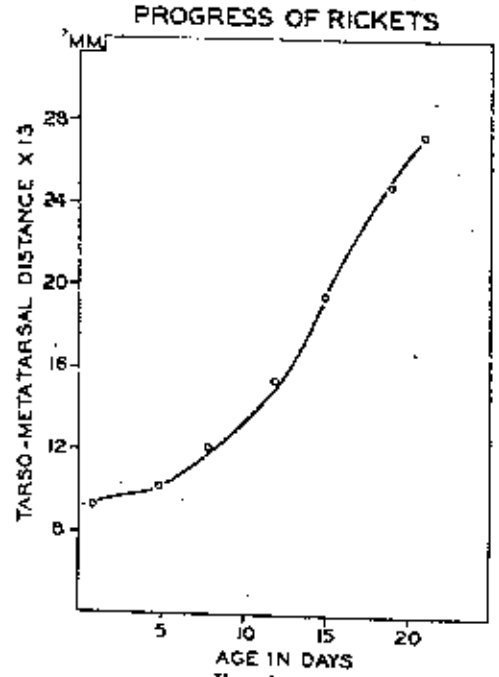

Fig. 1.

The experiments were conducted with one-, two- or three-week old chicks. The one-week old chicks were considered to be non-rachitic. At two weeks the rachitic condition is established. This is substantiated in Fig. 1, where the degree of rickets, as

measured by the tarso-metatarsal distance in an X-ray plate of the joint, is plotted against time.

The results of the first series of experiments are shown in Table I. The data illustrate quite conclusively that, in the non-rachitic one-week old chicks, vitamin D does not exert any influence on the net excretion of calcium or phosphorus. The effect of calcium ingestion on phosphate excretion is quite marked in that it definitely decreases the loss of phosphorus *via* the excreta.

TABLE I

Excretion of Calcium and Phosphorus in Starving Chicks

Experiment no.	Age	Calcium			Necessary diff. at $p=.01$
		Corn oil	Vitamin D	Corn oil and phosphate	
	wk.	mg.	mg.	mg.	
133	1	48	52	—	18.2
136	1	53	47	53	11.9
137	2	55	33	66	18.5
140	2	62	37	55	26.7
138	3	89	46	84	37.0

Experiment no.	Age	Phosphorus			Necessary diff. at $p=.01$
		Corn oil	Vitamin D	Corn oil and calcium	
	wk.	mg.	mg.	mg.	
133	1	110	99	47	18.3
136	1	143	136	72	18.2
137	2	207	183	116	34.1
140	2	232	216	161	27.8
138	3	304	244	211	91.0

The data with two- and three-week old chicks, which are rachitic, illustrate that vitamin D decreases the amount of calcium lost in the excreta but exerts very little effect on the phosphorus. The effect of calcium ingestion is the same as with non-rachitic one-week old chicks, namely the loss of phosphate is decreased. Also phosphate ingestion exerts no effect on the excretion of calcium.

If the effect of vitamin D, noted above, is not one of reabsorption, we must conclude that vitamin D influences the retention of calcium and its influence on absorption of calcium is a secondary effect.

To establish whether the decrease in calcium of the excreta is due to retention or reabsorption, the chicks, in which radioactive calcium was deposited, were used.

Since the Ca^{45} was deposited in all the chicks prior to treatment with vitamin D we assume that the specific radioactivity of the calcium in all chicks is the same. If

reabsorption caused the decreased amount of calcium in the excreta the specific radioactivity of the calcium in the excreta would remain unchanged. If retention caused the decrease, the specific radioactivity of the excreta calcium would be less if the retention took place in tissues of higher specific activity and greater if retention took place in tissues of lower specific activity. Therefore, if the specific radioactivity of bone calcium were higher than that for other tissues, and if the specific radioactivity of excreta calcium were less in the vitamin D-treated chicks than in the controls, we must conclude that vitamin D influences the retention of calcium in the bone.

The results of the excretion of Ca^{45} are shown in Table II. It is seen that the specific radioactivity of the excreta calcium from vitamin D-treated chicks is much less than that of the controls.

TABLE II

Effect of Vitamin D on Excretion of Ca^{45}

Expt. no.	Age	mg. Calcium		Nec. diff. at $p=.01$	c.p.m.[a] Ca^{45}/mg. Ca		Nec. diff. at $p=.01$
		Corn oil	Vitamin D		Corn oil	Vitamin D	
	wk.						
144	2	41	26	13	216	102	82
147	2	47	34	20	197	81	73

[a] Counts/min.

Analyses of the carcasses of chicks and of the tibiae are shown in Tables III and IV, respectively. It is seen that bone has a higher specific activity than other tissues and also that the activity of newly formed bone, *i.e.*, the epiphyses, is greater than the activity of the diaphyses, thereby leading us to the conclusion that vitamin D exerts a retentive effect on the calcium of newly formed bone.

TABLE III

Specific Activity (Ca^{45}) of the Chick Carcass One Week after Dosing with Ca^{45}

Tissue	c.p.m.[a]/mg. Ca	
	Corn oil	Vitamin D
Skin	200	250
G. I. tract	123	112
Muscle	254	274
Bone	319	334
Remainder	230	237

[a] Counts/min.

Note: Values represent the mean of 4 groups of 2 chicks/group.

Analysis of variance reveals that the variance for tissues is significant at $p=.01$ whereas the variance for treatment is not significant.

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TABLE IV

Specific Activity (Ca^{45}) in Epiphyses and Diaphyses of Chick Tibiae

	c.p.m.*/mg. Calcium	
	Corn oil	Vitamin D
Epiphyses	555	546
Diaphyses	425	465

* Counts/min.

Note: The values represent the mean of 4 groups of 10 chicks/group.

The analysis of variance reveals that the variance for tissue is significant at $p = .01$ whereas the variance for treatment is not significant.

Examination of the results in Tables III and IV reveals that there is no difference in specific radioactivity of the calcium in the tissues of vitamin D-treated and negative control chicks. This is the expected result, since the Ca^{45} was deposited prior to vitamin D treatment.

Conclusions

The results of this investigation on chicks lead us to a different conclusion about the action of vitamin D from that which Greenberg (2) arrived at working with rats. He concluded that vitamin D promotes the absorption of calcium and exerts a direct effect on the mineralization of bone. We conclude that the effect on the mineralization of bone is indirect in that it prevents the solution of bone. Rickets results because bone calcium is being dissolved. This may be prevented by feeding vitamin D or rectifying the level and ratio of calcium and phosphorus in the diet (4). It is a fact that, unless the calcium and phosphorus in the diet are unbalanced, rickets will not result in rats, or, in chicks, only a mild degree of rickets will occur.

We can explain the absorption effect of vitamin D in that, if vitamin D prevents the entrance of bone calcium into the metabolic pool, the tendency is to extract calcium from the gut.

Summary

Experiments on excretion of calcium and phosphorus of starving chicks indicate that vitamin D decreases the loss of calcium *via* the excreta, and has little effect on the phosphorus.

Calcium ingestion decreases the loss of phosphate.

When chicks which were previously fed radioactive calcium were used, vitamin D decreased the specific activity of the excreta calcium, indicating a retention of calcium in a tissue of high specific activity, namely, newly formed bone.

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CALCIUM ABSORPTION AND VITAMIN D[1]

By B. B. Migicovsky and J. W. S. Jamieson

Abstract

The rate at which Ca^{45} administered as an oral dose appears in blood and bone of chicks is markedly affected by vitamin D. Absorption of Ca^{45} from an intramuscular dose is not influenced by either vitamin D or the calcium content of the diet. The interaction of the amount of Ca^{45} in the oral dose and vitamin D on the amount of calcium absorbed indicated that the vitamin D effect on the absorption is a function of the quantity of calcium presented for absorption. Absorption experiments carried out with chicks which were fed diets of varying calcium content showed that vitamin D enabled the chicks to adapt the capacity to absorb calcium to different dietary intakes of calcium.

Introduction

Knowledge of the mode of action of vitamin D has been reviewed by Nicolaysen and Egg-Larsen (4). Although the "primary" effects of the vitamin have been established, neither the initial effect nor its mode of action has been defined satisfactorily. It is generally agreed that the initial site of action is on either the calcium absorption mechanism or the bone. The effect at both sites has been demonstrated repeatedly. The nature of the vitamin effect, increased absorption, and mineral deposition is such that a change in one can elicit a change in the other.

It is hoped that detailed studies of the absorption and deposition mechanisms may aid in establishing the initial target for vitamin D action.

Hansard *et al.* (1) found that the ability of rats to absorb calcium was increased after a short period of time on a low calcium diet. Nicolaysen (3) observed that rats were able to adapt the ability to absorb calcium, and concluded that this adaptation ability was associated with vitamin D. This led to the proposal (4) of an "endogenous factor", which is responsible for regulating the efficiency of absorption and is inoperable in the absence of vitamin D.

In this report we are presenting some studies on calcium absorption with chicks, using the Ca^{45} method of measuring relative absorption as described by Migicovsky and Nielson (2).

Experimental

One-day-old chicks were taken at random and fed an A.O.A.C. rachitogenic diet either with or without vitamin D. When vitamin D was given, it was mixed into the diet at the rate of 1 unit per gram feed. The calcium absorption measurements were made on two- or three-week-old chicks.

The first experiment was a study of the rate at which an oral dose of Ca^{45} appeared in blood and bone and the effect of vitamin D thereon. At two weeks of age one half of the groups were given vitamin D in the diet. When

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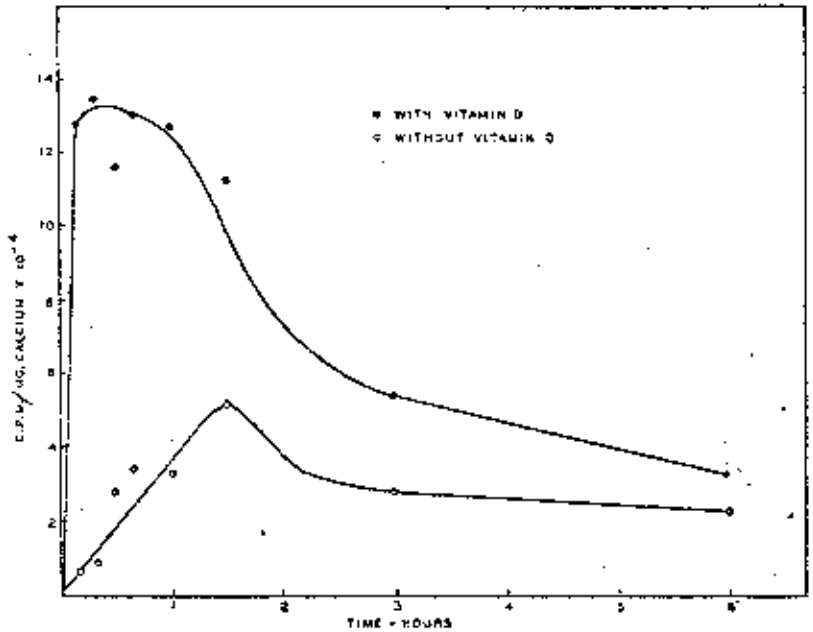

Fig. 1. Specific activity of blood calcium vs. time.

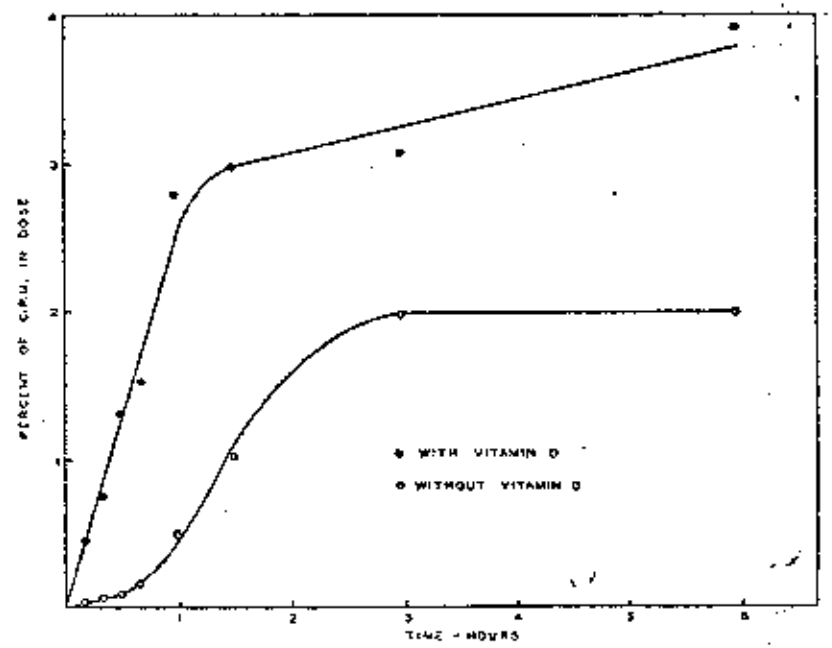

Fig. 2. Accumulation of an oral dose of Ca^{45} in tibia vs. time.

the chicks [illegible] three weeks of age, feed was removed. After 24-hr. starvation, all the chicks were given an oral dose of Ca^{45} equivalent to 5.0 mgm. calcium and 4.2×10^6 counts per minute. At varying intervals after the dose of Ca^{45}, groups were bled and the left tibiae removed. The analytical data for blood and bone are shown in Figs. 1 and 2, respectively.

The data illustrate that vitamin D exerts a positive effect on the rate at which calcium appears in the blood, that is, the rate of calcium absorption is increased by the presence of vitamin D.

The amount of Ca^{45} appearing in bone is a reflection of the calcium concentration in the blood in the intervening time. Previous work by Migicovsky and Nielson (2) showed that vitamin D did not affect the appearance of Ca^{45} in bone from an intramuscular dose, and a straight line relationship existed between Ca^{45} in the intramuscular dose and the Ca^{45} in bone. Data in Table I substantiate this finding and, in addition, illustrate that the calcium content of the chicks' diet prior to the 24-hr. starvation period does not affect the Ca^{45} content of bone after an intramuscular dose. A summary of the analysis of variance of the data in Table I is shown in Table II.

TABLE I

EFFECT OF DIETARY CALCIUM AND VITAMIN D ON PERCENTAGE OF INTRAMUSCULAR DOSE OF Ca^{45} IN TIBIA*

Diet		Vitamin D	No vitamin D
Ca, %	P, %	% Ca^{45} dose in tibia	
0.30	0.99	4.69	4.95
0.53	0.97	4.59	4.60
1.25	0.95	4.54	4.48
2.18	0.90	4.27	4.38

* *Each value represents the mean of three groups of three chicks per group.*

TABLE II

ANALYSIS OF VARIANCE OF DATA IN TABLE I

Source	Degrees of freedom	Mean square
Total	23	
Vitamin D	1	0.03[a]
Diet	3	0.25[a]
Diet × vit. D	3	0.03[a]
Error	16	0.11

[a] *Not significant at P = .05.*

As a result of these observations, the percentage of an oral dose of Ca^{45} appearing in bone may be taken as an index of the capacity of the organism to absorb calcium. A relative measure of the amount of calcium absorbed from any given dose is then the

$$\frac{\text{counts per minute/tibia}}{\text{counts per minute/dose}} \times 100 \times (\text{mgm. Ca/dose}).$$

The next series of experiments represented a study of the change in the absorption capacity with varying doses of calcium. Having established that vitamin D acts on the absorption mechanism by altering the rate of the calcium absorption reaction, the question arises whether the vitamin effect is a function of the amount of calcium presented to the absorption mechanism.

One day-old chicks were taken at random and raised as described above, one half of the chicks receiving vitamin D for two weeks prior to the experiment. When the chicks were three weeks of age, the feed was removed. Twenty-four hours later the dose of Ca^{45} was administered. Twenty-four hours later the chicks were killed and tibiae were removed for analysis.

Data obtained with different doses of calcium are shown in Table III. They indicate that the percentage of Ca^{45} dose per tibia fell with increasing doses of calcium but the effect of vitamin D was comparatively constant. The relative quantity of calcium absorbed, which is percentage of Ca^{45} dose per tibia × mgm. Ca per dose, is greater with increasing doses and the effect of vitamin D thereon also increases with increasing calcium dose. Analysis of variance of the data in Table III is shown in Table IV.

TABLE III

INTERACTION OF VITAMIN D AND AMOUNT OF CALCIUM IN DOSE ON THE PERCENTAGE OF THE Ca^{45} DOSE PER TIBIA

Trial No.*	Ca/dose, mgm.	Vit. D	No vit. D	Vit. D effect†	
		% Ca^{45} per tibia		$\frac{\text{c.p.m./tibia}}{\text{c.p.m./dose}} \times 100$	$\frac{\text{c.p.m./tibia}}{\text{c.p.m./dose}} \times 100 \times (\text{mgm. Ca/dose})$
65	0.7	5.26	4.19	1.07	.0075
	1.3	4.95	4.06	0.89	.0116
	2.5	4.98	3.51	1.47	.0368
	5.1	3.80	2.96	0.84	.0428
70	0.7	5.34	3.99	1.35	.0095
	2.8	6.41	3.79	2.62	.0734
	3.6	6.46	3.58	2.88	.1037
	8.4	5.20	2.85	2.35	.1974
	11.0	5.62	2.81	2.81	.3091
67	1.3	6.39	4.96	1.43	.0186
	3.1	5.87	3.94	1.93	.0598
	7.4	4.40	2.64	1.76	.1302
	15.3	3.69	2.61	1.08	.1652
	20.0	3.14	1.77	1.37	.2740

† *Value for vitamin D treated groups minus value for vitamin D deficient groups.*
* *Each value represents the mean of four groups of three chicks per group.*

TABLE IV

ANALYSIS OF VARIANCE OF DATA IN TABLE III

Source	Expt. 65		Expt. 70		Expt. 67	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square	Degrees of freedom	Mean square
Total	31		39		39	
Vitamin D	1	9.04[b]	1	57.6[b]	1	22.9[b]
Ca^{45} dose	3	2.78[b]	4	1.8[b]	4	13.9[b]
Ca^{45} dose × vit. D	3	0.16[a]	4	0.78[a]	4	0.22[a]
Error	24	0.21	30	0.39	30	0.20

[a] *Not significant.*
[b] *Significant at P = .01.*

These experiments indicate that irrespective of the amount of calcium in the dose, vitamin D increases the proportion that is absorbed by a constant factor. This leads to the conclusion that the amount of calcium that vitamin D causes to be absorbed is a function of the amount of calcium presented to the absorption mechanism.

The final series of experiments dealt with the adaptation mechanism referred to by Nicolaysen *et al.* (5). Chicks were reared on diets varying in calcium with and without vitamin D. After a period of one week the measurement of the percentage of Ca^{45} dose per tibia was made as described above. The results are shown in Table V. Accompanying the data are the values for the amount of calcium per tibia, which is a criterion of the degree of mineralization. Analysis of variance of the data is shown in Table VI.

It is clear that the effect of vitamin D on the absorption mechanism was greater after a low calcium diet than after a high calcium diet. In the

TABLE V

INTERACTION OF DIETARY CALCIUM AND VITAMIN D ON THE PERCENTAGE OF ORAL Ca^{45} DOSE IN TIBIA*

Diet		Vitamin D		No vitamin D	
Ca, %	P, %	$\frac{\text{c.p.m./tibia}}{\text{c.p.m./dose}} \times 100$	Ca/tibia, mgm.	$\frac{\text{c.p.m./tibia}}{\text{c.p.m./dose}} \times 100$	Ca/tibia, mgm.
0.30	0.99	4.25	19.2	1.93	12.9
0.53	0.97	4.32	25.3	1.66	15.1
1.25	0.95	2.89	27.1	1.65	19.1
2.18	0.90	2.32	26.2	1.59	21.1

* *Each value in the table represents the mean of three groups of three chicks per group.*

TABLE VI

ANALYSIS OF VARIANCE OF DATA IN TABLE V

Source	Per cent of Ca^{45} dose/tibia		Ca/tibia	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
Total	23		23	
Vitamin D	1	18.1[b]	1	325.0[b]
Diets with vit. D	3	2.95[b]	3	38.1[b]
Diets with no vit. D	3	0.07[a]	3	41.0[b]
Error	16	0.06	16	1.78

[a] *Not significant.*
[b] *Significant at P = .01.*

absence of vitamin D, the chicks did not adapt the absorption mechanism to a low calcium intake; whereas in the presence of vitamin D this adaptation was made. It may be noted also that, irrespective of the diet, vitamin D always exerted a significant effect on the absorption mechanism.

Discussion

It is generally felt that the action of vitamin D on both the absorption and deposition mechanisms is a primary effect. Nicolaysen *et al.* (5) invoke an endogenous factor to explain the absorption effect. There is no direct evidence for the presence of an endogenous factor and we feel that a plausible explanation for the action of vitamin D can be given without recourse to such a factor.

If we may be permitted to speculate, the action of vitamin D may be explained by a reaction of the vitamin or a derivative thereof with calcium at the absorption barrier. The reaction may be pictured as Ca + vit. D→[Ca D].

With this type of a reaction in mind, the amount of calcium bound to vitamin D in the presence of an excess of the vitamin would be a function of the amount of calcium presented to the absorption mechanism. This is borne out by the results of the first series of experiments presented above.

The adaptation effect, i.e., the differential effect of vitamin D after a period of variable calcium feeding, may be explained by the degree of accumulation of the bound calcium. That is, after a week on a high calcium diet plus vitamin D, the amount of bound calcium in the blood would be higher than after a period on a low calcium diet. The increased concentration of bound calcium would serve to decrease the rate of the reaction pictured above. This could account for results obtained with experiments which illustrate the adaptation phenomenon.

The mineralization effect of vitamin D, quantitative and qualitative, could be explained by the assumption that bound calcium is deposited more

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readily and in a more regular fashion. In the absence of vitamin D, where a high calcium diet yields a more mineralized but histologically abnormal bone, the deposition of calcium probably depends upon the irregular excess of calcium that is delivered to the blood via the intestinal barrier.

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Biological Activity of a Vitamin D Metabolite[1]

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A metabolite of vitamin D which is obtained from the carcasses of rats given ^{3}H-vitamin D_3 has been isolated by silicic acid column chromatography and tested for its effectiveness in curing rickets, stimulation of calcium transport by everted intestinal sacs, and in the elevation of serum calcium concentration. The metabolite was as effective as vitamin D in all three systems. In addition it stimulated intestinal transport of calcium within 8–10 hours after its oral administration to vitamin D-deficient rats, whereas vitamin D itself required a 20-hour lag for its action. The physiologic significance of these findings is discussed.

The idea that vitamin D may be converted to a metabolically active form has recently gained momentum with the isolation of vitamin D metabolites which are capable of curing rickets in rats (1, 2). One of these metabolites is found in large quantities in bone, liver and blood, and is at least as active in curing rickets as is the parent vitamin (2). Further experiments demonstrated that as the dosage of vitamin D was decreased to a truly physiologic dose, the proportion of this metabolite increased to the point where it is the major form of the vitamin present (2). Kinetics of its formation *in vivo* shows that it appears in quantity before an observable physiologic effect of the vitamin is obvious (Neville, Prees, and DeLuca, in preparation). Although this metabolite has not yet been identified, its very interesting *in vivo* characteristics have prompted our further investigation of its physiologic effects.

It will be demonstrated in this manuscript that this polar metabolite(s) of vitamin D not only cures rickets but activates calcium transport in the intestine and elevates serum calcium presumably by activating bone mineral mobilization. Furthermore, this metabolite(s) of vitamin D acts much more rapidly than does vitamin D itself in stimulating calcium transport by everted intestinal sacs.

MATERIALS AND METHODS

Preparation of rats. Male weanling rats were obtained from the Holtzman Co. of Madison, Wisconsin. They were maintained in hanging wire cages and fed *ad libitum* a purified, vitamin D-deficient diet described earlier (3). This diet, which contains 0.47% Ca and 0.3% P, does not induce rickets in rats but produces a severe vitamin D deficiency in 3–4 weeks characterized by low serum Ca^{++} and reduced growth (4). When the rats reached this degree of deficiency they were used in the following experiments.

Production of metabolite fraction. The vitamin D-deficient rats were given 8000 IU each of either 1,2-^{3}H-vitamin D_3 (5) or randomly labeled ^{3}H-vitamin D_3 (6), both of which were purified just before use and were judged radiochemically pure. The radioactive vitamin was dissolved in cottonseed oil and given orally in 0.1 ml. Seventeen hours later the rats were killed, their blood was collected and the gastrointestinal tract was removed and discarded. The carcass was skinned, freed of most the muscle, ground in a meat grinder, and extracted with methanol and chloroform as described earlier (2). The blood was also extracted by these solvents as previously described (2). The chloroform extracts to which 1 mg α-tocopherol was added as an antioxidant were dried in a flash evap-

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[2] Postdoctorate fellow of the U.S. Public Health Service, No. 5-F2 AM 25,034-02.

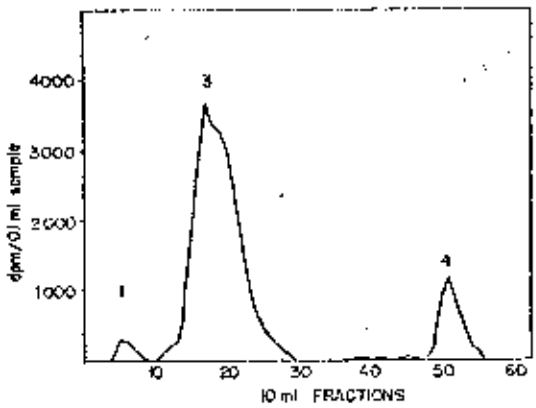

FIG. 1. A silicic acid chromatographic profile of a chloroform extract of the carcass of a rat given 10,000 IU of ^{3}H-vitamin D_3 17 hours previously. O, Radioactivity.

orator under a stream of N_2. The residue was dissolved in Skellysolve B (a petroleum fraction boiling at 65-67°) and chromatographed on silicic acid columns using gradient elution already described (1,2). Four components were usually obtained (Fig. 1). The peak 4 obtained from these chromatograms was once again rechromatographed before use. The peak 4 fraction was then dissolved in cottonseed oil (Wesson). The dosages administered were calculated on the assumption that the specific activity of the metabolite is the same as that of the original radioactive vitamin. For example, if the original ^{3}H-vitamin D_3 contained 800 dpm/1U, then the number of units of metabolite was calculated by dividing its total dpm by 800.

Line test assay or rickets cure test. Weanling rats of either sex were obtained from the Holtzman Co. and were fed the rachitogenic diet of Steenbock and Black (7) for 21 days. The diet was modified by the substitution of egg white protein for wheat gluten and by the addition of water-soluble vitamin at the levels described earlier (3). After the 21-day depletion period, a single 4 IU dose of either standard vitamin D or metabolite was administered. Controls received the oil vehicle alone. Seven days later the rats were killed and the line test was performed on sectioned radii and ulnae of individual rats. The biological activity was ascertained as described in the U.S. Pharmacopeia (8).

Serum calcium response. Vitamin D-deficient rats fed the purified diet as described above were used in all experiments. When their serum calcium concentrations fell to 1-5 mg % they were used in the following experiments.

The deficient rats received 5 IU of peak 4 metabolite every day for 4 days or a total of 20 IU. At the end of this period, the rats and others which had not received the metabolite fraction were killed and their serum calcium concentrations were determined by the method of Webster (9).

Everted intestinal sacs. The vitamin D-deficient rats were given a single 5 IU dose of metabolite orally in oil solution while the controls received the oil vehicle alone. After 20 or 24 hours the rats were killed and calcium transport by everted intestinal sacs as described earlier was carried out (9). The data are expressed as a ratio of the ^{45}Ca concentration on the serosal side over that on the mucosal side.

In another experiment vitamin D-deficient rats were given a single dose of 10 IU of metabolite or vitamin D_3 in oil solution. The rats were then killed at the times indicated for the determination of calcium transport by everted intestinal sacs and serum calcium concentration.

RESULTS

It was once again confirmed that biologically active metabolites of vitamin D do exist in the tissues of rats given vitamin D. Figure 1 shows a typical silicic acid column chromatogram profile of a chloroform ex-

TABLE 1

RELATIVE ABILITY OF METABOLITES FROM VARIOUS TISSUES[a] TO CURE RICKETS IN RATS

Tissue	Biological activity[b]			
	1	2	3	4
Kidney	1.7	2.5	4.0	2.0
Intestinal mucosa		—	4.0	2.5
Serum	0	1.9	4.5	3.7
Liver	1.2	0.5	4.0	3.8
Bone	—	—	3.8	3.5

[a] One to four represent the peaks eluted as shown in Fig. 1 from silicic acid column chromatography of chloroform extracts of tissues of rats which had been maintained for 3-4 weeks on a vitamin D-free diet and sacrificed 24 hours after administration of 500 IU of ^{3}H-vitamin D_3.

[b] Test animals received amounts of metabolites corresponding to 4 IU of parent vitamin, according to radioactivity. Animals which received 4 IU of standard vitamin D_3 gave an average value of 4. Each value in the table is the average of eight assay rats. Peak 1 fractions from kidney and intestine were pooled for assay. The assays were carried out according to the line test method described in the U.S. Pharmacopeia (8).

TABLE II

SERUM CALCIUM RISE IN RESPONSE TO PEAK 4 METABOLITE

Vitamin D-deficient rats received 0.1 ml of cottonseed oil containing 5 IU of peak 4 or oil only at 0, 24, 48, and 72 hours. After 96 hours, the animals were sacrificed and the serum calcium levels were determined. Control animals received the oil vehicle alone. The numbers in parentheses represent the number of animals in each group.

	Control (mg %)	Peak 4 (mg %)
Serum Ca (mg/100 ml)	4.1 ± 0.2[a] (5)	6.5 ± 0.3[b] (5)

[a] ± Standard error of the mean.
[b] $p < .001$

TABLE III

STIMULATION OF CALCIUM TRANSPORT IN EVERTED INTESTINAL SACS BY A VITAMIN D METABOLITE

Time after dosage	Deficient control; ratio[a]	Vitamin D_3; ratio[a]	Peak 4 metabolite; ratio[a]
20	1.44 ± 0.10[b] (4)	3.49 ± 0.11[b, c] (6)	2.53 ± 0.08[b, c] (4)
24	1.57 ± 0.11 (4)	2.58 ± 0.13[c] (3)	2.44 ± 0.11[c] (5)
21	1.64 ± 0.08 (4)	3.48 ± 0.12[c] (3)	2.00 ± 0.13[c] (4)

[a] Vitamin D-deficient rats were given either 5 IU of vitamin D_3 or 5 IU of metabolite orally in 0.1 ml of cottonseed oil, and the controls received the oil vehicle alone. At the times indicated, the rats were killed and everted sacs were prepared from their duodenum. Calcium-45 transport expressed as a ratio of ^{45}Ca (serosal side)/^{45}Ca (mucosal) was measured as described in the text.
[b] Standard deviation. The figures in parentheses represent the number of animals in each group.
[c] $p < .001$

tract of a whole rat which had received ^3H-vitamin D_3 17 hours before it had been killed. Again the four radioactive fractions described previously (1, 2) appeared. Peak 1 has already been identified as an ester of vitamin D and long-chain fatty acids (10). Peak 3 is unaltered vitamin D_3 (1) while peaks 2 and 4 are as yet unidentified.

Table I summarizes the results of line test assays of these fractions prepared from different tissues of rats given ^3H-vitamin D_3. Clearly all four fractions were able to cure rickets to some extent. Most important is the fact that peak 4 was in some cases as effective as was the parent vitamin (peak 3).

TABLE IV

EFFECT OF ORAL ADMINISTRATION OF 10 IU VITAMIN D_3 AND AN EQUIVALENT AMOUNT OF METABOLITE (PEAK 4) OF VITAMIN D_3 ON CA TRANSPORT BY EVERTED INTESTINAL SACS

The experiments were carried out as described in Table III except that a 10 IU dose of either vitamin D_3 or peak 4 metabolite was used.

Hours after administration	^{45}Ca Serosal/^{45}Ca Mucosal: 10 IU vitamin D_3	Peak 4 metabolite
Control	0.98 ± 0.03 (31)[a]	
2	—	1.09 ± 0.04 (3)
4	—	1.09 ± 0.23 (3)
6	0.79 ± 0.03 (4)	0.97 ± 0.15 (3)
8	1.17 ± 0.13 (4)	1.30 ± 0.11 (3)[b]
10	1.13 ± 0.09 (9)	1.86 ± 0.19 (7)[c]
12	0.99 ± 0.03 (5)	1.71 ± 0.15 (3)[c]
16	1.09 ± 0.14 (5)	1.78 ± 0.11 (3)
20	1.73 ± 0.13 (16)[c]	1.78 ± 0.15 (10)
24	1.53 ± 0.19 (3)[c]	—
48	2.23 ± 0.12 (3)[c]	—
72	2.61 ± 0.07 (3)[c]	—
96	1.09 ± 0.27 (3)[c]	—

[a] ± Standard error of the mean. Numbers in parentheses show the numbers of animals in each group.
[b] $p < .025$ above control.
[c] $p < .005$ above control.
[d] $p < .001$ above control.

The effectiveness of peak 4 metabolite in curing rickets by the line test method prompted our examination of its ability to carry out the other two well known physiologic actions of the vitamin, namely the elevation of serum calcium concentration by its bone mobilization action and the increased calcium transport by intestine. Table II demonstrates that the peak 4 metabolite is capable of bringing about the rise in serum calcium concentration, and Table III shows that it is effective in stimulating the transport of calcium in everted small intestinal sacs. These effects of the metabolite have been repeated many times. Thus two additional parameters have been measured which

demonstrate the biological activity of the peak 4 metabolite.

Perhaps most interesting is that the peak 4 metabolite acts much more rapidly than does the parent vitamin in stimulating calcium transport by small intestine (Table IV). A statistically significant effect of peak 4 metabolite on calcium transport can be observed as early as 8 hours after its oral administration, whereas 20 hours is required for comparable action by the parent vitamin D_3.

DISCUSSION

The present communication establishes firmly by three independent criteria the biological activity of peak 4 metabolite formed *in vivo* from vitamin D_3. There is little doubt that this metabolite can (*a*) cure rickets, (*b*) stimulate the transport of calcium across small intestine, and (*c*) increase serum calcium concentration presumably by stimulating bone mineral mobilization. Thus its action most certainly mimics those of the parent vitamin.

Of greatest significance is the fact that the metabolite acts much more rapidly than does the parent vitamin in initiating calcium transport in intestine. Clearly the metabolite fraction is effective within 8 hours after oral administration, whereas vitamin D itself is effective only after 20 hours. This in itself certainly constitutes evidence to support the idea that this fraction is or contains the metabolically active form of the vitamin. It has already been demonstrated that as the dose of vitamin D given per animal approaches the level considered truly physiological, the proportion of the radioactivity in the tissues that exists as peak 4 becomes greater, approaching 80% (Neville, Press, and DeLuca, unpublished results; 2). Additional experiments demonstrate that the peak 4 metabolite is formed rapidly and appears in maximal quantity long before the first biochemical effects of the vitamin occur including the stimulation of ^{3}H-orotic acid incorporation into RNA (11). Despite the evidence cited, it is not possible as yet to conclude that the peak 4 metabolite is in fact the metabolically active form of the vitamin. This can only be established when the biochemical systems responsible for vitamin D actions are identified and shown to utilize this metabolite to the exclusion of vitamin D itself.

The nature of the peak 4 metabolite is not known at the present time. That it is in fact a metabolite and not an artifact has been clearly established (2). Recent work with new chromatographic systems has revealed that the peak 4 fraction is not homogenous but contains more than one component (Neville and DeLuca, unpublished results). Preparative scale production of the components of the peak 4 region is currently in progress with the aim of identification of those which are biologically active.

Only two components which appear in the silicic acid chromatogram profile have as yet been identified. Peak 1 has been shown to be an ester of vitamin D and a long-chain fatty acid (usually palmitic) (Lund, Horsting, and DeLuca, submitted for publication). Peak 3 has been shown to be primarily the unaltered vitamin D (1), although it may contain an additional metabolite. Peak 2 is as yet unidentified although the possibility that it might be pre-vitamin D_3 has been ruled out (12).

Peak 4 metabolite is a normal component of human serum and has been shown to be formed from ^{3}H-vitamin D_3 (DeLuca, Lund, Rosenblum, and Lobeck, submitted for publication). It is also formed by rachitic chicks much as in the case of rats (Imrie, Drescher, and DeLuca, unpublished results). Thus the metabolite 4 is not confined to a single species but is of general occurrence.

Although it is not possible to conclude that metabolite(s) 4 is the metabolically active form of the vitamin, it is nevertheless highly significant that a metabolite of vitamin D has been shown to be biologically active by three distinct parameters covering all known physiologic actions of the vitamin. Regardless of the final position the metabolite holds in the sequence of events resulting in the physiologic expression of vitamin D action, its identification should at least add to our knowledge of modifications of the vitamin D molecule which are possible to permit expression of the vitamin's action.

ACKNOWLEDGMENT

We are grateful to Roberta Press for her excellent technical assistance.

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Consumer Exp Info

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Health and Welfare Canada | Santé et Bien-être social Canada

Health Protection Branch | Direction générale de la protection de la santé

November 26, 1973.

Tunney's Pasture,
Ottawa, Ontario,
K1A 0L2.

Dr. H. F. Sassoon,
Tracor Jitco, Inc.,
1100 East Gude Drive,
Rockville, Maryland, U.S.A.
20851.

Your file Votre référence

Our file Notre référence

Dear Dr. Sassoon:

Your letter of November 12, 1973, to Dr. W.B. Bennett, Scientific Counsellor, Canadian Embassy, Washington, D.C., concerning the regulatory status of Vitamin D in Canada has been referred to this Branch for reply.

I am enclosing a photostat copy of the relevant pages from the Canada Gazette containing regulations respecting Vitamin D. The Canada Gazette is the official source of information concerning Federal regulations. I am also enclosing copies of pages from the Office Consolidation of the Food and Drug Regulations containing references respecting Vitamin D. The latter affords easier and more convenient reading and, of course, contains the same information as in the Canada Gazette.

May I direct your attention particularly to the following:

Section D.01.009 - in which a person is prohibited from selling a food to which Vitamin D has been added unless a reasonable daily intake of that food would result in an intake of not less than 300 International Units. (I.U.'s)

Under Section D.01.005 a person may advertise or label a food as "an excellent source" or "an excellent dietary source" of Vitamin D if a reasonable daily intake of that food would contribute not less than 300 I.U.'s.

Section D.01.011 prohibits the sale of a food to which Vitamin D is added if a reasonable daily intake of that food contributes more than 400 I.U.'s.

The reasonable daily intake for various foods is contained in Schedule K and the foods to which Vitamin D may be added are outlined in Division 3 of Part D.

.../2

To: Dr. H. F. Sassoon,
Rockville, Maryland.

Re: Regulatory Status of Vitamin D in Canada.

A person is prohibited from selling a drug represented as containing Vitamin D if the smallest recommended daily dose of the drug would result in a daily intake of less than 200 I.U.'s of Vitamin D. Section D.04.009.

Under Section D.04.011 the largest recommended daily dosage of Vitamin D in drugs that are advertised to the general public may not exceed 400 I.U.'s.

If the recommended daily dosage is greater than 400 and not more than 1,000 I.U.'s the drug may be sold to the general public but may not be advertised and it must be labelled for therapeutic use only .

A drug that is represented as containing Vitamin D in which the largest recommended daily dosage shown on the label would result in a daily intake of more than 1,000 I.U.'s, is restricted to sale only on prescription.

Please do not hesitate to inform me if additional information is needed.

Yours sincerely,

A. B. Morrison, Ph.D.,
Assistant Deputy Minister,
Health Protection Branch.

Encl.

Can. J. Biochem. Physiol. 41: 1785-92, (1963)

STUDIES OF DIETARY FATS AND VITAMIN D TOXICITY IN THE RAT

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Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada

Received March 12, 1963

Abstract

The possible relationship between vitamin D toxicity and linoleate intake was investigated. Linoleate increased the survival time of essential fatty acid deficient rats given lethal doses of vitamin D but this effect was probably unspecific. Plasma calcium and kidney calification, increased by vitamin D, was not influenced by linoleate intake while the fecal excretion of calcium was decreased by all fat supplements. Body fat composition was altered by a small daily intake of linoleate but not by large doses of vitamin D. Weight gains of rats given fat supplements and vitamin D were so variable that they were considered unsatisfactory criteria for judging the effect of linoleate intake on vitamin D toxicity.

Sinclair suggested in 1956 (1) that idiopathic hypercalcemia of infancy was related to a deficiency of linoleic acid. In support of this theory he cited, without detail, his own observation that rats given linoleic acid survived doses of vitamin D that were fatal to linoleic acid deficient rats. In a study of blood lipids and calcium balance in three cases of idiopathic hypercalcemia, James *et al.* (2) were unable to show any relationship between this condition and essential fatty acid deficiency. Since the etiology of idiopathic hypercalcemia was not clear it seemed possible that linoleic acid might not be involved in this condition and yet afford some protection against the toxicity of vitamin D. The following experiments were conducted on the rat to investigate the latter possibility. The effect of daily doses of linoleate and of vitamin D on the fatty acid composition of carcass and liver was also examined.

Materials and Methods

Inbred rats of the Food and Drug Colony (Wistar strain) were housed in individual cages and supplied with food and water ad libitum. The fat-free diet had the following composition in percent: casein (vitamin-free) 19, sucrose 72, vitamin mixture (3) 1, salt mixture (U.S.P. XIV) 4, alphacel 4. The dustiness of the diet was reduced in the following manner.* Two hundred milliliters 95% ethanol, 3 ml glycerol, and 100 ml water were mixed and added to 300 ml of 0.5% agar warmed to 55–60° C. The warm alcoholic solution was sprinkled over the dry diet which was thoroughly mixed and air-dried. Weight gain and food consumption were recorded weekly. Vitamin D (calciferol) in propylene glycol was administered orally 5 days a week, while control groups received propylene glycol alone. Fat supplements were given on the same days and in the same manner.

The rats were killed by decapitation and samples of blood were collected in

*This modification was suggested by Dr. C. C. Lucas, University of Toronto.

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heparinized tubes. Calcium was determined in the plasma by the method of Harrison and Harrison (1) and in feces after ashing for 3 hours at 550° C by a modification of the same method. The fatty acid composition of liver and degutted carcass was obtained by gas liquid chromatography as previously described (5). Kidneys were examined histologically after fixing in alcoholic formalin and staining by the method of von Kossa (6). Calcification was rated on an arbitrary 0–5 scale.

Procedures and Results

Weanling rats were fed the fat-free diet until they showed signs of essential fatty acid deficiency of the paws and tail and had ceased to gain weight. They were divided into four groups of 10, each with a mean weight of about 205 g. The daily supplements and the survival times of the various groups are shown in Table I. None of the rats given vitamin D survived for more than 2

TABLE I

Effect of methyl linoleate on the survival time of rats given massive doses of vitamin D

Linoleate supplement	Vitamin D dose (I.U./100 g body wt. per day)	
	10,000	40,000
	Survival time (days)	
None	8.0±0.6*	6.6±0.3
100 mg/day	10.9±0.9	6.4±0.3

*Mean ± S.E.

weeks, and the survival time of those given 40,000 I.U. vitamin D per 100 g body weight was not influenced by linoleate. The survival time of rats given 10,000 I.U. per 100 g body weight was, however, increased by linoleate.

Further studies with adult rats, deprived of essential fatty acids for many weeks, were invalidated by a striking incidence of pneumonia among the deficient animals. Young rats were selected in subsequent experiments.

Eighty weanling males were fed the fat-free, dust-free diet for 2 weeks and then distributed in a randomized block design of four groups. The following doses were given 5 days a week: (1) 0.1 ml propylene glycol, (2) 100 mg methyl linoleate, (3) 2000 I.U. calciferol per 100 g body weight in 0.1 ml propylene glycol, (4) doses (2) and (3). The vitamin D dose was increased to 5000 I.U. per 100 g body weight for the last 2 weeks of the 4-week experiment. The results, exclusive of the fatty acid analyses, are shown in Table II. Vitamin D alone depressed and linoleate alone stimulated growth in the young rats. When both supplements were given, weight gains fell between the two extremes and were not significantly different from those of rats given only the fat-free diet. Liver weights varied with body weights. The kidneys of rats given vitamin D

TABLE II

Effect of vitamin D and of methyl linoleate on weight gain, liver weight, kidney weight, and plasma calcium

Treatment	Food intake	Wt. gain	Liver wt. (g)	Kidney wt. (g)	Plasma Ca (mg/100 ml)
None	272±6*	79±3	6.40±0.20	1.36±0.01	9.87±0.15
Linoleate	280±6	91±3	7.86±0.28	1.39±0.05	9.81±0.14
Vit. D	218±7	60±4	6.24±0.24	1.31±0.04	10.87±0.14
Linoleate + vit. D	260±4	81±2	6.92±0.18	1.47±0.03	10.17±0.18

*Mean ± S.E.

alone were as large as those of rats given no supplement although the body weights were significantly lower. On the other hand, differences in kidney weights of rats given linoleate with or without vitamin D were accounted for by body weight. Plasma calcium, a more specific indication of vitamin D intake, was increased significantly by vitamin D but this increase was prevented by concurrent doses of linoleate.

The fatty acids of the carcass and liver fats are shown in Tables III and IV.

TABLE III

Fatty acid composition of carcasses

Fatty acid	% total fatty acids			
	Fat-free diet	Fat-free diet − linoleate	Fat-free diet + vit. D	Fat-free diet + linoleate + vit. D
$C_{16:0}$	0.1 (0.1–0.1)*	0.1 (0.1–0.1)	0.1 (0.0–0.1)	0.1 (0.0–0.1)
$C_{12:0}$	0.5 (0.4–0.6)	0.6 (0.3–1.0)	0.3 (0.3–0.4)	0.4 (0.3–0.6)
$C_{14:0}$	2.7 (2.6–2.8)	2.7 (2.4–3.4)	2.4 (2.0–3.0)	2.4 (2.3–2.6)
$C_{16:1}$	0.5 (0.3–0.6)	0.5 (0.1–1.0)	0.5 (0.4–0.6)	0.5 (0.3–0.6)
$C_{16:0}$	0.2 (0.1–0.2)	0.1 (0.1–0.2)	0.1 (0.0–0.2)	0.1 (0.1–0.2)
$C_{16:0}$	27.2 (25.4–28.7)	27.9 (23.0–31.2)	27.6 (21.0–30.3)	28.0 (27.0–29.3)
$C_{18:1}$	15.5 (14.8–17.1)	14.3 (12.3–16.4)	15.8 (14.3–18.2)	15.3 (14.6–16.4)
$C_{17:0}$	0.4 (0.2–0.5)	0.2 (0.0–0.5)	0.5 (0.3–0.7)	0.5 (0.2–0.6)
$C_{18:0}$	0.5 (0.2–0.8)	0.4 (0.0–0.6)	0.4 (0.0–0.2)	0.3 (0.0–0.5)
$C_{18:0}$	3.3 (2.5–4.3)	3.4 (2.9–4.7)	3.4 (2.6–4.0)	3.5 (3.3–4.1)
$C_{18:1}$	41.4 (42.7–45.6)	42.0 (40.1–46.7)	43.8 (43.8–51.7)	41.0 (40.0–41.7)
$C_{18:2}$	2.5 (2.2–2.7)	6.4 (6.1–9.0)	1.9 (1.4–2.7)	5.5 (5.2–5.7)
$C_{18:3}$	0.4 (0.0–0.6)	0.5 (0.2–0.8)	0.2 (0.0–0.7)	0.3 (0.0–0.6)
$C_{20:1}$	0.8 (0.5–1.2)	0.2 (0.0–0.6)	0.3 (0.0–0.6)	0.7 (0.4–1.2)
$C_{20:2}$	0.7 (0.6–0.8)	0.1 (0.0–0.3)	0.1 (0.0–0.7)	0.2 (0.0–0.5)
$C_{20:4}$	0.7 (0.6–0.9)	0.9 (0.7–1.2)	0.6 (0.4–0.9)	1.1 (0.1–1.8)

*Mean of five determinations of four rats each (range).

Rats receiving a fat-free diet, with or without a small supplement of methyl linoleate, contained proportionately more palmitic, palmitoleic, and oleic acids than were previously found in rats fed a diet containing 20% fat (5). The daily administration of 100 mg of methyl linoleate increased the concentration of linoleic acid in the carcass and liver, and of arachidonic and docosatetraenoic in the liver. In the absence of linoleate, the proportion of docosatrienoic was increased in the liver. The ratio of trienoic to tetraenoic acids proposed by

TABLE IV

Fatty acid composition of livers

Fatty acid	% total fatty acids			
	Fat-free diet	Fat-free diet + linoleate	Fat-free diet + vit. D	Fat-free diet + linoleate + vit. D
$C_{14:0}$	1.2 (0.8–1.4)*	1.4 (0.8–1.7)	1.1 (1.0–1.3)	1.1 (0.9–1.2)
$C_{16:1}$	0.5 (0.1–0.7)	0.5 (0.1–0.8)	0.5 (0.2–0.8)	0.3 (0.2–0.5)
$C_{16:0}$	20.8 (18.1–24.2)	20.2 (14.1–23.1)	21.5 (19.4–23.4)	21.1 (17.7–23.5)
$C_{18:1}$	12.8 (9.4–18.0)	9.8 (9.1–11.8)	13.0 (9.2–15.0)	10.0 (8.3–11.7)
$C_{18:0}$	0.6 (0.5–1.0)	0.7 (0.2–1.0)	0.6 (0.1–1.0)	0.8 (0.6–0.9)
$C_{18:2}$	0.9 (0.3–1.3)	0.8 (0.0–1.3)	0.8 (0.5–1.1)	1.0 (0.7–1.3)
$C_{18:3}$	8.8 (6.7–10.0)	15.0 (7.0–11.6)	10.0 (8.8–13.0)	10.1 (7.0–12.1)
$C_{20:1}$	33.5 (31.0–38.4)	26.6 (27.4–32.1)	33.8 (31.6–36.2)	29.8 (27.1–33.0)
$C_{20:2}$	3.5 (2.8–4.2)	4.7 (1.1–5.1)	3.3 (2.1–3.8)	5.6 (4.7–8.6)
$C_{20:3}$	0.1 (0.0–0.2)	0.6 (0.0–1.1)	0.3 (0.0–0.8)	0.5 (0.0–1.1)
$C_{20:4}$	0.6 (0.3–1.1)	0.6 (0.0–1.0)	0.2 (0.0–0.7)	0.6 (0.0–0.8)
$C_{20:5}$	7.0 (7.0–9.0)	4.5 (2.0–5.4)	8.2 (7.5–9.1)	4.4 (2.2–5.9)
$C_{22:4}$	4.1 (3.0–5.5)	10.7 (10.1–12.1)	5.1 (3.6–6.5)	10.3 (5.8–12.9)
$C_{22:5}$	0.9 (0.1–1.4)	2.5 (2.1–2.8)	0.6 (0.0–1.0)	2.2 (1.1–3.5)
$C_{22:6}$	1.0 (0.0–1.5)	0.7 (0.0–1.6)	0.9 (0.7–1.7)	1.1 (0.7–1.5)
$C_{20:3}/C_{20:4}$	1.9	0.4	1.6	0.4

*Mean of five determinations of four rats each (range).

Holman (7) as an indication of essential fatty acid deficiency is also shown in Table IV. Large doses of vitamin D had no influence on fatty acid composition.

In a third experiment young male rats were fed the fat-free diet and 0.1 ml of methyl palmitate, methyl erucate, methyl linoleate, or safflower oil daily. The safflower oil, which contained about 70% linoleate, was administered in an amount which provided the equivalent of 0.1 ml linoleate. Ten rats of each group of 20 received 5000 I.U. vitamin D per day. The results of this experiment, terminated after 3 weeks, are shown in Table V. In the presence or absence of vitamin D, linoleate or safflower oil promoted significantly greater

TABLE V

Effect of vitamin D and various fat supplements on weight gain, plasma calcium, and kidney calcification

Supplements		Food cons. (g)	Wt. gain (g)	Plasma Ca (mg/100 ml)	Calcific.
Fat (0.1 ml/day)	Vitamin D (5000 I.U./day)				
Palmitate	−	201±9*	49±4	8.78±0.36	0.5
Palmitate	+	181±6	31±3	11.21±0.45	2.5
Erucate	−	213±8	52±4	9.37±0.35	0.6
Erucate	+	180±9	31±4	9.51±0.32	4.0
Linoleate	−	221±7	68±4	8.17±0.34	0.8
Linoleate	+	204±6	47±3	11.00±0.46	3.1
Safflower	−	221±3	73±2	9.76±0.34	1.2
Safflower	+	213±8	62±5	11.30±0.36	3.6

*Mean ± S.E.

weight gains than did palmitate or erucate. Only safflower oil, however, prevented the decrease in weight gain caused by vitamin D, and only methyl erucate prevented an increase in plasma calcium. The kidney calcification which accompanied the administration of vitamin D was not reduced by any of the dietary oils.

The effect of triglycerides was further examined by feeding young male rats the fat-free diet supplemented daily with 0.1 ml butter fat, olive oil, safflower oil, or menhaden oil. Ten of the 20 rats receiving each oil were given 5000 I.U. vitamin D per day. Feces for calcium analysis were collected from all rats during the third week of the 5-week experiment. Safflower oil and menhaden oil produced significantly greater weight gains than did the fat-free diet, butter, or olive oil (Table VI). Vitamin D significantly depressed the growth of rats

TABLE VI

Effect of vitamin D and triglycerides on weight gain, plasma and fecal calcium, and kidney calcification

Supplements		Food cons. (g)	Wt. gain (g)	Plasma Ca (mg/100 ml)	Fecal Ca (mg/day)
Fat (0.1 ml)	Vitamin D (5000 I.U./day)				
−	−	520	104±6*	8.76±0.3	25.12±1.2
−	+	458	73±6	10.43±0.5	11.80±1.0
Butter	−	512	110±5	9.05±0.2	22.30±1.1
Butter	+	465	88±6	11.17±0.5	8.96±0.8
Olive	−	513	111±4	9.31±0.2	24.24±1.1
Olive	+	503	113±3	10.30±0.4	7.02±0.6
Safflower	−	550	138±5	9.45±0.5	22.50±1.2
Safflower	+	482	112±7	10.31±0.5	7.97±0.8
Menhaden	−	525	125±5	9.87±0.4	22.88±1.0
Menhaden	+	522	118±7	11.22±0.9	10.78±1.2

*Mean ± S.E.

fed the fat-free diet alone or supplemented with butter or safflower oil but not of those given olive or menhaden oil. None of the oils prevented the rise in plasma calcium caused by vitamin D. Fecal excretion of calcium was reduced by vitamin D, especially in rats given butter, olive oil, and safflower oil. In another similar experiment neither olive oil nor a mixture of olive oil and methyl linoleate (25 mg linoleate/0.1 ml of mixture) prevented the decrease in weight gain caused by 5000 I.U. vitamin D per day.

The effect of various fat supplements on linoleic-acid-depleted rats given non-lethal doses of vitamin D was measured. Male rats were depleted as described above except that respiratory tract infections were kept in check by the addition of terramycin (180 mg/liter) to the drinking water during the last 4 weeks of the depletion period. One hundred such rats were divided in five groups of 20, and each received daily 0.1 ml of butter oil, olive oil, or a mixture of olive oil and linoleate which provided 25 mg or 50 mg linoleate. A further group received no fat supplement. Ten rats of each group received, in addition, 5000 I.U. vitamin D per day. After 4 weeks the rats were killed, and plasma calcium

TABLE VII

Effect of vitamin D, butter, olive oil, and linoleate on weight, plasma calcium, and kidney calcification in essential fatty acid deficient rats

Supplements					
Fat (0.1 ml)	Vitamin D (5000 I.U./day)	Food cons. (g)	Wt. gain (g)	Plasma Ca (mg/100 ml)	Kidney calcific.
—	—	404±13*	14±5	9.25±0.17	1.0
—	+	276±12	−39±4	11.61±0.30	2.0
Butter	—	422±11	33±3	9.42±0.21	0.5
Butter	+	277±14	−27±6	11.90±0.20	2.2
Olive	—	412±14	42±5	9.20±0.28	0.6
Olive	+	279±15	−15±5	11.08±0.24	2.5
Olive and linoleate†	—	406±12	42±2	8.89±0.27	0.3
Olive and linoleate†	+	289±15	−10±6	11.32±0.29	3.0
Olive and linoleate‡	—	428±11	59±4	9.06±0.30	1.0
Olive and linoleate‡	+	323±17	−12±5	10.04±0.54	2.3

*Mean ± S.E.
†25 mg methyl linoleate per 0.1 ml.
‡50 mg methyl linoleate per 0.1 ml.

and kidney calcification determined. The results are shown in Table VII. Weight gain increased with the intake of linoleate and decreased significantly in all groups given vitamin D. None of the oil supplements influenced plasma calcium levels or kidney calcification.

Discussion

These experiments confirmed Sinclair's observation (1) that linoleate reduced mortality among rats given large doses of vitamin D. It seems likely, however, that this effect was non-specific and that a stress other than massive doses of the vitamin would influence more adversely rats deficient in an essential factor such as linoleate. The marked susceptibility of such animals to pneumonia was perhaps an example of this.

In the experiments with young rats, vitamin D alone always depressed weight gain significantly and this was not prevented by daily supplements of the pure fatty acids, linoleate, palmitate, erucate, nor by butter oil. The response to other triglycerides was variable; olive oil, safflower oil, and menhaden oil permitted normal weight gain in rats given vitamin D in one experiment each but these results could not be confirmed in subsequent experiments. The reason for these erratic results was not apparent and no conclusions could be drawn from the weight gain data.

In the entire series of experiments the increase in plasma calcium which accompanied ingestion of vitamin D was prevented on only two occasions. The oil supplements given on these occasions were linoleate and erucate and subsequent experiments failed to confirm this effect. Kidney calcification was always increased by vitamin D and none of the oil supplements had any effect on this

process. Vitamin D also markedly decreased fecal excretion of calcium and this effect was enhanced, not reduced, by fat supplements. The linoleate content of the supplements was not a factor in this.

While linoleate provided no protection against the toxicity of vitamin D in the experiments discussed above, the rats were not, strictly speaking, deficient in essential fatty acids. In one experiment with deficient rats those given olive oil or olive oil plus linoleate gained more weight than those given butter or no fat supplement but the difference in weight gain between those which received vitamin D and those which did not was as great or greater in the groups given linoleate as in those which were not.

In carcass and liver fatty acids of rats deprived of dietary fat, the increase in saturated, oleic, and palmitoleic acids was similar to that reported by Mead (8), and was not affected by the small daily supplements of linoleate. The lack of dietary linoleate was associated with a higher level in the liver of eicosatrienoic acid, a fatty acid which was identified by Mead and Slaton (9), and shown by Falco and Mead (10) to be derived from oleic acid. The small intake of linoleate significantly increased the level of that acid in the carcass fatty acids, and of arachidonic acid and docosatetraenoic acid in the liver fatty acids. Since arachidonic acid is synthesized from linoleic acid (11), it appeared that docosatetraenoic acid may have the same origin. Enser and Bailey (12), however, noted the appearance of docosatetraenoic acid in the fatty acids of the intestines of essential fatty acid deficient rats, but not in the control animals. Vitamin D had no influence on fatty acid composition, including the ratio of trienes to tetraenes (13) which has been used as an indication of essential fatty acid deficiency. Dietary cholesterol has been shown to aggravate a deficiency of essential fatty acids when it comprised 1% of the diet, but it was not possible to test a comparable level of vitamin D.

It can be concluded from these results that linoleate in the amounts used in these experiments did not significantly affect the toxicity of vitamin D for the rat. In several instances fat supplements permitted normal weight gain in the presence of large doses of vitamin D but the variability of the weight gain data made it impossible to form conclusions on this basis. Changes in plasma calcium, kidney calcification, fecal excretion of calcium, or body fat composition did not suggest any relationship between vitamin D and linoleate.

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Calcium-Utilization and Requirement

SUMMARY

In general, the body can adapt to varying calcium intakes by regulating the intestinal absorption and renal excretion of calcium. A number of factors influence calcium absorption, although the mechanisms are not clearly understood. Vitamin D is by far the most important exogenous factor affecting calcium absorption. The vitamin D metabolite, 1,25-dihydroxycholecalciferol, is recognized as the "hormonal form" of vitamin D responsible for the maintenance of serum calcium; it may also be involved in the synthesis of an intestinal calcium-binding protein. The extent to which this vitamin D-induced calcium-binding protein participates in calcium mobilization is a matter for speculation. The effect of both dietary phosphorus and the dietary ratio of calcium-to-phosphorus on calcium utilization is not firmly established. Dietary protein is directly correlated with urinary calcium excretion, although the long-term effect of protein on calcium retention remains to be determined. In the presence of lactose calcium absorption is increased. The interactions of phosphorus, magnesium, fat, oxalic acid, and phytic acid with calcium are discussed. The above nutrient interrelationships and the reports of increased calcium needs for those individuals predisposed to osteoporosis and those who do not readily adapt to varying calcium intakes should not be overlooked in the consideration of the optimal intake of calcium. It is obvious that there are considerable gaps in our information on the metabolism of calcium. Research on many areas of calcium metabolism, in particular the interrelationship of calcium with other nutrients, is a necessity.

In 1968 the Recommended Dietary Allowance (RDA) for calcium was reaffirmed at 0.8 g daily for the adult (1). This allowance was based on data available at the time relating to mandatory endogenous losses and true absorption of calcium. The Food and Nutrition Board (1) also recognized that the prevalence of osteoporosis (reduction in the quantity of bone) in the elderly could be attributed to long-term suboptimal calcium intakes and suggested that a daily allowance of 0.8 g calcium may grant little margin of safety for such individuals. The above RDA is expressed without reference to some of the dietary components recently suggested to affect calcium utilization.

It will be the purpose of this *Digest* to review current research concerning (a) some of the biochemical interrelationships, specifically between calcium and vitamin D, and (b) the nutritional interrelationships such as phosphorus, calcium-to-phosphorus ratio, protein, and lactose which may influence calcium utilization. The significance of the above factors in formulating a revised calcium allowance will also be discussed.

BIOCHEMICAL INTERRELATIONSHIPS

It is well established that calcium absorption is dependent upon the calcium needs of the body (2-6). That is, by increasing the absorptive capacity of the gut and by regulating renal excretion, the body can adapt itself to restricted dietary intakes of calcium and/or an increased requirement for calcium. With increasing calcium intake the percentage of calcium absorbed decreases; however, the actual amount of calcium absorbed is increased (6).

Vitamin D. One of the most important factors affecting calcium metabolism is an adequate supply of vitamin D, whether from the diet or from exposure to ultraviolet radiation (3,7,8). In northern latitudes and/or smoggy cities where ultraviolet radiation is limited or blocked, the dietary source of vitamin D becomes very important. Nutritional or conditional deficiencies of vitamin D and their effect on calcium metabolism are well documented (9).

Recent attention has been given to the active metabolites of vitamin D and the feedback regulation involved in

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its conversion to various forms. Cholecalciferol, the storage form of vitamin D_3 in man, is synthesized in the epidermal layer of the skin from 7-dehydrocholesterol by ultraviolet irradiation. Exposure to total solar or ultraviolet radiation influences the synthesis of endogenous cholecalciferol and subsequently calcium absorption. Cholecalciferol is further metabolized to its respective 25-hydroxylated compounds which are responsible for its actions on the intestine, skin, and bone. In the liver cholecalciferol is converted to 25-hydroxycholecalciferol (25-HCC), the major circulating metabolite (3,7,8,10). A direct correlation exists between individual 25-HCC concentrations, dietary vitamin D, exposure to sunlight, and total serum-calcium levels (7). In the kidney 25-HCC has been shown to be hydroxylated to 1,25-dihydroxycholecalciferol (1,25-DHCC) and 24,25-dihydroxycholecalciferol (24,25-DHCC). The conversion to 1,25-DHCC, the most active metabolite, is regulated by circulating calcium, parathyroid hormone, and calcitonin. The present theory holds that low serum-calcium levels stimulate the secretion of parathyroid hormone, the latter increasing the synthesis of 1,25-DHCC which controls the intestinal absorption of calcium and the remodeling of bone. Conversely, increased serum-calcium concentrations would cause a decreased release of parathyroid hormone and an increased release of calcitonin. The effect of the above reactions would be a decreased production of 1,25-DHCC, an increased production of 24,25-DHCC, and a decrease in the intestinal absorption of calcium and bone mobilization (3,10,11). According to some investigators (8,12) 1,25-DHCC represents the "hormonal form" of vitamin D responsible for the maintenance of serum calcium.

The calcium-transport system of the intestine may involve a vitamin D-induced calcium-binding protein (CaBP). In the intestine of several species a vitamin D-induced CaBP has been found (13-18) and preliminary studies have indicated the presence of two CaBPs in the human small intestine (3). However, the extent to which CaBP participates in calcium mobilization is subject to speculation (8).

NUTRITIONAL INTERRELATIONSHIPS

There are many interrelationships between calcium and dietary nutrients, some of which are receiving increased attention.

Phosphorus and the Calcium-to-Phosphorus Ratio. It is believed that an association exists between the intestinal absorption of calcium and phosphorus; however, the relationship is not clearly established (3). If the diet contains adequate calcium, large variations in dietary phosphorus may not influence intestinal calcium absorption (3,6,19-22).

Serum calcium concentration is influenced more by the dietary calcium-to-phosphorus (Ca:P) ratio than by the absolute intake of dietary calcium. Several investigators (6,19-22) have studied the effect of varying Ca:P ratios on calcium metabolism in animals. Excess dietary phosphorus or a low Ca:P ratio (less than 2:1) accelerates the rate of bone resorption in adult animals by a parathyroid-hormone mediated mechanism. Thus skeletal changes in animals may be induced by excess dietary phosphorus or insufficient dietary calcium.

It has not been firmly established whether the consumption of excess phosphorus induces mild chronic secondary hyperparathyroidism resulting in bone resorption in man. However in ordinary U.S. diets the intake of phosphorus invariably equals or exceeds the intake of calcium (1,23). The Ca:P ratio in U.S. diets has been estimated to be 1:2.8 (23), while the recommended ratio is 1:1 (1). During 1955-1965 the production in the U.S. of milk and milk products, which are the main source of dietary calcium, decreased by 10%. During the same period there was a 10% increase in terms of the average consumption of phosphorus-containing foods. The intake of phosphorus and/or the Ca:P ratio may not be in accord with what is recommended. The influence of the dietary Ca:P ratio in man is an area where considerable research is needed.

Protein. A direct correlation between dietary protein and urinary calcium excretion has been demonstrated (24-26). Urinary calcium excretion is increased in subjects fed high-protein diets. However, the effect of dietary protein on the apparent absorption and/or retention of calcium is not as well established as the effect on urinary calcium.

Lee et al (27) in a study with rats noted that a greater retention of calcium resulted when dietary protein was increased from 8.5% to 10%. In a study with adolescent boys the mean apparent calcium absorption appeared to be enhanced by a high-protein diet but no conclusions could be made regarding the retention data due to the high variability in the study (26).

Other investigators (24,25) provide data suggesting that high-protein diets decrease the absorption and retention of calcium and thus may be detrimental to calcium retention. Johnson et al (24) studied the effect of increasing protein intake from 48 to 141 g daily on calcium balance in six young men. Calcium and phosphorus intakes were maintained at 1400 mg daily. The substantial increase in urinary calcium following the high-protein intake was not attended by a comparable increase in calcium absorption, as measured by metabolic balance studies. It was suggested that hypercalciuria, if significant and chronic, could contribute to a negative calcium balance with secondary effects on the skeleton. Walker and Linkswiler (25) also demonstrated that high-protein intakes can result in negative calcium balance in adult males. In this

study calcium intake was maintained at 800 mg and the effect of three levels of dietary protein (47, 95, and 142 g) on calcium balance was examined. The subjects lost substantial amounts of calcium when fed the 142 g protein diet but retained calcium when they consumed the 47 or 95 g protein diet. When calcium is consumed within the range of 800 to 1400 mg, protein intake has a greater influence than calcium intake on urinary calcium excretion (24,25).

The possible effect of high-protein intakes on the pathogenesis of osteoporosis has been considered (24,25). It may be that high-protein intakes are detrimental to calcium retention under some conditions but not others, depending upon the amounts of dietary calcium and the composition of the remainder of the diet. It is possible that the effect of protein on calcium metabolism is only transitory and that long-term studies would demonstrate quite different results. The relationship between protein intake and calcium retention in the adult human should be further investigated (24-27).

Lactose. In the presence of lactose, the disaccharide of milk origin, calcium absorption has been shown to be enhanced (2,18,28-32). Condon et al (30) found that lactose ingestion improved calcium absorption in lactose-tolerant but not lactose-intolerant subjects. Thus, the improved absorption of calcium depended on the activity of normal intestinal lactase. In lactose-fed rats there was an increase in calcium retention as well as improved bone calcification; the beneficial effect of lactose was confined to the absorptive process of calcium and was more pronounced when dietary calcium was limited (31,32). The mechanism by which lactose improves calcium absorption has been an area of considerable investigation. Whether the effect of lactose is direct or dependent upon another variable is unknown (2,18). Additional studies of a long-term duration are required to discern whether the effect of lactose on calcium absorption is temporary or permanent.

Other Food Components. The effects of magnesium on calcium metabolism are controversial depending upon the experimental conditions (5,18). Some investigators have shown that magnesium increases the absorption, balance, and retention of calcium (33-35); whereas, others have found no effect of dietary magnesium on calcium absorption (24,26).

Decreased fat, bile, and bile salts may lessen the availability of calcium for absorption directly by the formation of insoluble calcium salts and indirectly by retarding exogenous cholecalciferol absorption (3).

Oxalic acid and phytic acid may interfere with the intestinal absorption of calcium by forming insoluble calcium salts within the intestinal lumen (1-3). Oxalic acid is found in rhubarb, spinach, chard, and beet greens; phytic acid is found mainly in the outer husks of cereal grain and is high in wholemeal flour. Many cereals contain the enzyme phytase which destroys phytic acid during the leavening of bread; thus unleavened bread contains phytic acid in greater amounts than leavened bread. Also the intestinal tract may contain a phytate-splitting enzyme. Although the levels of these compounds in the usual human diet may not be of practical significance, the increased concern and usage of "natural" foods and unleavened products made of wholemeal flour may result in sufficient increases of phytic acid in some dietaries to cause concern.

REQUIREMENTS NECESSARY FOR MAN

The evidence for the calcium allowance of 800 mg for the adult (1) is viewed with considerable uncertainty (2,5,36). Calcium is one nutrient in which dietary intakes are likely to be insufficient. It has been assumed (37) that well-defined disabilities attributable to low calcium intakes do not exist, but although the relationship to calcium intake varies from series to series, osteoporosis is found in all parts of the world. The dietary calcium allowance is 800 mg/day in the U.S. (1); 500 mg/day in Canada and the United Kingdom (36-38); and a range of 400 to 500 mg/day has been recommended by the Food and Agriculture Organization World Health Organization (36). The variation in allowances demonstrates how available data can be differently interpreted.

Most of the evidence relating to calcium requirements has come from: (a) metabolic balance studies (intake minus fecal, skin, sweat, and urinary output); (b) radioisotopic studies; and (c) surveys of dietary intakes (1,5, 36,39,40). Metabolic balance studies have been criticized due to (i) the ability of the body to adapt to ranging calcium intakes, (ii) the effect of prior calcium intakes, and (iii) the degree of body saturation and skeletal calcification at the time of the study (1,5,36,40). However, Whedon (39) claims that balance studies can be meticulously executed and offers many suggestions to achieve high reproducibility of results. It is emphasized that calcium balance studies must be of sufficient duration to allow early adaptation to occur if it will (5,39).

Whedon (39) using data compiled from several precise balance and radioisotopic studies calculated, by the factorial method, a dietary calcium allowance of 1086 mg based on an approximate upper limit of range of endogenous calcium loss of 380 mg (urinary output, 200 mg; fecal output, 150 mg; and dermal losses of 30 mg) and an absorption of 35%. The Food and Nutrition Board (1) arrived at their allowance of 800 mg for the adult on the basis of an estimate of 320 mg endogenous calcium loss (urinary output, 175 mg; endogenous fecal output, 125 mg; and dermal losses, 20 mg) and a 40% absorption. The allowance (1) was designed to provide a

buffer against the added needs of various stresses. Other countries (36-38) which recommend a lower allowance for calcium emphasize the adaptative aspect of intestinal calcium absorption and in many cases base their recommendation on the customary level of calcium in the diet. It is possible that many populations readily adapt to low calcium intakes with greater efficiency than others (39). Also, these populations may have relatively low protein and/or low phosphorus intakes. An allowance of 400 to 500 mg of calcium may not provide a very significant margin of safety if endogenous losses closely approximate the allowance (39). The problem is that it is not established whether the endogenous loss is fixed, or whether it varies with calcium intake. If the endogenous calcium loss varies with calcium intake then there may be no basis for using it in the calculations to determine the dietary calcium allowance. Whedon (39) states that allowances of 400 to 500 mg of calcium cannot be justified solely by the claim that deficiency diseases have not been observed in populations subsisting on calcium intakes of 300 to 400 mg when surveys have not adequately been conducted to elucidate the prevalence of osteoporosis.

Although some investigators (2,36,41) suggest the safety of a calcium allowance below 800 mg/day for the adult, there is also support for a calcium allowance of 800 mg or greater (23,39,42-44). In individual cases it cannot be assumed that adaptation to calcium intakes invariably occurs (39).

The suggestion is often reported that an intake of calcium of 800 to 1000 mg/day over the years may afford some protection against the development of osteoporosis in man (23,42-44); however, this hypothesis has still not received the critical study that such an important concept deserves. Whedon (39) observed that intakes of calcium greater than 800 mg/day were necessary to achieve calcium balance in most of the patients studied with postmenopausal or senile osteoporosis. Similarily Spencer et al (43) found that patients with osteoporosis had a decreased ability to absorb calcium from the intestine and thus greater calcium intakes were necessary to achieve calcium balance in these patients compared with control subjects.

Krook et al (23) and Lutwak et al (44) support a calcium allowance of at least 800 mg as a result of preliminary studies on human periodontal disease. These investigators have suggested that human periodontal disease is manifested by accentuated bone loss induced by dietary calcium insufficiency and/or excess dietary phosphorus. The explanation presented is that the bones of the skeleton vary in response to dietary calcium; alveolar bone being initially affected, followed by the vertebra, and finally long bones. It is suggested that bone loss in the jaws may be a precursor of osteoporosis. Studies on this aspect of calcium metabolism are being continued.

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THE DETERMINATION AND SOURCES OF VITAMIN D

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This article and others recently published or to be published comprise a new series on the present status of our knowledge of the vitamins. They have been prepared under the general auspices of the Council on Pharmacy and Chemistry and the Council on Foods. The opinions expressed are those of the authors and not necessarily the opinions of either council. Reprints are not available but the articles will be published later in book form. Ed.

The observations that rickets can be produced in rats[1] and that healing of induced rickets occurs in the proliferative zone of the cartilage led to the development of quantitative methods for the determination of vitamin D by the so-called line test procedure.[2] The basis for this procedure is the fact that within certain limits and under properly controlled conditions the degree of healing induced is proportional to the quantity of vitamin D fed. Severe rickets will develop in young rats in about three weeks, and curative periods of from five to ten days are sufficient to produce healing which can be evaluated. The degree of healing can be determined roentgenographically, but the most common procedure is to kill the animal and section and stain one of the leg bones.

Prior to the adoption of the U. S. P. method in 1934 the vitamin D content of pharmaceutical products in this country was indicated in three different units (Steenbock, A. D. M. A. and Poulsson, or Oslo), whose relative value to one another could only be approximated. Each one of these units was defined in terms of a method of assay bearing the same name. Fundamentally the procedures were the same and they were about equally serviceable, but the use of units of different values was responsible for confusion and misunderstanding. The elimination of this confusion was accomplished through the adoption in 1931 by the Health Organization of the League of Nations of an International standard for vitamin D[3] and an International unit, which was defined in terms of a definite quantity of the standard. A similar vitamin D standard had been used in Great Britain prior to that time. The International standard for vitamin D is irradiated ergosterol, prepared under specified conditions, dissolved in olive oil. The International unit is defined as "the vitamin D activity of 1 milligram of the International standard solution of irradiated ergosterol, which has been found equal to that of 0.025 microgram of crystalline vitamin D."

The International standards for vitamins are prepared under the auspices of the Health Organization of the League of Nations, and allotments are sent without charge to a central distributing agency in each country. With the extensive commerce in vitamin preparations in this country and consequent heavy demands for standards, it seemed desirable to conserve the supply of International standards. Accordingly the United States Pharmacopeia adopted (1934 Interim Revision) as a standard for vitamins A and D a specimen of cod liver oil and designated it the U. S. P. (Pharmacopeia) Reference Cod Liver Oil. A suitable quantity of a specially prepared cod liver oil was put up in ounce bottles. The oil was then assayed against the International standards for vitamins A and D. A U. S. P. unit of vitamin D is defined as "equal, in antirachitic potency for the rat, to one International Unit of Vitamin D as defined and adopted by the Conference of Vitamin Standards of the Permanent Commission on Biological Standardization of the League of Nations in June of 1931."

The method of assay referred to is the same as that given in the current Pharmacopeia and, while specific for cod liver oil, with slight modifications is suitable for the assay of a wide variety of vitamin D preparations. The method provides for comparing the vitamin D content of the oil under assay with the U. S. P. Reference Cod Liver Oil by the line test procedure and specifies the conditions for raising the experimental animals, the limits of the period for producing rickets, the duration of the assay period, the number of animals and their age and weight limits, feeding methods, a method for evaluating the degree of healing, the data that must be recorded, and how the vitamin D potency of the oil shall be calculated.

In applying this method to various types of products, due recognition must be given to its limitations. Success in developing experimental rickets is due in part to the feeding of a high proportion of calcium as compared to phosphorus. A disturbance of the prescribed calcium-phosphorus ratio will influence the degree of healing. The rate of healing is also influenced by change in the acid-base balance of the diet, ingestion of products which affect the rate of growth or compounds which influence the assimilation of calcium or phosphorus. For these reasons it is difficult and sometimes impossible to obtain satisfactory assays on food products by direct feeding. With foods fortified with vitamin D these problems can usually be met by extraction of the vitamin with suitable solvents or modification of the basal diet of the control animals.

While the U. S. P. or similar methods involving the line test are most extensively used for vitamin D determinations, the percentage of ash in the fat-free leg bones of the rat may also be used as a criterion in vitamin D assays. This procedure was used by Bethke, Steenbock and Nelson[4] as early as 1923 but has never received wide acceptance. It is more tedious than the line test but, being objective, could probably be developed to a greater degree of precision. However, efforts in this direction have not produced particularly promising results. Bone ash is also used as a criterion in vitamin D assays, with baby chicks in the tentative A. O. A. C. method.[5] The fact that rats and chicks do not respond in the same manner to different types of vitamin D[6] makes it possible by duplicate tests with rats and chicks to differentiate between vitamin D from irradiated ergosterol and from cod liver oil. While 20 U. S. P. units of vitamin D from cod liver oil per hundred grams of feed will permit development of chicks with normal bone ash, from 500 to 2,500 units of vitamin D in the form of

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activated ergosterol per hundred grams of feed may be required to provide the same degree of protection. The ratio widens with lengthening of the test period.

Biologic methods for some of the other vitamins are being supplemented by physical and chemical methods, but the latter are of practically no value in the determination of vitamin D. They can of course be applied to preparations of a high degree of purity and therefore have application in certain manufacturing processes. Evidence is accumulating that there are several forms of naturally occurring vitamin D and that their physiologic properties may differ. Vitamin D is a substance having great physiologic activity. A barrel of average grade cod liver oil contains approximately 0.25 Gm. of vitamin D. Pure vitamin D made by irradiation of ergosterol contains forty million units of vitamin D per gram. The chemist needs only one millionth of a gram for a biologic assay. Owing to the small quantities of vitamin D present in products, chemical tests are practically limited to color tests. When one considers that vitamin D belongs to the ubiquitous sterols, the probability of developing color tests with a high degree of specificity seems rather remote.

FOOD SOURCES OF VITAMIN D

There appears to be no proof that vitamin D exists in living plant tissue, and fresh green vegetables are generally considered entirely devoid of this vitamin. Plant tissue which is no longer living will, at least in some instances, acquire significant vitamin D potency when exposed to the sun, as is evidenced by the presence of vitamin D in hays[7] and cacao shells.[8] Natural foods which contain vitamin D are of animal origin.[9] Fish which contain much body oil, such as salmon, sardines and herring, are the richest natural sources; eggs are next in importance, and milk fat and meat products contain some vitamin D. Since this vitamin is not found in appreciable quantities in so many of the foods ordinarily consumed and since evidence of acute vitamin D deficiency occurs only in infants, it appears that the vitamin D requirement of man is very low or that his needs are usually provided for by exposure to sunshine.

Of the common foods fortified with vitamin D, only milk needs to receive serious consideration. The remainder are all too frequently transients and usually emblazoned with statements of their alleged virtues in a manner that cannot escape notice. If the use of foods fortified with vitamin D is contemplated, the labels of the products should be examined to determine whether their vitamin content is stated in units so that the claims can be checked. The types of vitamin D milk that are acceptable to the Council on Foods and the allowable claims for such milk have been published in THE JOURNAL.[10] Approximately one half of the evaporated milk manufactured is fortified with vitamin D, and approximately 700 dairies sell milk similarly fortified.

PHARMACEUTICAL PREPARATIONS

The vitamin D of pharmaceutical products is derived either from natural fish liver oils or artificially produced activated sterols. Some of the fish liver oils are offered as the pure oil, such as cod liver oil and halibut liver oil, and viosterol in oil contains only activated ergosterol in an edible vegetable oil, but a great many of the products offered are combinations of several of the sources of vitamin D. In addition the vitamin D preparations take the form of emulsions, tablets, capsules and malt preparations. The fact that the vitamins of fish liver oils can be removed in the unsaponifiable fraction, concentrated by direct extraction of the oil, or even distilled from the oil under a high vacuum makes it possible to manufacture preparations which have advantages in ease of administration. Products which are either tasteless or very palatable are readily available.

With an ever increasing array of vitamin D preparations before him, what guide does the physician have in the selection of a vitamin D preparation with respect to vitamin D content? There are U. S. P. standards for only three vitamin D preparations. "Cod Liver Oil" is required by the U. S. Pharmacopeia to contain at least 85 U. S. P. units of vitamin D per gram. A teaspoonful contains at least 312 units, calculated on the basis that a teaspoonful is 4 cc. and the oil has a specific gravity of 0.92. A product labeled "Emulsion of Cod Liver Oil" must contain at least 50 per cent cod liver oil by volume, and the vitamin D potency of the oil used must be equal at least to the minimum U. S. P. standard. "Viosterol in Oil" must contain not less than 10,000 units of vitamin D per gram. In addition there are National Formulary standards for three cod liver oil emulsions. "Emulsion of Cod Liver Oil with Hypophosphites" and "Emulsion of Cod Liver Oil with Egg" must contain 50 per cent cod liver oil by volume, and "Emulsion of Cod Liver Oil with Malt" must contain 30 per cent cod liver oil by volume. The Federal Food and Drugs Act provides that a drug sold under or by a name recognized in the U. S. Pharmacopeia or National Formulary must conform to the standard of strength required by the Pharmacopeia or National Formulary. The only vitamin D preparations listed in these publications are those named. The Federal Food and Drugs Act also requires that a drug must meet the standard of strength under which it is sold. Statements of vitamin potency are therefore subject to check by the Food and Drug Administration. The readers of THE JOURNAL are familiar with the vitamin preparations listed in New and Nonofficial Remedies and the requirements for their acceptance. This publication should be consulted for useful information concerning not only classes of products but individual products as well.

During the past few years a large number of new types of vitamin D preparations have appeared on the market. With the discovery of new sources of vitamin D and new methods of preparing vitamin concentrates and the application of new scientific methods in the synthesis of the vitamin, more types of products must be expected before the industry becomes stabilized.

SUMMARY

Within the past few years the method of expressing vitamin D potency has become standardized. Vitamin D content is expressed either in U. S. P. or International units, and these units are equal in value. Biologic methods for the determination of vitamin D are now available which permit satisfactory control over preparations intended for use by the physician. There are at present no physical or chemical methods which give promise of replacing biologic assays.

Most foods appear to be devoid of demonstrable quantities of vitamin D. Fish which contain much body

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oil, such as salmon, sardines and herring, are the richest natural sources, eggs are next in importance, and milk fat and meat products contain some vitamin D. Vitamin D milk is now being used extensively as a dependable dietary source of vitamin D.

A great variety of pharmaceutical preparations of vitamin D are available for therapeutic use, and new types can be expected to make their appearance. Since there are minimum standards for vitamin D content for only a few of these preparations, statements of vitamin potency must be relied on for their intelligent use.

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COUNCIL ON FOODS AND NUTRITION

The Council has authorized publication of the following report.

PHILIP L. WHITE, Sc.D., *Secretary.*

DETERIORATION OF VITAMIN D WHEN MIXED WITH CALCIUM SALTS

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It has been known for fully 25 years that vitamin D is not stable when mixed with a preparation containing 25% or more of minerals. In 1932, the observation was made that, when mixed with limestone, cod liver oil did not retain its vitamin D.[1] Cod liver oil was added to ground limestone and the mixture stored in glass jars in the laboratory. When examined two weeks later, the mixture was devoid of the vitamin. With further investigation, researchers found that cod liver oil lost vitamin D rapidly when added to a salt mixture containing all of the mineral elements necessary for growth of the rat. However, the loss was just as rapid when the oil was mixed with a preparation of silica of the same degree of fineness. Loss of vitamin D at temperatures ranging from 85 to 100 F was more rapid than at room temperature, and less rapid in mixtures kept under refrigeration. Since no catalytic effect of the minerals could be demonstrated, it was concluded that the loss was due primarily to the greater surface area and a resulting increased exposure to air.

Since that time, we have examined a large number of vitamin D preparations on the market containing various calcium salts. In a sample of 50 preparations we assayed, 4 were devoid of vitamin D entirely, 5 had an excess of vitamin D of 100% or more, 20 contained a substantially greater excess of vitamin D than did the nonmineral vitamin D products, and the remainder were of satisfactory composition at the time of examination. Both vitamin D_2 and vitamin D_3 have been found, frequently in combination with di-calcium phosphate, but a variety of other salts and salt mixtures have been used. Products of this type generally are offered for use during pregnancy and are prescribed to a considerable extent by dentists.

The annual report of the Federal Security Agency for 1948 carried this statement: "Vitamin D is generally regarded as stable, but it deteriorates rapidly in the presence of minerals. This fact was first observed many years ago in mineral feeds, and feed manufacturers are now generally aware of the difficulty. Some pharmaceutical manufacturers continue to incorporate the calcium phosphate and other minerals in vitamin D tablets. Almost invariably these products are found low in vitamin D within a month or two of the time of manufacture."

Since that report was published, there have been some important developments, but at the present time the situation is far from satisfactory. Some manufacturers have tried to compensate for this deterioration by adding vitamin D in excess of the amount declared, but this is not a practical method of providing a satisfactory product due to the uncertain rate of loss. About two years ago, different forms of the so-called stabilized vitamin D came into use. The vitamin D in powdered form is coated in such a way that it is protected from air, even when used in a powdered mineral preparation. We do not know how well these coated preparations withstand the pill-making process in which the walls of the coated particles conceivably may be ruptured.

Pharmaceutical manufacturers usually have been prompt in discontinuing distribution of unsatisfactory preparations, but some have been reluctant to do so. With a chemical method for detection of vitamin D now available,[2] there should be no reason for continued distribution of unstable products of this type.

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From the Division of Nutrition, Food and Drug Administration, Department of Health, Education, and Welfare.

†Dr. Nelson died Dec. 24, 1958.

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Actions of Vitamin D Independent of the Parathyroid Glands

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ABSTRACT. Vitamin D was shown to decrease tubular reabsorption of P in rachitic dogs that were parathyroidectomized. This effect was not consistently preceded by an increase in the serum Ca. The actions of vitamin D were much slower in onset than similar effects of parathyroid extract, and this could not be attributed to a delay in absorption of the vitamin. Finally, unlike parathyroid hormone, vitamin D failed to increase OHPr excretion. (*Endocrinology* **82:** 760, 1968)

PREVIOUS studies showed that vitamin D produces an increase in the renal tubular reabsorption of phosphorus (P) in rachitic dogs with intact parathyroid glands (1). These results could have been due to a direct action of vitamin D on the tubules, or they could have been due to a suppression of parathyroid function resulting from the effect of the vitamin to increase the serum calcium (Ca). In view of these limitations in studying the actions of vitamin D in the presence of the parathyroid glands, it was of interest to observe the effects of vitamin D in the state of hypoparathyroidism. Albright found that vitamin D produced phosphaturia in patients with hypoparathyroidism, and, although clearance studies were not performed, the results suggested that a decrease in tubular reabsorption of P had occurred (2). Crawford, Gribetz and Talbot, utilizing clearance studies in the parathyroidectomized rat (3), demonstrated that vitamin D decreases tubular reabsorption of P.

Our experiments have examined certain actions of vitamin D independent of the parathyroid glands in the dog. Animals were made deficient in vitamin D and parathyroidectomized; observations made under these conditions served as a reference for serial observations made during the subsequent administration of vitamin D. These studies were performed with three purposes in mind, namely: a) to provide an explanation for the previous divergent results (1–3) with regard to the effects of vitamin D on tubular reabsorption of P, by performing clearance studies in parathyroidectomized dogs; b) to determine whether effects of vitamin D on the tubules are consistently preceded by, and therefore perhaps dependent upon, vitamin-induced changes in calcium metabolism; and c) to compare the independent actions of vitamin D with those of parathyroid hormone. In the latter case, we compared the results of the present study with vitamin D with our previous experiments (4) in which parathyroid hormone was given to vitamin D-deficient, parathyroidectomized dogs under similar experimental conditions.

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Materials and Methods

Thirteen mongrel pups were placed on a constant vitamin D-deficient diet, the components of which have been described previously (4). The animals were housed in cages free from ultraviolet light. After approximately 3 months, the animals manifested the clinical and chemical abnormalities of rickets (4). In addition, vitamin D activity was not detectable in their serum, with the lower limit of sensitivity of the bioassay being 25 U/100 ml (Table 1). In contrast, vitamin D activity was consistently demonstrable in the serum of normal pups of comparable age (4). When vitamin D deficiency was established, the animals underwent thyroparathyroidectomy (following which each animal received 65 mg desiccated thyroid daily). Clearance studies were per-

formed 6 8 hr postoperatively. Five of the rachitic, parathyroidectomized pups were then given an intravenous infusion of vitamin D_2[1] at a dosage of 500 U/min for 2 hr, during which time clearance studies were repeated. Four of these pups and 5 additional rachitic, parathyroidectomized animals (which had not received vitamin D infusions) were given 100,000 U of vitamin D_2[2] intramuscularly, and clearance studies were performed 24 hr later. Finally, 5 animals were studied after they had received vitamin D_2[3] orally at a dosage of 30,000 U daily for 6 days or more. Since the action of vitamin D in increasing the serum calcium was slow in onset (see below), it was occasionally necessary to administer calcium gluconate as an intravenous or intramuscular injection (providing 90 mg of calcium) for the treatment of tetany; however, no such injections were given for at least 1 day prior to the performance of clearance studies. Several animals also received in injections of 100 U of parathyroid extract[4] for the treatment of tetany; however, these injections were withheld for at least 2 days prior to the performance of clearance studies. Two rachitic animals were maintained after parathyroidectomy on Ca lactate 20 g daily, but without vitamin D.

The animals were fasted for 18 hr prior to the clearance studies. Alpha-chloralose was employed as the anesthetic during the performance of the clearances. Each of the clearance periods was 20 min in duration, and whenever possible observations were made for 3 or more successive periods at each phase of the studies. Indwelling bladder catheters were used for the urine collections, and a blood specimen was obtained in the middle of each clearance period.

Serum and urinary Ca were measured with the flame photometer (5). Serum and urinary P were determined by the method of Fiske and Subba Row (6). Urinary hydroxyproline (OHPr) was measured by the method of Prockop and Udenfriend (7). Inulin was infused for the estimation of glomerular filtration rate as previously described (8); serum and urinary inulin were determined by the method of Walser, Davidson and Orloff (9). Serum vitamin D assays utilized the epiphyseal line test in the vitamin D-deficient rat (10).

The data were analyzed with the Student t test, using paired values for each animal in order to determine the significance of changes under various experimental conditions (11).

TABLE 1. Serum vitamin D concentrations in rachitic, parathyroidectomized pups before and during vitamin D administration (dosage and route of administration of vitamin D are described in detail in the text under Materials and Methods)

Dog	Serum vitamin D U/100 ml			
	Before	←—Vit. D administration—→		
		2 hr	24 hr	>6 days
827E	<25		222	934
T933	<25		346	
526D				1975
617A	<25		293	1260
832A	<25		260	
X781	<25	1200		
486L	<25	1200		
T782	<25		235	
Mean	<25	1200	271	1390

Results

Effects of vitamin D on tubular reabsorption of P. In our previous studies, we showed that tubular reabsorption of P was decreased in rachitic pups, but this increased markedly after parathyroidectomy (4). In the present experiments, tubular reabsorption of P was similarly virtually complete in the rachitic, parathyroidectomized pups, and consequently P excretion was negligible (Table 2). The intravenous infusion of vitamin D_2 over a two-hour period was without effect. However, 24 hours after the intramuscular injection of vitamin D_2, a decrease in the percentage of the filtered P which was reabsorbed by the tubules (% TRP) was observed in seven of nine animals. Furthermore, the % TRP was decreased in each of five animals that received vitamin D_2 30,000 U daily for 6 days or more. The increased excretion of P was not consistently accompanied by an increase in serum P (Table 3), nor was it accompanied by an increase in the glomerular

[1] Crystalline vitamin D_2 (40,000 U/mg) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. This preparation was dissolved in 1 ml Tween 80, and then diluted to an appropriate volume with saline for intravenous infusion. The diluted preparation was fully potent when assayed in the rachitic rat (10).

[2] Vitamin D_2 for intramuscular injection (Hi-Derated) was obtained from Brewer and Co., Worcester, Mass.

[3] Vitamin D_2 for oral administration (Drisdol) was obtained from Winthrop Laboratories, New York, N. Y.

[4] Parathyroid extract was obtained from Eli Lilly Co., Indianapolis, Ind.

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filtration rate [the latter actually showed a tendency to decrease (Table 4)]. The increase in P excretion was therefore not a function of an increase in the filtered load of P. Of the ten pups that received vitamin D for 24 hours or more, phosphaturia was eventually observed in eight, an effect which could be ascribed to a decrease in the quantity of P reabsorbed by the renal tubules ($p < .01$ for the combined data). Two rachitic animals were maintained after parathyroidectomy on oral supplements of 20 g of calcium lactate daily, but without vitamin D. In one of these animals, a TRP of 99% was found ten days after parathyroidectomy, and a similar value was found in the second animal 26 days after parathyroidectomy.

It was of interest that the vitamin D-induced decrease in tubular reabsorption of P was often evident prior to an effect of vitamin D to increase the serum Ca (Table 5). Thus, the reabsorption of P was decreased in animals T 933, 617A, 473P, and 65N within 24 hours of the administration of vitamin D (Table 2), but the serum calcium had shown no increase at this time. Although only limited observations of urinary Ca were made (Table 5), this also did not consistently increase prior to a decrease in tubular reabsorption of P.

Studies of OHPr excretion. We have previously shown (4) that OHPr excretion is increased in rachitic pups, that it decreases after parathyroidectomy, and that it is again increased with the infusion of parathyroid extract. These results suggested an important role of the parathyroids in regulating OHPr excretion, presumably reflecting an effect on bone collagen metabolism. It was therefore of interest to determine whether vitamin D, like parathyroid extract, would increase OHPr excretion in the rachitic, parathyroidectomized pups. Table 6 shows that such an effect of vitamin D was not demonstrable.

Serum vitamin D assays. It was note-

TABLE 2. Phosphorus (P) filtered, excreted, reabsorbed and the percentage of filtered P reabsorbed (% TRP) in rachitic, parathyroidectomized pups before and during vitamin D administration

Dog	P filtered µg/min				P excreted µg/min				P reabsorbed µg/min				% TRP			
	Before vit. D	←Vit. D administration→ 2 hr	24 hr	>6 days	Before vit. D	←Vit. D administration→ 2 hr	24 hr	>6 days	Before vit. D	←Vit. D administration→ 2 hr	24 hr	>6 days	Before vit. D	←Vit. D administration→ 2 hr	24 hr	>6 days
827E	2360 (4)	—	3780 (4)	2350 (4)	5 (4)	—	451 (4)	101 (4)	2555 (4)	—	3321 (4)	2240 (4)	100 (4)	—	88 (4)	96 (4)
T933	2519 (4)	—	2320 (4)	—	10 (4)	—	127 (4)	—	2530 (4)	—	1933 (4)	—	100 (4)	—	94 (4)	—
526D	2710 (1)	—	—	2090 (2)	6 (1)	—	—	295 (2)	2736 (1)	—	—	1785 (2)	100 (1)	—	—	86 (2)
617A	1979 (1)	—	1206 (4)	1080 (3)	98 (1)	—	235 (4)	181 (3)	1872 (1)	—	1061 (4)	899 (3)	95 (1)	—	82 (4)	83 (3)
832A	592 (4)	—	594 (4)	—	2 (4)	—	2 (4)	—	560 (4)	—	592 (4)	—	100 (4)	—	100 (4)	—
N781	2810 (3)	2680 (3)	2850 (3)	1830 (4)	17 (3)	6 (3)	217 (3)	217 (4)	2823 (3)	2654 (3)	2633 (3)	1608 (4)	99 (3)	100 (3)	92 (3)	88 (4)
486L	1172 (3)	1280 (3)	—	—	17 (3)	26 (3)	—	—	1455 (3)	1254 (3)	—	—	99 (3)	98 (3)	—	—
817N	1152 (3)	1308 (2)	1476 (3)	2134 (3)	2 (3)	1 (2)	215 (3)	230 (3)	1150 (3)	1387 (2)	1260 (3)	1474 (3)	100 (3)	100 (2)	87 (3)	89 (3)
473P	1701 (2)	1512 (3)	1365 (3)	—	1 (3)	2 (3)	415 (3)	—	1700 (3)	1510 (3)	950 (3)	—	100 (3)	100 (3)	70 (3)	—
65N	1068 (4)	1068 (3)	940 (2)	—	37 (4)	51 (3)	435 (2)	—	1041 (3)	1917 (4)	501 (2)	—	99 (3)	98 (3)	53 (2)	—
T782	2070 (4)	—	3230 (3)	—	3 (4)	—	5 (3)	—	2937 (4)	—	3325 (3)	—	100 (4)	—	100 (3)	—
Mean	1901	1758	1906	1893	18	17	255	205	1913	1741	1731	1688	99	99	85	88
± SEM	±101	±256	±370	±220	±8	±10	±58	±32	±215	±254	±376	±222	+1	±1	±5	±2

Values are the mean of the observations at each phase of the studies. Number of observations is in parentheses. Dosage schedules of vitamin D are described in the text.

TABLE 3. Serum phosphorus in rachitic, parathyroidectomized pups before and during vitamin D administration

Dog	Serum phosphorus mg/100 ml			
	Before vit. D	Vit. D administration		
		2 hr	24 hr	>6 days
827E	6.4 (4)		7.7 (4)	6.9 (4)
T933	4.1 (4)		4.9 (4)	
526D	6.1 (1)			6.5 (2)
617A	4.2 (1)		3.6 (4)	4.7 (3)
832A	3.3 (4)		3.3 (4)	
X781	7.1 (3)	6.8 (3)	6.1 (3)	6.5 (4)
486L	6.4 (3)	6.4 (3)		
817N	4.8 (3)	5.7 (2)	5.9 (3)	9.7 (3)
473P	6.3 (3)	7.2 (3)	6.5 (3)	
65N	8.2 (3)	8.2 (3)	7.3 (2)	
T782	5.3 (4)		6.8 (3)	
Mean ±SEM	5.6±0.4	6.9±0.4	6.0±0.5	6.9±0.8

Values are the mean of the observations at each phase of the studies. Number of observations is in parentheses. Dosage schedules of vitamin D are described in the text.

worthy that the actions of vitamin D on both P and Ca metabolism were much slower in onset than similar effects of parathyroid extract (4). It was possible that vitamin D was slowly absorbed into the circulation. This possibility was partly excluded by the studies in which vitamin D was given intravenously, but produced no effects. In addition, vitamin D activity was present in the serum, often in concentrations that were elevated as compared to those of normal pups (4), after it was given intravenously, intramuscularly, or orally (Table 1).

Discussion

The present experiments in the dog confirm previous results in the rat (3) in showing that vitamin D exerts an effect to decrease tubular reabsorption of P. This effect was evident in the absence of the parathyroids. In experiments in the dog in which the parathyroid glands were intact (1), the predominant effect may have been the suppression of parathyroid function, thereby masking the independent effect of the vitamin on the tubules and leading eventually to a net increase in tubular re-

TABLE 4. Inulin clearances in rachitic, parathyroidectomized pups before and during vitamin D administration

Dog	Inulin clearance ml/min			
	Before vit. D	Vit. D administration		
		2 hr	24 hr	>6 days
827E	47 (4)		49 (4)	34 (4)
T933	62 (4)	42	42 (4)	
526D	45 (1)			32 (2)
617A	47 (1)		36 (4)	23 (3)
832A	17 (4)		18 (4)	
X781	40 (3)	39 (3)	47 (3)	28 (4)
486L	23 (3)	20 (3)		
817N	24 (3)	24 (2)	25 (3)	22 (3)
473P	27 (3)	21 (3)	21 (3)	
65N	24 (3)	24 (3)	13 (2)	
T782	59 (4)		49 (3)	
Mean ±SEM	36±4	26±3	33±6	28±2

Values are the mean of the observations at each phase of the studies. Number of observations is in parentheses. Dosage schedules of vitamin D are described in the text.

TABLE 5. Serum and urinary calcium in rachitic, parathyroidectomized pups before and during vitamin D administration

Dog	Before vit. D	Vit. D administration 2 hr	24 hr	>6 days
		Serum Ca mg/100 ml		
827E	7.4 (4)		5.0 (4)	6.3 (4)
T933	6.1 (4)		4.3 (3)	
526D	6.7 (2)			8.2 (2)
617A	9.0 (1)		9.4 (2)	10.8 (2)
832A	8.9 (2)		8.3 (2)	
X781	9.9 (3)	7.8 (1)	11.6 (2)	11.2 (4)
817N	6.2 (3)	7.9 (3)	6.8 (2)	6.8 (2)
473P	5.6 (2)	5.7 (1)	4.3 (2)	
65N	4.6 (2)	4.8 (2)	4.5 (2)	
T782	9.4 (3)		7.2 (3)	
Mean ± SEM	7.4 ± 0.6	6.6 ± 0.8	6.8 ± 0.9	8.7 ± 0.8
		Urinary Ca μg/min		
X781	660 (2)	210 (2)	230 (2)	362 (2)
817N	415 (2)	305 (2)		673 (2)
473P	448 (2)	104 (2)	352 (2)	
65N	515 (2)	473 (2)	588 (2)	
Mean ± SEM	510 ± 54	273 ± 78	390 ± 105	518 ± 156

Values are the mean of the observations at each phase of the studies. The number of observations is indicated in parentheses. The dosage schedules of vitamin D are described in the text.

absorption of P. It was of interest that, despite a deficiency of vitamin D, tubular conservation of P was almost complete after parathyroidectomy (Table 2). This finding is in keeping with the interpretation that vitamin D does not maintain reabsorption of P through a direct tubular effect; in all probability, the decreased reabsorption of P in vitamin D deficiency is due to a secondary hyperparathyroidism (4), and hence this is corrected when the parathyroids are removed.

Since an increase in serum Ca in itself has been shown to decrease tubular reabsorption of P (12), it was of interest to determine whether the tubular effects of the vitamin were consistently preceded by changes in serum Ca. In several animals, at least, the vitamin produced a phosphaturia prior to an increase in serum or urinary Ca.

It was not possible to study rachitic animals after parathyroidectomy without any treatment at all, since the animals did not survive in the untreated state for more than about 12 hours. We therefore cannot completely exclude the possibility that the decrease in tubular reabsorption of P during vitamin D administration was a spontaneous occurrence not dependent on vitamin D. However, the administration of calcium supplements without vitamin D to rachitic, parathyroidectomized pups failed to produce a phosphaturia. Albright (2) also noted that the administration of calcium either intravenously or orally, without vitamin D, failed to produce phosphaturia in patients with hypoparathyroidism. Further, spontaneous increases in P excretion have not been found in untreated patients with hypoparathyroidism (2). Thus, it appears likely that the effects on tubular reabsorption of P are attributable to the vitamin D.

It was also of interest to compare the actions of vitamin D with those of parathyroid hormone. In previous experiments (4), we gave parathyroid extract to rachitic, parathyroidectomized dogs under conditions similar to those of the present study. While the effect of parathyroid extract in decreasing tubular reabsorption of P occurred within approximately 30 minutes (13), this action of vitamin D was much slower in onset. The delay could not be attributed to a failure of the vitamin in reaching the circulation (Table 1). It is

TABLE 6. Urinary OHPr in rachitic, parathyroidectomized pups before and during vitamin D administration

Dog	Urinary OHPr μg/min			
	Before vit. D	Vit. D administration		
		2 hr	24 hr	>6 days
827E	41 (2)			
T933	46 (2)			20 (2)
526D	17 (3)		34 (2)	
617A	5 (1)			7 (3)
832A	3 (2)		17 (2)	11 (2)
X781	40 (2)	24 (2)	3 (2)	
486L	11 (2)	9 (2)	43 (2)	18 (1)
817N	18 (1)			
473P	39 (1)	16 (1)	8 (1)	20 (1)
65N	20 (1)	15 (1)	12 (1)	
T782	43 (2)		14 (1)	
Mean ± SEM	25 ± 5	16 ± 3	20 (2) 19 ± 5	15 ± 3

Values are the mean of the observations at each phase of the studies. Number of observations is indicated in parentheses. Dosage schedules of vitamin D are described in the text.

still possible, however, that vitamin D was slower in reaching a common cellular receptor with parathyroid extract; alternatively, the two substances might act through different mechanisms. Vitamin D was also slower in increasing the serum Ca, and exerted this effect only after a period of several days (Table 5). It is not possible to determine from these studies whether this effect on Ca metabolism resulted from mobilization of bone mineral, or from an increase in intestinal absorption of Ca (or, indeed, from a combination of these effects). Recent studies by Toverud (14) have suggested that the vitamin does increase mobilization of bone mineral, in addition to its known action on intestinal absorption of Ca. However, it would seem that vitamin D and parathyroid hormone may affect bone metabolism through different mechanisms. This is suggested by the finding that the vitamin does not appear to affect collagen metabolism [as reflected in OHPr excretion (Table 6)], while parathyroid hormone clearly does so (4, 15–17).

The present experiments do not allow a conclusion as to whether the decrease in tubular reabsorption of P that occurs during vitamin D administration is due to a direct effect on the tubules, or whether it is secondary to some other effect associated with the administration of the vitamin. Finally, it is recognized that large doses of vitamin D were utilized in the present experiments; this was necessary for the maintenance of the animals in the state of hypoparathyroidism. This is analogous to the requirement for large doses of vitamin D in human subjects with hypoparathyroidism. The much smaller doses which are required when the parathyroids are intact might not have demonstrable tubular effects.

Acknowledgment

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Second International Seminar on Reproductive Physiology and Sexual Endocrinology

The Second International Seminar on Reproductive Physiology and Sexual Endocrinology will be held in Brussels, Belgium, May 30–June 1, 1968, under the auspices of the Fondation Jean Snoeck and the Ford Foundation, and will be held in the Department of Obstetrics and Gynecology, Hôpital Universitaire Saint-Pierre, Bruxelles. The main program topics will be Ovo-Implantation and Human Gonadotropins and Prolactin.

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Prof. P. O. Hubinont
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Actions of Parathyroid Hormone in the Vitamin D-deficient Dog *

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The effects of parathyroid hormone on bone and kidney have been well documented (1). It has not been settled, however, whether the hormone exerts similar actions in the rachitic animal. Rasmussen and associates have found that parathyroid hormone lowers the serum phosphorus in the vitamin D-depleted rat (2). Harrison and Harrison, however, observed that vitamin D-depleted rats did not respond to parathyroid hormone with either an increase in urinary phosphorus or a decrease in serum phosphorus (3). In both of these studies, the serum calcium of vitamin D-depleted rats failed to increase after an injection of parathyroid extract at a dosage that elevated the serum calcium of control animals (2, 3). In contrast, in recent studies by Tverud (4, 5), it was found that the serum calcium decreases after parathyroidectomy in vitamin D-depleted rats; an increase in serum calcium was observed after the administration of parathyroid extract to the vitamin D-depleted, parathyroidectomized animals. Earlier investigations (6) had shown that parathyroid extract could increase the serum calcium in the rachitic dog, although the effect was not as great as that in normal dogs and bore no consistent relationship to dosage. However, the rachitic dogs were not parathyroidectomized before the administration of parathyroid extract. It was therefore possible that the animals were under the influence of large quantities of endogenous parathyroid hormone, which obscured the action of exogenous extract. In the present studies, serial observations have been made of calcium and phosphorus metabolism and of hydroxyproline excretion in the vitamin D-depleted dog. Animals were studied before and after parathyroidectomy and with the subsequent administration of parathyroid extract.

Methods

Seventeen mongrel pups were placed on a rachitogenic diet at the time of weaning. The daily diet for each animal contained 400 g of oatmeal, 100 g of powdered skimmed milk, 7 g of brewer's yeast, 3.5 g of NaCl, 10 mg of vitamin C, 1,000 U of vitamin A, and 15 ml of peanut oil. The diet provided 200 mg of calcium and 800 mg of phosphorus daily. Five control pups of comparable age were studied while on an identical diet; these animals also received 500 U of vitamin D_2 daily. These additional control pups were maintained on a constant diet of standard dog food, providing 300 mg of calcium and 1,000 mg of phosphorus daily. All animals were kept in a room protected from sunlight, illuminated by an ultraviolet free light source.

Phosphorus and inulin clearances were measured in 14 rachitic dogs while they were on diet alone. The studies were repeated in 12 animals 3 to 6 hours after thyroparathyroidectomy, and in six of these, clearance studies were performed again with the subsequent infusion of parathyroid extract. Phosphorus clearances were measured in six control dogs while on constant diet alone, and in three of these inulin clearance was also measured. Animals were fasted 16 hours before the studies. The anesthesia employed was 1% alpha chloralose in 0.9% NaCl. Inulin and phosphorus clearance studies were performed as described previously (7). Each clearance period was 30 minutes in duration, and blood was obtained in the middle of the period. Whenever possible, data were obtained for three or more successive periods in each phase of the study.

Serum and urinary phosphorus as inorganic phosphate were measured by the method of Fiske and Subbarow (8). Serum and urinary calcium were determined with the flame photometer (9). Urinary hydroxyproline was

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TABLE I

Comparison of data for normal and rachitic dogs

	Normal Mean±SEM	Rickets Mean±SEM	p value
Serum vitamin D *U/100 ml*	47.2 ± 5.5 (7)*	<25 (7)	<0.001
Serum alkaline phosphatase *King-Armstrong units*	13 ± 2 (4)	47 ± 7 (9)	<0.02
Serum calcium *mg/100 ml*	11.7 ± 0.4 (10)	8.5 ± 0.5 (12)	<0.005
Serum phosphorus *mg/100 ml*	8.3 ± 0.5 (8)	4.7 ± 0.2 (14)	<0.001
Urinary phosphorus *μg/minute*	159 ± 71 (6)	206 ± 26 (14)	<0.50
Clearance phosphorus *ml/minute*	2.2 ± 0.4 (6)	4.6 ± 0.6 (11)	<0.02
Clearance inulin *ml/minute*	43 ± 7 (5)	34 ± 5 (8)	<0.40
Urinary hydroxyproline *μg/minute*	32 ± 3 (5)	48 ± 6 (8)	<0.05

* The number of animals is indicated in parentheses.

measured by the method of Prockop and Udenfriend (10). The coefficient of variation was 2.2, 3.0, and 2.3% for the calcium, phosphorus, and hydroxyproline methods, respectively (11). Inulin was measured in blood and urine by the method of Walser, Davidson, and Orloff (12). Serum alkaline phosphatase was measured by the method of King and Armstrong (13). Serum vitamin D assays[1] were performed in the rachitic rat according to the method of Shue, Friedman, and Tolle (14). This method is capable of detecting as little as 1 U of vitamin D. Since the maximal amount of serum that was administered to a rat was 1 ml, the lower limit of sensitivity of the assay was 25 U per 100 ml of serum.

When the data for the rachitic pups were compared with those for the control pups, the significance of differences between the two groups was determined by the Student *t* test (15). When rachitic animals were studied serially, e.g., before and after parathyroidectomy, the observations for each animal were paired. The *t* test was then performed on the paired observations for the group of animals.

Results

Comparison of rachitic and control animals. After 8 to 12 weeks on the rachitogenic diet, all 17 pups demonstrated severe clinical rickets, with characteristic physical findings in the extremities, skull, and ribs. Rickets was documented radiologically, and in a few instances by histological examination of bone. No vitamin D activity was detectable in the serum of the rachitic animals, whereas the mean activity in the serum of control animals was 420 U per 100 ml (Table I). Serum alkaline phosphatase was markedly elevated in the rachitic pups (Table I).

Abnormalities in phosphorus metabolism in the rachitic animals are apparent from the data listed in Table I. The rachitic dogs demonstrated significant hypophosphatemia. Although mean urinary phosphorus for the rachitic animals was greater than that for the controls, the difference was not significant. Renal phosphorus clearance for the rachitic animals, however, was more than twice that for the controls. Since glomerular filtration rate (measured with inulin) was not different for the two groups, the greater phosphorus clearance of the rachitic dogs resulted from a decrease of tubular conservation of phosphorus.

Phosphorus metabolism in rachitic dogs after parathyroidectomy and with the subsequent administration of parathyroid extract. One to seven days after the base-line clearance studies, 12 of the rachitic dogs underwent thyroparathyroidectomy. Between 3 and 6 hours after surgery, clearances were repeated. These clearances were performed at about the same time of day as the base-line studies. Urinary phosphorus and phosphorus clearance had decreased strikingly in every animal (Table II). These changes could not be attributed to variations in glomerular filtration rate (Table III). Serum phosphorus rose in eight of the animals, the mean for the group increasing from 4.7 to 5.5 mg per 100 ml ($p \leq 0.05$). In three additional rachitic animals, serum phosphorus was determined before and 24 hours after parathyroidectomy; clearances were not performed at these times. Serum phosphorus in these animals was 4.2, 5.7, and 4.9 mg per 100 ml before parathyroidectomy, and had increased to 5.8, 9.1, and 7.6 mg per 100 ml, respectively, 24 hours after surgery. Thus, in rachitic animals greater increases in serum phosphorus were observed with time after parathyroidectomy. These findings indicate that the hypophosphatemia and the decreased renal tubular conservation of phosphorus of the rachitic dogs are dependent on parathyroid activity.

Four rachitic animals subsequently received 2-hour infusions of parathyroid extract[2] at a rate of 10 U per minute. Two additional animals re-

[1] The authors are indebted to Mr. Walter Hooper and Mr. Glen Shue of the U. S. Food and Drug Administration, Washington, D. C., for the performance of the vitamin D assays.

[2] Generously supplied by Dr. G. Irwin of the Eli Lilly Company, Indianapolis, Ind.

TABLE II

Phosphorus metabolism in rachitic dogs before and after parathyroidectomy and with the subsequent administration of parathyroid extract

Dog	Serum phosphorus: Base line	Serum phosphorus: Parathyroidectomy	Serum phosphorus: Parathyroid extract	Urinary phosphorus: Base line	Urinary phosphorus: Parathyroidectomy	Urinary phosphorus: Parathyroid extract	Phosphorus clearance: Base line	Phosphorus clearance: Parathyroidectomy	Phosphorus clearance: Parathyroid extract
		mg/100 ml*			μg/minute*			ml/minute*	
381C†	4.8 ± 0.1 (8)‡	7.0 ± 0.0 (6)	6.3 ± 0.2 (6)	129 ± 21 (8)	5 ± 0 (6)	301 ± 65 (6)	7.6 ± 0.4 (8)	0.1 ± 0.0 (6)	5.0 ± 1.1 (6)
526D†	5.4 ± 0.3 (4)	6.0 ± 0.1 (2)	5.1 ± 0.0 (3)	201 ± 26 (4)	4 ± 0 (4)	383 ± 40 (3)	3.3 ± 0.3 (1)	0.1 (1)	7.5 ± 0.9 (3)
887C†	5.1 ± 0.1 (4)	5.6 ± 0.2 (3)	4.8 ± 0.2 (4)	72 ± 17 (4)	[illegible] (3)	619 ± 38 (4)	1.4 ± 0.4 (1)	0.2 ± 0.0 (3)	12.9 ± 0.9 (4)
100A§	5.[illegible] ± 0.0 (4)	5.0 ± 0.1 (4)	4.7 ± 0.1 (3)	197 ± 41 (4)	9 ± 1 (4)	503 ± 50 (3)	3.9 ± 0.8 (4)	0.2 ± 0.0 (4)	10.7 ± 1.1 (3)
677F§	4.7 ± 0.2 (4)	4.6 ± 0.3 (3)	4.2 ± 0.2 (4)	307 ± 23 (4)	10 ± 5 (2)	498 ± 11 (4)	6.7 ± 0.7 (4)	0.2 ± 0.1 (2)	11.8 ± 0.7 (4)
X278†	4.4 ± 0.0 (3)	4.6 ± 0.1 (5)	4.5 ± 0.0 (4)	183 ± 36 (3)	2 ± 0 (3)	533 ± 32 (4)	4.1 ± 0.8 (4)	0.1 ± 0.0 (3)	11.9 ± 0.7 (4)
337D	3.0 ± 0.1 (3)	6.3 (1)		67 ± 15 (3)	10 (1)		2.2 ± 0.7 (3)	0.2 (1)	
348C	3.8 ± 0.1 (4)	3.9 ± 0.2 (5)		178 ± 33 (4)	4 ± 0 (3)		4.6 ± 0.8 (4)	0.1 ± 0.0 (5)	
1[illegible]*	5.4 ± 0.9 (4)	6.6 ± 0.2 (4)		109 ± 30 (4)	8 ± 2 (4)		2.6 ± 0.7 (4)	0.1 ± 0.0 (4)	
827E	4.0 ± 0.2 (4)	6.4 ± 0.0 (4)		309 ± 22 (4)	5 ± 1 (4)		8.0 ± 0.9 (4)	0.1 ± 0.0 (4)	
T782	4.6 ± 0.2 (4)	5.3 ± 0.1 (4)		361 ± 16 (4)	3 ± 0 (4)		7.9 ± 0.3 (4)	0.1 ± 0.0 (4)	
T933	4.4 ± 0.0 (4)	4.1 ± 0.0 (4)		198 ± 48 (4)	10 ± 3 (4)		4.5 ± 1.0 (4)	0.3 ± 0.1 (4)	
141E	5.1 ± 0.0 (4)			363 ± 54 (4)			7.2 ± 1.1 (4)		
693B	4.3 ± 0.2 (3)			229 ± 15 (3)			5.3 ± 0.6 (3)		
Mean ± SEM	4.7 ± 0.2	5.5 ± 0.3	4.9 ± 0.3	207 ± 26	7 ± 1	473 ± 46	4.6 ± 0.6	0.1 ± 0.0	10.0 ± 1.3
p value		<0.05	<0.01		<0.001	<0.001		<0.001	<0.001

* Mean ± SEM.
† These animals received parathyroid extract from the Eli Lilly Co., Indianapolis, Ind.
‡ Numbers in parentheses indicate the number of clearance periods from which the mean values and standard errors are derived.
§ These animals received partially purified parathyroid extract (200 U per kg) provided by Dr. G. Aurbach.

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TABLE III

Inulin clearances before and after parathyroidectomy and with the subsequent administration of parathyroid extract

Dog	Inulin clearance: Before	After parathyroidectomy	With parathyroid extract
	ml/min*		
351C†	49 ± 3 (8)‡	11 ± 3 (6)	51 ± 3 (5)
526D†	44 ± 1 (4)	45 (1)	68 ± 1 (3)
1103S§	10 ± 2 (4)	19 ± 1 (3)	27 ± 2 (3)
677F§	39 ± 7 (1)	28 ± 7 (3)	79 ± 7 (4)
X278§	25 ± 0 (3)	19 ± 1 (3)	26 ± 2 (4)
827B	34 ± 2 (4)	47 ± 8 (4)	
T787	48 ± 1 (1)	39 ± 3 (4)	
T933	50 ± 9 (4)	62 ± 5 (4)	
LP	26 ± 2 (3)	30 ± 4 (4)	
1411S	25 ± 2 (4)		
p value		>0.5	<0.10

* Mean ± S.E.M.

† These animals received parathyroid extract from the Eli Lilly Co.

‡ The number of clearance periods is indicated in parentheses.

§ These animals received parathyroid extract (200 U per mg) prepared by Dr. G. Aurbach.

ceived a more purified parathyroid extract[3] at a rate of 5 U per minute (Tables II and III, dogs 100 A and 677 F). Marked increases were observed in urinary phosphorus and in phosphorus clearance in every animal. In the five dogs in which the measurement was made, there was a tendency for the inulin clearance to increase ($p < 0.10$, Table III). Therefore, although endogenous parathyroid hormone had maintained phosphaturia through a tubular effect in the vitamin D-deficient animals (see above), the phosphaturia resulting from exogenous extract may have been partly due to increases in filtered phosphorus.

Calcium metabolism in rachitic dogs after parathyroidectomy and with the subsequent administration of parathyroid extract. Serum calcium in the rachitic dogs was lower than that of controls (mean values, 8.5 vs. 11.7 mg per 100 ml, Table I). After parathyroidectomy, however, further decreases in serum calcium were noted in the vitamin D deficient animals within 3 to 6 hours in 11 of 12 instances (Table IV), a change that was highly significant ($p < 0.001$). In the three additional rachitic animals, serum calcium was 10.4, 10.2, and 8.5 mg per 100 ml before parathyroidectomy, and it had decreased to 4.5, 5.6, and 4.2 mg per 100 ml, respectively, 24 hours after surgery; clearances were not performed at these times. Thus, in rachitic animals after parathyroidectomy, greater decreases in serum calcium were observed with time. These findings suggest that endogenous parathyroid hormone was exerting a calcium-mobilizing action in the vitamin D depleted dog.

With the administration of parathyroid extract to the rachitic, parathyroidectomized dogs, serum calcium increased in every instance ($p < 0.01$, Table IV). Urinary calcium was determined at the different phases of the study for several of the animals (Table IV). The fall in serum calcium after parathyroidectomy was not accompanied by a consistent increase in urinary calcium; the means for the group before and after parathyroidectomy did not differ significantly ($p \geq 0.50$). Similarly, the rise in serum calcium with the administration of parathyroid extract was not accompanied by a significant decrease in urinary calcium ($p < 0.40$). These findings indicate that the changes in serum calcium were not a reflection of changes in urinary calcium.

Urinary hydroxyproline in rachitic dogs before and after parathyroidectomy and with the subsequent administration of parathyroid extract. In the rachitic dogs, urinary hydroxyproline was significantly greater than that of the controls (Table I). After parathyroidectomy, hydroxyproline peptides decreased in seven of eight rachitic animals (Table V), a finding that suggested that the initial high values in these animals resulted from excess of endogenous parathyroid hormone. When parathyroid extract was administered in six of the rachitic parathyroidectomized dogs, urinary hydroxyproline increased in every instance.

Discussion

The present studies indicate that the hypophosphatemia of rachitic dogs is associated with, and is

[3] Parathyroid extract with a potency of 200 U per mg was kindly provided by Dr. G. Aurbach. The methods of preparation of this extract have been previously published (16).

perhaps a consequence of, decreased renal tubular conservation of phosphorus. That these modifications in phosphorus metabolism are not the direct result of a deficiency of vitamin D is evident from the findings after parathyroidectomy. As urinary phosphorus and phosphorus clearance fell strikingly, it is clear that the renal tubules were able to conserve phosphorus despite deficiency of vitamin D. Furthermore, serum phosphorus increased progressively with time after parathyroidectomy. These data also indicate that the renal tubules of the vitamin D-deficient dogs were responsive to endogenous parathyroid hormone, and that the preoperative hypophosphatemia and increased phosphorus clearance were dependent on the tubular effect of the hormone. It has been suggested that impairment in calcium absorption is the primary physiological abnormality in the rachitic animal (17). It may be postulated that the resulting hypocalcemia stimulates parathyroid activity, leading to the observed abnormalities in phosphorus metabolism.

The present studies also suggest that parathyroid hormone influences the mobilization of bone mineral in the vitamin D-deficient dog. After parathyroidectomy, serum calcium fell significantly, indicating that endogenous hormone was exerting a calcium mobilizing effect. The serum calcium had decreased further 24 hours after parathyroidectomy. The administration of parathyroid extract increased the serum calcium in each rachitic parathyroidectomized animal. The changes

TABLE IV

Calcium metabolism in rachitic dogs before and after parathyroidectomy and with the subsequent administration of parathyroid extract

Dog	Serum calcium: Pre-Op	Serum calcium: Parathyroidectomy	Serum calcium: Parathyroid extract	Urinary calcium: Base line	Urinary calcium: Parathyroidectomy	Urinary calcium: Parathyroid extract
	mg/100 ml			*μg/minute**		
381C†	9.5 ± 0.3 (4)‡	5.3 ± 0.3 (5)	6.4 ± 0.3 (6)			
5261D†	7.2 ± 0.1 (2)	6.7 ± 0.0 (2)	7.3 (1)			
887C†	6.5 ± 0.3 (2)	5.1 ± 0.2 (2)	5.7 ± 0.8 (2)			
109AS§	9.7 ± 0.2 (2)	7.8 ± 0.1 (2)	9.7 ± 0.0 (2)	32 ± 13 (2)	35 ± 6 (2)	43 ± 17 (2)
677VS§	8.1 ± 0.1 (2)	6.4 ± 0.1 (4)	7.6 ± 0.0 (3)	83 ± 37 (2)	29 ± 7 (3)	94 ± 21 (2)
x278§	7.8 ± 0.1 (2)	6.2 ± 0.1 (2)	7.9 ± 0.2 (2)	56 ± 7 (2)	37 ± 3 (2)	59 ± 0 (2)
335D	6.0 ± 0.1 (2)	6.1 (1)				
318C	8.0 ± 0.1 (2)	7.5 ± 0.1 (2)				
LP	9.5 ± 0.5 (2)	6.3 ± 0.7 (2)				
8276	10.1 ± 0.5 (4)	7.4 ± 0.1 (1)		57 ± 15 (2)	142 ± 35 (2)	
1783	11.5 ± 0.6 (4)	10.1 ± 0.7 (1)		161 ± 35 (2)	176 ± 20 (2)	
T0.53	8.1 ± 0.7 (1)	6.1 ± 0.1 (3)		141 ± 18 (2)	187 ± 23 (2)	
Mean	8.5 ± 0.5	6.8 ± 0.4	7.4 ± 0.6	88 ± 24	101 ± 31	65 ± 15
p value	<0.001	<0.01		>0.5	<0.10	

* Mean ± SEM.

† These animals received parathyroid extract from the Eli Lilly Co.

‡ Numbers in parentheses indicate the number of consecutive observations from which the mean values and standard errors are derived.

§ These animals received parathyroid extract (200 U per mg) prepared by Dr. G. Aurbach.

TABLE V

Urinary hydroxyproline before and after parathyroidectomy and with the subsequent administration of parathyroid extract

Dog	Urinary hydroxyproline: Base-line	Urinary hydroxyproline: Parathyroidectomy	Urinary hydroxyproline: Parathyroid extract
		*μg/minute**	
381C‡	62 ± 7 (1)‡	20 ± 4 (1)	68 ± 10 (4)
526D†	49 ± 7 (3)	17 ± 6 (3)	61 ± 7 (3)
887C†	67 ± 4 (1)	46 ± 3 (3)	115 ± 8 (4)
100A§	25 ± 3 (4)	21 ± 2 (4)	58 ± 3 (4)
F677§	39 ± 2 (4)	23 ± 2 (4)	34 ± 6 (4)
x278†	16 ± 3 (3)	23 ± 2 (4)	44 ± 3 (4)
827E	38 ± 1 (2)	42 ± 6 (2)	
T782	59 ± 6 (2)	13 ± 3 (2)	
Mean	48 ± 6	29 ± 4	63 ± 11
p value		$p < 0.005$	$p < 0.025$

* Mean ± SEM.
† These animals received parathyroid extract from the Eli Lilly Co.
‡ The number of observations is indicated in parentheses.
§ These animals received parathyroid extract (200 U per mg) prepared by Dr. G. Aurbach.

in serum calcium concentration could not be attributed to changes in urinary calcium. Previous studies have shown that parathyroid hormone exerts a direct action on bone (18, 19). It has also been shown that the increases in serum calcium observed with the administration of parathyroid extract are largely a reflection of this action on bone (20). It is likely, therefore, that the changes in serum calcium in the rachitic dogs resulted from the action of parathyroid hormone on bone mineral.

The results of the present experiments in the dog confirm the observations of Toverud in the rat (4, 5) and indicate that parathyroid hormone is capable of mobilizing bone mineral despite a deficiency of vitamin D. Other investigators (2, 3), however, have not observed a decrease in serum calcium after parathyroidectomy in vitamin D depleted rats. The reason for these varying results is not apparent at present. It may be that the animals that showed no decrease in serum calcium after parathyroidectomy were more completely depleted of vitamin D. It is possible that vitamin D is required for the actions of parathyroid hormone on bone, as has been shown for the action of the hormone on kidney mitochondria (21), but that only minute, perhaps unmeasurable, quantities of the vitamin are required for this effect. In the present studies, antirachitic activity could not be detected in the serum of the rachitic dogs with a bioassay technique (Table I). However, it will be necessary to apply more sensitive and specific methods for measuring vitamin D in blood and tissues to resolve this question.

Since the rachitic pups demonstrated base-line hypocalcemia, they appeared to be less responsive than normal to the calcium-mobilizing action of endogenous hormone. This may have resulted from a decrease in the quantities of calcium at specific bone sites where the hormone exerts its effect, a possibility previously suggested by Munson, Hirsch, and Tashjian (22). Alternatively, as mentioned above, it may have resulted from a requirement for greater quantities of vitamin D. To assess in a quantitative manner the role of vitamin D in the responsiveness to the calcium-mobilizing action of parathyroid hormone, it will be necessary to measure the responses to graded doses of parathyroid extract in animals on graded dosages of vitamin D.

Urinary hydroxyproline decreased after parathyroidectomy in the rachitic dogs and subsequently increased with the administration of parathyroid extract (Table V). Available evidence suggests that urinary hydroxyproline peptides reflect collagen turnover (23). Parathyroid extract has been shown to increase release of hydroxyproline from bone *in vitro* (24). Recent studies by Walker, Lapiere, and Gross (25) have shown that parathyroid extract stimulates a collagenolytic factor in bone, thereby causing the release of hydroxyproline peptides *in vitro* (25). It has also been shown that parathyroid extract increases excretion of hydroxyproline in human subjects (26). The present studies suggest that parathyroid hormone influences bone collagen turnover despite a deficiency of vitamin D.

Summary

Calcium and phosphorus clearances and hydroxyproline excretion were measured in rachitic

dogs before parathyroidectomy, after parathyroidectomy alone, and with the administration of parathyroid extract after parathyroidectomy. In the rachitic dogs, serum phosphorus was decreased and phosphorus clearance was increased, as compared to controls. After parathyroidectomy of the rachitic animals, urinary phosphorus and phosphorus clearance decreased strikingly, indicating the ability of the renal tubules to conserve phosphorus despite vitamin D deficiency. These results also showed that endogenous parathyroid hormone was exerting a tubular effect to decrease phosphorus conservation despite a lack of vitamin D, and was responsible for the abnormalities in phosphorus metabolism in the rachitic dogs.

After parathyroidectomy, serum calcium fell in 11 of 12 rachitic animals, and it increased with the subsequent administration of parathyroid extract. These changes could not be attributed to changes in urinary calcium, and they suggested an action of parathyroid hormone on bone. Urinary hydroxyproline decreased after parathyroidectomy and increased with the subsequent administration of parathyroid extract, suggesting an action of parathyroid hormone on collagen metabolism in the vitamin D-deficient animals.

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Studies on calciferol metabolism. III. Comparison of species distribution and chromatographic separation of vitamin D metabolites

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The majority of the radioactivity in the plasma and intestine exists as 3 polar metabolites 15 hours after the intracardiac administration of a physiologic dose (10 IU) of radioactive vitamin D_3 (cholecalciferol) to rachitic chicks. These are designated as 4A (known to be 25-hydroxycholecalciferol), 4B (known to be 1,25-hydroxycholecalciferol) and 4C on the basis of their chromatographic mobility on silicic acid columns. In the intestine, the primary metabolite is 4B; in the plasma, the predominant metabolite is 25-hydroxycholecalciferol (25-OH-CC). A detailed comparison was made of the pattern of intestinal metabolites of vitamin D_3 produced by 4 separate and different silicic acid column chromatography systems. Each chromatographic system produced essentially identical amounts of metabolites 25-OH-CC and 4B. Additionally, the presence of metabolites 25-OH-CC and 4B, produced in vivo from vitamin D_3, was detected in chick skeleton, laying hen uterus, and the intestinal mucosa of the chick, rat, rabbit, frog, and monkey.

One basic approach utilized to elucidate the primary sequence of biomical events initiated by vitamin D (calciferol), has been to trace the fate physiologic amounts† of radioactive preparations of this steroid. Much recent

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†One international unit of vitamin D_3 [illegible] mmole) or vitamin D_2 [illegible] mmole) is equivalent to 0.025 μg. There is no formal international unit designation for dihydrotachysterol. Vitamin D_3 and vitamin D_2 have an equal biologic activity in the rat; in the chick vitamin D_3 is approximately 10 times more active than vitamin D_2 and 140 times more active than dihydrotachysterol.[16]

work from this laboratory[1-7] and other laboratories[8-9] supports the concept that the vitamin must first be metabolized to a new compound prior to the generation of its characteristic intestinal response, the enhancement of calcium transport. The appearance of the physiologic response is believed to be dependent upon the activation of genetic information by the new metabolite. The search for this new compound is the subject of intensive interest in a number of laboratories.

One major reason for this interest is the several pathologic conditions which have been postulated to be due in some manner to an altered metabolism of vitamin D. Reports have appeared in the literature which relate to the glucocorticoid antagonism of vitamin D action,[10] vitamin D resistant rickets,[11] the apparent abnormal sensitivity to vitamin D associated with sarcoidosis,[12] and the renal osteodystrophy and abnormalities in calcium metabolism associated with uremia.[13] Also, a number of reports have appeared which concern vitamin D metabolism in normal humans.[14, 15]

Several metabolites of vitamin D_3 appear to be of considerable interest in the search for the active form of this steroid. DeLuca and co-workers have isolated and characterized the compounds 25-hydroxycholecalciferol,[8] 21,25-dihydroxycholecalciferol,[17] 25,26-dihydroxycholecalciferol,[18] and 25-hydroxyergocalciferol from porcine serum.[19] 25-Hydroxycholecalciferol, which is 1.4 times as active as vitamin D_3 as a cure for rickets,[8] has been reported to be the "active form" of vitamin D_3.[20] Also, they reported the existence of at least 8 other metabolites of vitamin D.[21] Norman and Midgett[22] have reported the existence of 7 to 8 metabolites of vitamin D_3. Of major interest is an as yet uncharacterized compound, designated metabolite 4B, which is more polar than 25-OH-cholecalciferol.[1, 2] This compound, which is at least 4 times as active as vitamin D_3,[22] and which can be produced from 25-hydroxycholecalciferol, also has been suggested to be the "active form" of vitamin D in the intestine. Metabolite 4B has been shown to localize selectively within the nucleus, and its nuclear chromatin fraction of the target intestine.[1] In addition, Lawson, Wilson, and Kodicek,[9] and Mawer and Backhouse,[23] have reported the existence of other polar metabolites of vitamin D_3 in the chick and man, respectively.

Although the importance of vitamin D metabolism in the mode of action of this steroid seems certain, it is considerably more difficult to assess the identity and similarity of all the reported metabolites, due to the fact that each laboratory has utilized a different chromatography scheme and a different system of nomenclature. It is the purpose of this paper to report experiments in which the same sample of vitamin D metabolites was chromatographed in each of the major chromatography systems described in the literature. In addition, the results of studies on the species distribution of the 2 major metabolites of vitamin D_3, 25-hydroxycholecalciferol and 4B, and the comparative metabolism of vitamin D_2 and dihydrotachysterol$_2$ in the chick are reported.

Methods

Animals. White Leghorn cockerels (generously donated by H & N of California, Inc.) were raised on a rachitogenic diet.[10] The chicks were utilized in their fourth week, after they had become severely rachitic. In 1 experiment, several adult, white Leghorn hens

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were fed the rachitogenic diet for 5 weeks. Male, weanling rats (100 grams) of the Holtzman strain housed in individual hanging wire cages were fed the vitamin D free diet No. 11 of Guzoff and associates[4] for 5 days. At this time the rats were considered to have their vitamin D reserves lowered. Rabbits, turtles, and frogs were supplied by the Life Sciences Department, and a monkey species, *Saimiri sciureus*, was supplied by the Psychology Department of the University of California, Riverside. All these animals were fed on appropriate standard diets, in which the amount of vitamin D was not controlled. The exact amount of radioactive compound given to the chicks and rats is specified for each experiment. Two rabbits received 2 doses of 1,2-^{3}H-vitamin D_3 (3.75 nmoles) (50 IU) every other day prior to death. The monkey received of 6-^{3}H vitamin D_3 (22.5 nmoles) (750 IU) every day for 7 days prior to death. Nine frogs and 7 turtles received doses of 1,2-^{3}H-vitamin D_3 (0.13 nmole) (2 IU) every day for 4 days prior to death. At death, the intestines were immediately excised and placed in ice cold sucrose (0.25M).

Chemicals. The following steroids were utilized in the metabolism studies: 4-^{14}C-vitamin D_3 (Philips Duphar, Weesp, Netherlands) (specific activity 57.8 mCi per mmole); 1,2-^{3}H-vitamin D_3 (New England Nuclear, Boston, Mass.) (specific activity 29.8 mCi per mmole); 6-^{3}H-vitamin D_3 (specific activity 4.9 mCi per mmole); and 6-^{3}H-vitamin D_2 (specific activity 5.2 mCi per mmole), were prepared according to the procedure of Norman and Tolnai[23] and purified by Partion chromatography; 6-^{3}H-dihydrotachysterol$_2$ (2.5 mCi per mmole), was prepared by direct exposure of nonradioactive dihydrotachysterol$_2$ to tritium gas, followed by purification on columns of silicic acid and Partion. The appropriate amounts of these materials were dissolved in 1,2-propanediol (0.20 ml.) for convenient intracardial (chick), intraperitoneal (monkey, turtle, frogs), and oral (rat, rabbit) injection. Silicic acid (silica 325 mesh) was obtained from Calbiochem (Los Angeles, Calif.). All solvents and chemicals used were analytic grade.

Lipid extraction techniques. Lipid extraction of the intestine, plasma, skeleton, or egg-shell gland was carried out according to a modification of the procedure of Bligh and Dyer[22] as described previously.[2] The skeleton tissue consisted of chick femurs which were split longitudinally, and the marrow removed prior to lipid extraction.

Chromatography. The chloroform lipid extract of interest was brought to dryness under nitrogen, redissolved in petroleum ether, and dried over anhydrous Na_2SO_4. The petroleum ether was transferred to a second container and a small aliquot was taken for radioactivity determination; the remainder was available as the sample for column chromatographic analysis. In this report 3 different silicic acid column chromatography elution systems were utilized. They are separately described below. Each system employs varying amounts of silicic acid (silica 325 mesh) as the adsorbent. Elution systems I, II, and III employed a 250 ml. constant-volume mixing chamber to generate successive, exponential gradients of solvents with an increase in the dielectric constant. In each system, the mixing chamber was first filled with 250 ml. of petroleum ether. Elution system I was originally described by Norman, Lund, and DeLuca[23] and employs a column which contains heat activated silicic acid (30 Gm.) (1 by 80 cm.). The holding chamber was filled successively with 50 per cent (v/v) diethyl ether (300 ml.) in petroleum ether, 100 per cent diethyl ether (300 ml.), 100 per cent methanol (500 ml.). Elution system II (Riverside) was first described by Myrtle, Hayssler and Norman and employs a column which contains heat-activated silicic acid (30 Gm.) (1 by 80 cm.), to which the sample is applied and containing 0.5 ml. of ether. The holding chamber is filled successively with 100 per cent diethyl ether (300 ml.), 50 per cent (v/v) dichloroethane (300 ml.) in diethyl ether, 100 per cent dichloroethane (300 ml.), 100 per cent acetone (300 ml.), and 100 per cent methanol (300 ml.). Elution system III (Madison) was first described by Deluca and DeLuca[27] and employs a column which contains heat activated silicic acid (19.6 Gm.) (1 by 50 cm.). The holding chamber was filled successively with 75 per cent (v/v) diethyl ether (300 ml.) in petroleum ether, 100 per cent diethyl ether (100 ml.), 5 per cent (v/v) methanol (500 ml.) in diethyl ether, 50 per cent (v/v) methanol (300 ml.) in diethyl ether, and finally stripping the column directly with 100 per cent methanol (500 ml.). Elution system IV (Cambridge) originally described by Lawson, Wilson and Horlick[28] employs a column which contains

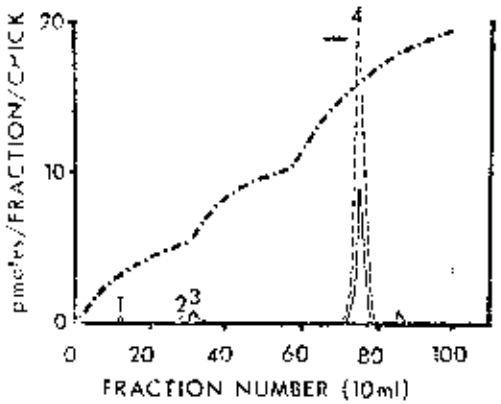

Fig. 1. Silicic acid chromatography of intestinal and plasma lipids with elution system I. Separate groups of 6 chicks received an intracardial dose of 4-^{14}C- or 1,2-^{3}H-vitamin D_3 (3.75 nmole) (10 IU) 15 hours prior to death. The intestines and plasma from each group of chicks were separately extracted for total lipids. The ^{3}H-intestinal lipids were mixed with the ^{14}C-plasma lipids and simultaneously chromatographed on a 30 Gm. (1 by 80 cm.) silicic acid column with the utilization of gradient elution system I. Peak 3 is unmetabolized vitamin D_3; ——, intestine; — — —, plasma; ———, gradients.

heat activated silicic acid (19.6 Gm.) (1 by 50 cm.). The column is eluted with a linear gradient of 0→20% per cent diethyl ether (600 ml.) (original solvent is petroleum ether), followed by batch elution with 20 per cent diethyl ether (200 ml.), followed by a linear gradient of 0→100 per cent methanol (300 ml.). Elution system V (Manchester) originally described by Mawer and Backhouse[29] employs a column which contains gonadical activated silicic acid (24 Gm.) (1 by 60 cm.). The column is successively batch eluted with 25 per cent (v/v) diethyl ether (200 ml.) in petroleum ether, 50 per cent (v/v) diethyl ether (60 ml.) in petroleum ether, 100 per cent diethyl ether (200 ml.), 5 per cent (v/v) methanol (200 ml.) in diethyl ether, 50 per cent (v/v) methanol (100 ml.) in diethyl ether, and 100 per cent methanol (110 ml.).

Radioactivity determinations. Liquid scintillation counting of tritium and ^{14}C-labeled steroids was carried out in a Packard CPM 200 counter. All of the samples were dissolved in 10 ml. of a counting solution which consists of phenylbiphenyl oxadiazole-1,3,4 (5 Gm.) per liter of toluene and counted to 2 per cent error. The number of disintegrations per minute of tritium and ^{14}C present in the sample was determined by the use of an external standard and a computer program designed to process single or double label counting. With the use of the specific activities of the parent vitamin D_3 and the number of chicks used in the experiment, this program also converts the disintegrations per minute in the sample to an absolute amount (pmole/dec) of steroid per sample per chick.

Results

Fig. 1 shows the pattern of metabolites of vitamin D present in both the plasma and intestinal mucosa of rachitic chicks when the combined lipid extract is simultaneously chromatographed on a silicic acid column with elution system I. The intestinal ^{3}H-lipids and the plasma ^{14}C-lipids were obtained 15 hours after an intracardial dose was given to separate groups of rachitic chicks with 0.05 nmole (10 IU) doses of ^{3}H-vitamin D_3 or ^{14}C-vitamin D_3. Elution system I was developed by Norman, Lund, and DeLuca[23] and represented the first attempt to study the metabolism of vitamin D with the use of a high specific activity, radio-

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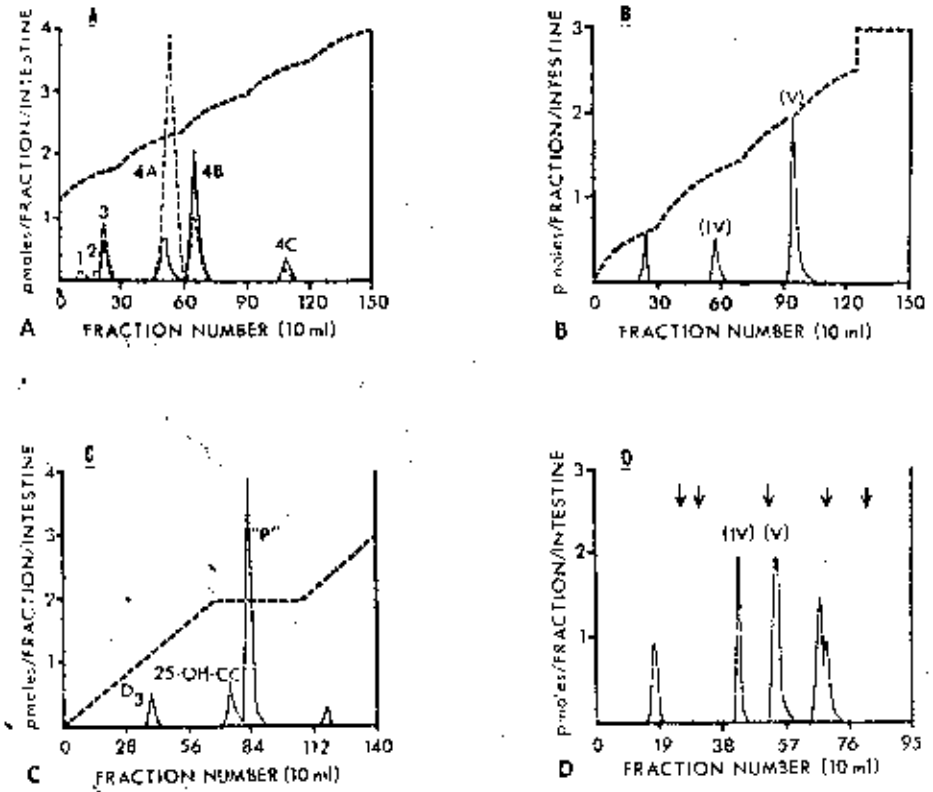

Fig. 2. Silicic acid chromatography of intestinal lipids with elution system II, Riverside (*A*), elution system III, Madison (*B*), elution system IV, Cambridge (*C*), and elution system V, Manchester (*D*). A group of 12 rachitic chicks received 1-^{3}H-vitamin D_3 (0.65 nmole) (10 μc) 15 hours before death. The intestinal lipids were extracted by the procedure of Bligh and Dyer,[27] the solvent removed under nitrogen and then replaced with hexane. Equal aliquots were applied to each column. The details of the solvent elution schedule for each elution system are described under Methods.

The pattern of intestinal metabolites observed is shown by ——. In *A*, ^{3}H-plasma lipids, designated by - - - -, were co-chromatographed on the same column. - - - - gradients. In *D*, Manchester, the successive changes of the solvents used for batch elution is designated by the arrows. All fractions were 10 ml.

active vitamin D, and modern chromatographic techniques. With elution system I, the bulk of the radioactivity in both the intestine (79 per cent) and plasma (92 per cent) migrates as an apparent single peak. This peak was designated Peak 4 by Norman and associates.[7] Peak 3 represents unmetabolized vitamin D and Peak 1 has been shown to be fatty acid esters of vitamin D.[29]

Since Peak 4 (system I) was eluted from the column with the initiation of the methanol gradient, it was suspected by Haussler, Myrtle, and Norman[1,2] that Peak 4 was possibly a composite of several metabolites "stripped off the column simultaneously." Accordingly, elution system I (Fig. 1) was modified to elution system II (Fig. 2, *A*) by the inclusion of exponential gradients of

0 → 50 per cent dichloroethane, 50 → 100 per cent dichloroethane, and 0 → 100 per cent acetone between the gradients of diethyl ether and methanol. Fig. 2, *A* shows the pattern of vitamin D metabolites present in both the intestinal mucosa and plasma when the combined ^{3}H-intestinal and ^{3}H-plasma lipids were chromatographed on the same column. Again, the intestinal ^{3}H-lipids and plasma ^{3}H-lipids were obtained 15 hours after an intracardial dose was given to separate groups of chicks with the appropriate radioactive vitamin D preparation. The polar metabolites were designated by Haussler and associates as Peaks 4A, 4B and 4C. The bulk (79 per cent) of the plasma radioactivity (dotted line) occurs as metabolite 4A and only 10 per cent as 4B. Serum metabolite 4A has been tentatively identified as being 25-hydroxycholecalciferol, on the basis of its migration with authentic 25-hydroxycholecalciferol during silicic acid column chromatography.[7] Also metabolite 4A has been shown[6,30] to have 2.04 ± 0.[illegible] (S.E.M.) times the biologic activity of standard vitamin D_3 in an assay which measures the intestinal calcium transport, in vivo.

In sharp contrast (Fig. 2, *A*) the bulk (65 per cent) of the target intestinal radioactivity (solid line) occurs as the polar metabolite 4B. This intestinal metabolite has previously been shown (1) to be homogeneous in 2 additional chromatographic systems,[1] (2) to bind specifically to the intestinal mucosa chromatin,[1] and (3) to have 4.74 ± 1.09 (S.E.M.) times the biologic activity of standard vitamin D_3 in an intestinal assay.[6,32] Unfortunately, the chemical structure of metabolite 4B is not yet known; this is in large part due to the fact that only extremely small amounts of 4B (6.5 pmoles) are found in 1 chick mucosa. Metabolite 4B has been chemically characterized as being 1,25-dihydroxycholecalciferol.

Since the introduction of elution system II (Riverside) by Haussler, Myrtle, and Norman[1,2] 3 other chromatographic systems have appeared in the literature all of which employ silicic acid columns, but each with different organic solvent elution schemes and a different system of nomenclature for naming the various peaks found.[6,31,33] Accordingly, it has become very difficult to assess the similarities and differences in the results from studies on the metabolism of vitamin D_3 obtained in these laboratories. Fig. 2 presents the pattern of metabolites of vitamin D_3 found in the intestinal mucosa which utilize elution system II (Riverside, Fig. 2, *A*), elution system III (Madison, Fig. 2, *B*), elution system IV (Cambridge, Fig. 2, *C*), and elution system V (Manchester, Fig. 2, *D*). Aliquots from the same sample of intestinal ^{3}H-lipids were chromatographed in each system. In each system there is a relatively small peak of unmetabolized vitamin D, followed by a series of polar metabolites. The Madison, Cambridge, and Manchester separation schemes all give a qualitative pattern of vitamin D metabolites similar to that produced by the Riverside system II. Each has a peak corresponding to 4A (25-hydroxycholecalciferol) and a second, more polar peak that corresponds to metabolite 4B.

Table I presents a summary of the nomenclature used by the various laboratories to name the various peaks, and a quantitative tabulation of the amounts of the 2 major metabolites, 4A and 4B, reported in each elution system in Fig. 2. Each elution system produced equivalent amounts of metabolite 4A (25-hydroxy

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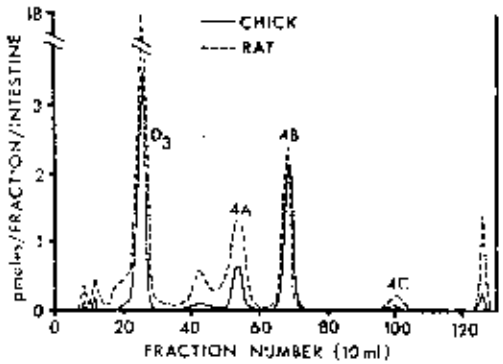

Fig. 4. Silicic acid chromatography of chick and rat intestinal lipids with elution system II. Five normal male rats (150 Grams) were placed on a vitamin D-deficient diet[22] for 5 days, then orally administered 4-^{14}C-vitamin D_3 (0.25 nmoles) (50 IU) daily for 7 days. A separate group of 5 rachitic chicks received 1.3 μl-vitamin D_3 (1.3 nmoles) (20 IU) intramuscularly 15 hours before killing. The rats were killed 24 hours after the final dose, and intestinal lipids from both the rats and chicks prepared, combined, and applied in hexane to a heat activated silicic acid column (30 Gm.) (1 by 50 cm.). The column was developed with elution system II. — , Chick intestine; . . . . . , rat intestine.

Table I. Summary of terminology of separation schemes for intestinal metabolites of vitamin D_3*

Gradient system	Laboratory	Reference	Peak: 25-OH-CC (4A)	Peak: 4B	Minor metabolites: 25-OH-CC (4A) (pmoles/mucosa)	Minor metabolites: 4B (pmoles/mucosa)
II	Riverside (Norman and associates)	6	4A	4B	3.21	8.4
III	Madison (DeLuca and associates)	8	Peak IV	Peak V	2.61	7.8
IV	Cambridge (Kodicek and associates)	9	25-OH-CC	Peak "p"	3.21	6.5
V	Manchester (Stanbury and associates)	7	Peak IV	Peak V	2.61	7.8

* All chromatographic systems were set up according to published procedures and utilized aliquots of the same sample of chick intestinal metabolites obtained 24 hours after 5 IU of D-3-H. The intestinal chromatographic patterns are shown in Fig. 4.

hydroxycalciferol), 2.6 to 3.2 nmoles per chick mucosa, and metabolite 4B, 6.5 to 8.4 nmoles per chick mucosa. It is clear that in each system, the major form of radioactivity present in the intestine is not 25-hydroxycholecalciferol, but is the compound designated as metabolite 4B. In experiments not reported here, we have obtained chick mucosal ^{3}H-lipids prepared in either Cambridge or Manchester and co-chromatographed in Riverside with some Riverside generated ^{14}C mucosal lipids by use of elution system II. Additionally, intestinal ^{14}C lipids produced in Riverside have been chromatographed in Cambridge and Manchester with identical results. In each instance, both the carbon-14 and ^{3}H-lipids con-

Table II. Species distribution of metabolites of vitamin D_3 in the intestine*

Species	Metabolites: 25-OH-CC (4A)	Metabolites: 4B
Chicken		
Duodenum	+	+
Skeleton	+	+
Egg shell gland	+	+
Cecum	−	+
Rabbit	+	+
Monkey	+	+
Turtle	−	−
Frog	+	+

* Combined by co-chromatography with standard chick intestinal metabolites of vitamin D_3 as separated on a silicic acid column. Typical results are given in Fig. 3.

tained the same number and pattern of metabolites as shown in Fig. 2, *A*. Thus, it is apparent that metabolites 4A and 4B are not artifacts of the conditions of chromatography. In view of the high biologic activity reported for these compounds,[7-9] it is highly probable that they play an important role in the mediation of the physiologic response of vitamin D in the chick intestine.

To further assess the biologic significance of these compounds, experiments were initiated to study the occurrence of metabolites 4A and 4B in the intestine of various species. The general protocol consisted of giving 4 to 5 animals several intraperitoneal or oral doses of ^{3}H-vitamin D_3 over a period of 1 week. Thus, although these animals were not vitamin D deficient, their body pool of vitamin D became radioactive, and in each instance resulted in the localization of radioactivity within the intestinal mucosa. The animals were killed, and after extraction of the intestinal ^{3}H-lipids, they were mixed with some standard chick intestinal ^{14}C-lipids and co-chromatographed on a silicic acid column by use of elution system II. Then the presence or absence of ^{3}H-4A or ^{3}H-4B can be carefully assessed in terms of both its existence and co-migration with the standard ^{14}C-4A and ^{14}C-4B. Fig. 3 presents typical results comparing the chick and rat pattern of intestinal metabolites. Both the chick and the rat produce the major metabolites 4A, 4B, and 4C plus a number of other minor metabolites. Interestingly, the rat has 6 times the amount of unmetabolized vitamin D_3 in the intestine as compared with the chick.

Table II presents a summary of the results obtained which concern the species distribution of metabolites of vitamin D_3. In each instance the experiments were carried out in a manner identical to that for the rat reported in Fig. 3. Of the various species tested, the rat, rabbit, monkey, frog, and turtle, all except the turtle produced polar compounds which migrated very similarly to those from chick intestine. In addition, evidence has been obtained via similar techniques for the presence of metabolites 4A and 4B in human intestinal mucosa.* Clearly, both metabolites 4A and 4B have a wide species distribution

* Unpublished observations of Drs. A. W. Norman, J. W. Coburn, and D. Hartenbower.

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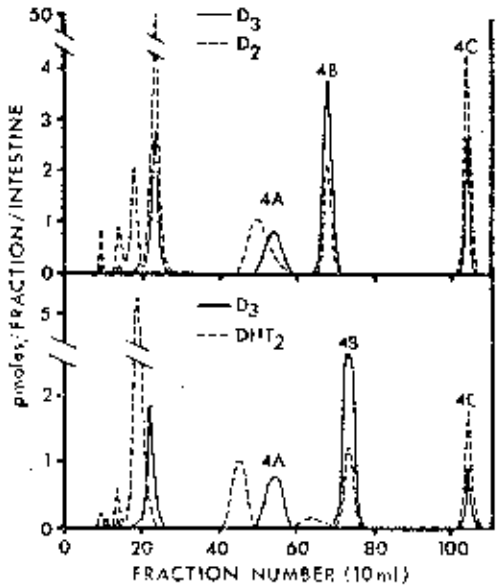

Fig. 4. Silicic acid chromatography of intestinal metabolites produced from vitamin D_2 (top) and dihydrotachysterol$_2$ (bottom) with elution system II. Separate groups of 5 rachitic chicks received intraperitoneal doses of G-^{3}H vitamin D_2 (5.2 nmoles, 80 IU), G-^{3}H-dihydrotachysterol$_2$ (0.9 nmoles, 110 IU) or 1-^{14}C-vitamin D_2 (1.3 nmoles, 20 IU) 15 hours before death. The intestinal Foods from each group were separately extracted by the procedure of Bligh and Dyer,[27] the solvent evaporated under nitrogen and then replaced with hexane. Aliquots of the ^{14}C-lipids were separately mixed with each group of ^{3}H-lipids. The two samples were applied to separate heat-activated silicic acid columns (30 Gm.) (1 by 80 cm.). The columns were developed with elution system II. ——, vitamin D_2; —·—, vitamin D_2 + metabolites (top) or dihydrotachysterol$_2$ + metabolites (bottom).

which is consistent with the earlier suggestion that they may play a prominent role in mediating intestinal calcium transport.

Other compounds related to vitamin D_2 often used clinically or experimentally include vitamin D_3 and dihydrotachysterol$_3$. Fig. 4 presents results which compare the intestinal pattern of metabolites produced from ^{3}H-vitamin D_2 and ^{3}H-dihydrotachysterol with that of the standard ^{14}C-vitamin D_2. Separate groups of rachitic chicks received 1-^{14}C vitamin D_2 (1.3 nmoles, 20 IU), G-^{3}H-vitamin D_2 (5.2 nmoles) (8.0 IU), or G-^{3}H-dihydrotachysterol (0.9 nmoles) (10 IU). Several important features are apparent. Both vitamin D_2 and dihydrotachysterol$_2$ are metabolized in a manner qualitatively similar to that of vitamin D_2; that is, each compound produces 4A-like, 4B-like, and 4C-like compounds. However, in view of the much larger administered doses of vitamin D_2 and dihydrotachysterol$_2$, it is apparent that they produced less of the metabolite 4B analogue than that produced from a 10 IU dose of vitamin D_2. This may be of importance in explaining the lower biologic activity of these compounds in the chick.*

*Unpublished observations.

Discussion

Reports of studies on the metabolism of vitamin D have become increasingly frequent in recent years.[1-26] This is in large part due to the routine availability of radioactive vitamin D with a high enough specific activity that small physiologic doses may be routinely administered.[1-26] All of these reports depend upon the availability of separation techniques which are capable of separating unmetabolized vitamin D from its metabolites. The first reports employed thin-layer chromatography to separate vitamin D from its metabolites which were present in various subcellular fractions of rat kidney and intestine. However, it soon became apparent that thin-layer chromatography was incapable of easily accommodating the large amounts of lipid present in the tissue samples. Therefore, Norman and associates[23] introduced a silicic acid column chromatography separation that employed successive exponential gradients of ether and methanol, (system I, this report). By use of this system, they demonstrated for the first time the existence of a biologically active polar metabolite of vitamin D, designated Peak 4. Subsequently, Lund and DeLuca[4] found Peak 4 material in the liver, serum, and skeleton of rats. They ultimately were able to isolate and characterize Peak 4 from porcine blood as being 25-hydroxycholecalciferol.[5] This compound was reported to be the active form of vitamin D.[7] More recently, 25,26-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol plasma metabolites which migrate in the peak V region (system III), have also been isolated from porcine plasma.[7,28] This latter compound may preferentially mediate bone resorption as compared to intestinal calcium absorption.

Unfortunately, with elution system I, all of the metabolites which are more polar than vitamin D are simultaneously eluted as a single peak by the methanol gradient. Hence, it is impossible to distinguish between the different patterns of metabolites found in the serum and intestine. As shown in Fig. 2A, elution system II (Riverside) is capable of resolving the Peak 4 of elution system I into at least 3 major components, 4A, 4B, and 4C, and a number of more minor components. Accordingly, it is necessary to carefully reconsider earlier metabolic studies[8-22] in which composite Peak 4 was treated as a single compound.

Also, it is important to note that whereas 25-hydroxycholecalciferol (metabolite 4A) is the predominant derivative of vitamin D found in the plasma, metabolite 4B is the predominant form present in the intestine. Both compounds have been shown to have biologic activity. Blunt, DeLuca, and Schnoes[9] found that 25-hydroxycholecalciferol was 1.4 times as active as vitamin D_3 in the rat line test. Myrtle, Haussler, and Norman[22] found that metabolite 4B obtained from chick intestines was over 4 times as effective as vitamin D and over 2 times as effective as 25-hydroxycholecalciferol in the stimulation of intestinal calcium transport 24 hours after administration.

More recently, reports on the detailed metabolism of vitamin D_3 have appeared from the laboratories of Myrtle, Haussler, and Norman,[7] DeLuca,[7] Lawson, Wilson, and Kodicek,[8] Mawer, Lund, and Stanbury,[17] Avioli and associates,[18,19] and Boerg, Kinberg, and Gerson.[21] Each of these reports has sig-

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lized 1 of 4 different silicic acid column chromatographic systems. Each system employs a different nomenclature system to identify the peaks. They are designated elution systems II through V in this paper. Their laboratory of origin and the nomenclature which concerns the key metabolites 4A and 4B are summarized in Table 1. Heretofore it has been impossible to directly correlate all the results from the laboratories which utilize these chromatography systems, but in view of the results presented in Fig. 2, it appears that metabolite 4A (Riverside) is synonymous with Peak IV (Madison), Peak IV (Manchester), and 25-hydroxycholecalciferol (Cambridge). Also, we have shown metabolite 4B (Riverside) to be synonymous with Peak V (Madison), Peak V (Manchester), and Peak "p"[18] (Cambridge). In addition, Haussler and associates[24] reported the existence of both metabolites 4A and 4B when the intestinal lipids were separated by the process of counter-current distribution. Thus, metabolites 4A and 4B are not artifacts of any one particular chromatographic separation scheme, nor is their detection dependent upon silicic acid chromatography.

A further test of the importance of these metabolites in initiating vitamin D-dependent intestinal calcium transport concerns their tissue distribution. Both metabolites 4A and 4B have been found in chick intestinal mucosa and skeleton, both of which are prime target organs for vitamin D action. Additionally 4A and 4B have been found in the uterus or egg-shell gland of a laying hen—again a prominent site of intensive calcium metabolism. Little information is available, however, which concerns the details of the involvement of vitamin D or its metabolites in the formation of the egg shell.

Also, virtually no information is available as concerns the species distribution of metabolites of vitamin D. The existence of 25-hydroxycholecalciferol (metabolite 4A) in the serum of rats by Poncher and DeLuca,[9] of chicks by Suda, DeLuca, and their associates,[10] and of hogs by Blunt, DeLuca, and Schnoes,[7] while Avioli[26] has reported the presence in human serum of a compound which migrated identically with 25-hydroxycholecalciferol. We have reported here, for the first time, the presence of metabolites 4A (25-hydroxycholecalciferol) and 4B in the intestinal mucosa of the chick, rat, rabbit, monkey, frog, and man. To the extent that the presence of these metabolites are indicative of a vitamin D-dependent, intestinal-calcium transport system, then it can be concluded that all these organisms have a vitamin D requirement. Certainly this is the case for the chick, rat, rabbit, monkey, and man. Thus, the combination of high specific activity radioactive vitamin D and a sensitive separation technique, which always has incorporated into it a standard sample of chick intestinal metabolites, provides a powerful and sensitive tool with which to further explore the metabolism of vitamin D in species whose nutritional calcium status or vitamin D dependency is not clearly delineated. On this basis we tentatively conclude either that the turtle does not have an intestinal requirement for vitamin D, since neither metabolite 4A nor 4B was detected, or that unaltered vitamin D is active.

The proposal[5,6,22] that metabolites 4A and 4B produced from vitamin D are important initiators of intestinal calcium transport would strongly infer that

similar metabolites may be produced from vitamin D_2 and dihydrotachysterol$_2$. Both of these compounds are known to stimulate intestinal calcium transport[16] and both are frequently employed in clinical studies where vitamin D_3 is either not available or contraindicated. As shown in Fig. 4, both vitamin D_2 and dihydrotachysterol$_2$ are metabolized to 4A-like and 4B-like compounds. Presumably, the 4A-like metabolites are the respective 25-hydroxy derivatives of vitamin D and dihydrotachysterol. Since both vitamin D_2 and dihydrotachysterol$_2$ are much less biologically active than vitamin D_3 in the chick,[2] it is interesting to speculate on the significance of the reduced amounts of the 4B-like compounds which were found in comparison to the amount of metabolite 4B produced from vitamin D_3. This is in spite of the fact that 6.3 nmole (400 IU) doses of the compound were given and only 0.65 nmole (10 IU) doses of vitamin D_3 were given. It is not possible to conclude from these experiments whether the lower biologic activity of these compounds is due to a slower rate of metabolism of the parent compound, or to a lowered affinity of the metabolite for its nuclear chromatin receptor. In any case, these represent the first reports of the metabolism of these 2 important calcium-oriented compounds.

All of the results reported here, when taken together with other reports from our laboratory that concern the biologic activity of metabolite 4B[5,22] and its nuclear localization[3,5] are consistent with the view that metabolite 4B is an important initiator of vitamin D-dependent events in the intestinal mucosa. However, confirmation of this fact must await chemical elucidation of the structure of 4B.[4] Metabolite 4B has been chemically characterized as being 1,25-dihydroxycholecalciferol.[22]

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devised as follows: To 1.7 mmole of *l*-cysteine-*N*-acetate in 5 ml of distilled water 1.5 mmole of nonradioactive phenylmercuric acetate in 40 ml of ethanol was added. After the solution was mixed thoroughly, it was acidified 10 ml of 1*N* H_2SO_4 and extracted 100 ml of benzene. Upon purification on a Florisil column the major reaction product was crystallized in *n*-hexane. This compound matched the major microbial metabolic product in six different thin layer chromatographic systems (the spot corresponding to an R_F value of 0.52 in the chromatogram in Fig. 3). The product was a white crystalline material which melted at 123° to 124°C. Elementary analyses showed that the material contained 42.13 percent carbon, 3.19 percent hydrogen, and no nitrogen (the mercury content was not analyzed). The mass spectrum of this compound showed the presence of two phenyl rings in addition to one mercury atom per molecule. Comparison of the infrared spectra of this material with that of an authentic sample of diphenylmercury unequivocally established that the crystalline material was diphenylmercury. On the average, diphenylmercury was responsible for [illegible] percent (for seven aquatic microorganisms) and 46.6 percent (for nine [illegible] microorganisms) of the total solvent-extractable radioactivity. The corresponding figures for the second major metabolic product (R_F = 0.46, Fig. 3) were 20.0 and 15.3 percent, respectively. The chemical identity of this second major metabolite, which could be observed in most of microbial incubation products, could not be established.

Since it is known that methylmercury and dimethylmercury are produced from various mercury sources, it was surprising that these microorganisms did not convert phenylmercuric acetate into methylmercury compounds. It is possible that a direct conversion of phenylmercury to methylmercury is not a common process in microorganisms, at least not under aerobic conditions. However, at least one species of microorganism is known (*14*) to be capable of directly converting phenylmercury to metallic mercury, a process which could eventually lead to formation of methylmercury [illegible]gh the conversion of Hg^0 to [illegible].

Diphenylmercury is the most apolar (and probably the most lipophilic) component of the metabolic products from phenylmercury. It has been suggested (*1, 15*) that the rate of absorption of mercury compounds by biological materials in water is related to the apolarity-lipophilicity of the compound. In view of the possibility that diphenylmercury can form in nature by the action of microorganisms, the toxicological implications of such a form of mercury in relation to its impact on various biospheres must be carefully examined.

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1,25-Dihydroxycholecalciferol: Identification of the Proposed Active Form of Vitamin D_3 in the Intestine

Abstract. *The major polar metabolite of cholecalciferol (vitamin D_3) present in chick intestinal mucosa has been chemically characterized by mass spectrometric analysis to have a molecular formula of $C_{27}H_{44}O_3$ and a structure of 1,25-dihydroxycholecalciferol. This compound, which is produced in the kidney from 25-hydroxycholecalciferol, has been previously shown to be from 4 to 13 times as active as cholecalciferol in stimulating intestinal calcium transport. 1,25-Dihydroxycholecalciferol (previously designated metabolite 4B in this laboratory) probably represents the biologically active form of cholecalciferol in the intestine.*

The concept has been developed in a number of laboratories that cholecalciferol (CC) (vitamin D_3) must first be metabolized to a more polar form prior to the stimulation of intestinal calcium transport (*1–3*). It has been conclusively shown that administration of radioactive CC to rachitic chicks or rats results in the production of two major polar metabolites and a number of other minor metabolites. The predominant metabolite present in the blood has been shown by Blunt *et al.* to be 25-hydroxycholecalciferol (25-OH-CC) (*4*). It is known to be produced by the liver (*5*). The major metabolite of CC present in the target intestine was shown by Norman and co-workers to be chemically different from both the parent vitamin and 25-OH-CC (*1, 2*). We have previously designated this substance metabolite 4B. The highly selective nature of the binding of metabolite 4B to the target intestine and the kinetics of appearance of this metabolite in relation to the well-known lag in action of vitamin D suggested to us that metabolite 4B played a prominent role in the development of this physiological response (*1, 6*). Lower concentrations of metabolite 4B were also found in bone and kidney of chicks, in the eggshell gland of the laying hen, other known sites of intensive calcium metabolism, and in the intestinal mucosa of the frog, rat, rabbit, monkey, and man (*7*).

We have carried out extensive studies on the relative biological activities of CC, 25-OH-CC, and highly purified metabolite 4B. Myrtle and Norman (*8*) reported that metabolite 4B is four to five times as effective as CC and over two times as effective as 25-OH-CC in stimulating intestinal calcium transport 24 hours after administration. After a considerable lag, CC and its 25-hydroxy derivative produce a maxi-

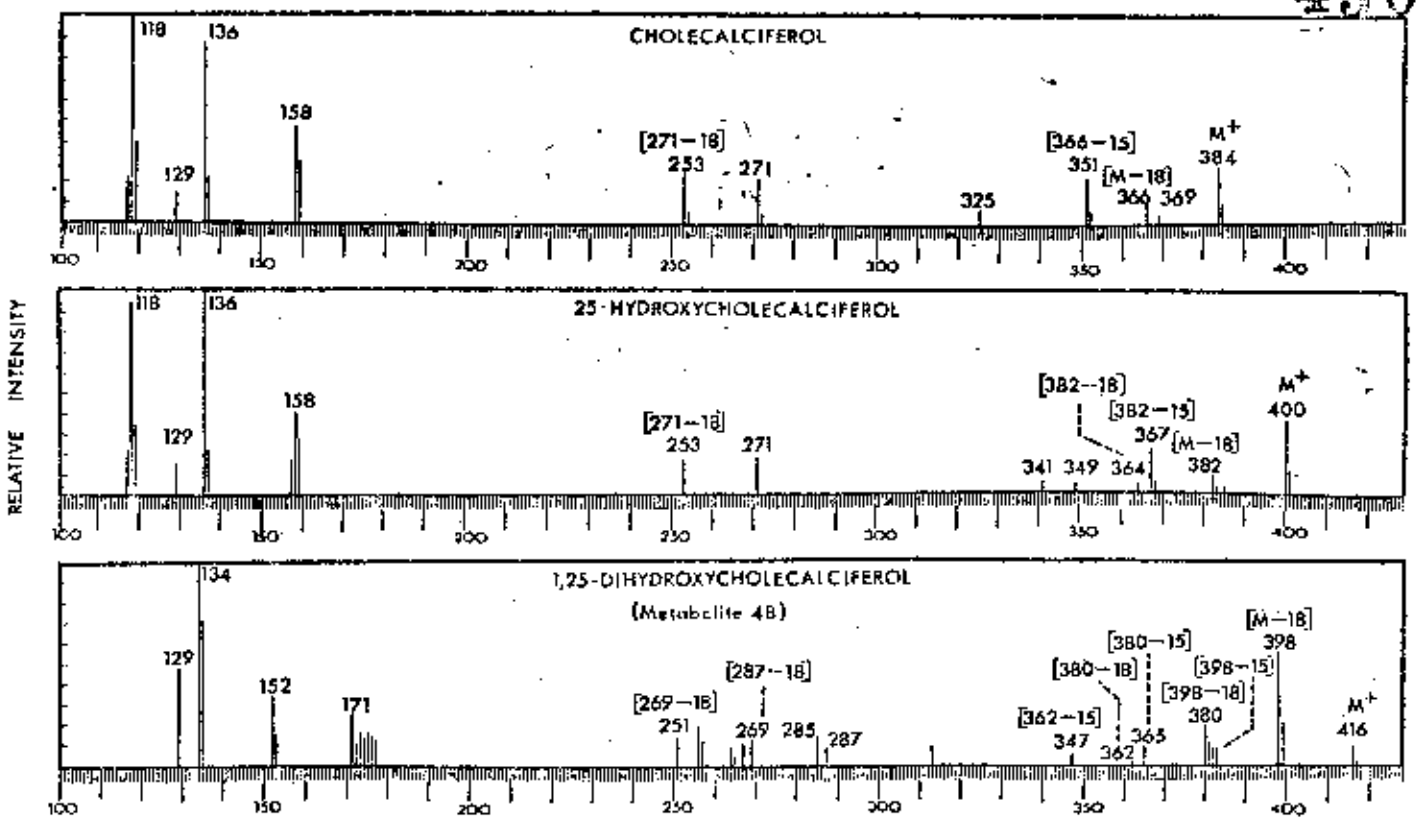


Fig. 1. Mass spectra of cholecalciferol, 25-hydroxycholecalciferol, and 1,25-dihydroxycholecalciferol.

mum stimulation of the transport response at 24 to 48 hours. Most significantly, metabolite 4B greatly shortens this lag, stimulating maximum calcium transport by 9 hours. At 9 hours this metabolite is at least 13 times as active as the parent vitamin (*8*). Similar results were also obtained by Haussler *et al.* (*9*).

Although the intestine is by far the richest source of metabolite 4B, owing to the low absolute amounts of the metabolite present (*10*) it has proved difficult to isolate enough metabolite 4B from the intestine to permit its chemical characterization. Accordingly a search was initiated for tissues which might be capable of producing metabolite 4B in an in vitro incubation. Thus Norman *et al.* (*11*) and Fraser and Kodicek (*12*) have recently reported that the kidney is the major if not exclusive site of production of metabolite 4B when 25-OH-CC is present as a substrate. It had previously been shown (*2*) that 25-OH-CC was an intermediate in the production, in vivo, of metabolite 4B. Furthermore, Norman *et al.* demonstrated that metabolite 4B produced from a kidney homogenate incubation is both chromatographically identical with and has biological activity equivalent to metabolite 4B obtained from chick intestinal mucosa (*11*).

This finding has permitted the production in vitro of large enough quantities of metabolite 4B to permit its chemical characterization. We now report that metabolite 4B has been identified to be 1,25-dihydroxycholecalciferol. While this report was in preparation, identical results were reported by two other laboratories (*13*).

Microgram quantities of metabolite 4B were prepared for mass spectrometric analysis in the following manner. Kidneys were obtained from groups of approximately 20 rachitic white Leghorn cockerels (24 days old), and 10 percent homogenates were made in 0.25*M* sucrose. With this homogenate a series of 50 incubations each containing 2.5 nmole of [26,27-^{3}H]25-OH-CC (specific activity 196 mc/mmole) and a reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system in 12 ml was performed as described previously (*11*). After incubation at 37°C for 2 hours, 45 ml of methanol-chloroform (2 : 1) was added to each incubation to obtain total lipids. This lipid extract was subjected to extensive chromatography (*14*) to obtain essentially pure metabolite 4B. The yield of metabolite 4B from such a series of incubations followed by chromatography has varied from 4 to 8 μg.

Mass spectra were obtained with an AEI MS-902 high-resolution mass spectrometer. The samples were introduced into the ion source on a direct insertion probe at source temperatures between 150° and 230°C above ambient. The mass spectra of metabolite 4B samples were obtained by increasing the ion source temperature up to 230°C so as to fractionate the sample while at the same time continuously scanning the molecular ion region for the appearance of the *m/e* 416 (molecular ion 416). High-resolution spectra of metabolite 4B were taken at a resolving power of 1 : 10,000. Typical mass spectra of CC and 25-OH-CC and metabolite 4B (1,25-diOH-CC) are shown in Fig. 1. The data for 1,25-diOH-CC is the observed spectrum from which contributing unknown contaminants have been omitted. In Fig. 2 are presented the structures of the three compounds and a tabulation of the important fragments produced in the mass spectrometer.

It was known that CC and 25-OH-CC have one and two oxygen atoms, respectively. Thus the finding that the parent molecular ions of CC, 25-OH-CC, and metabolite 4B were at *m/e* of 384, 400, and 416, respectively, suggested that metabolite 4B had only one more oxygen atom than 25-OH-CC and two more oxygen atoms than CC. The presence of three oxygen atoms was

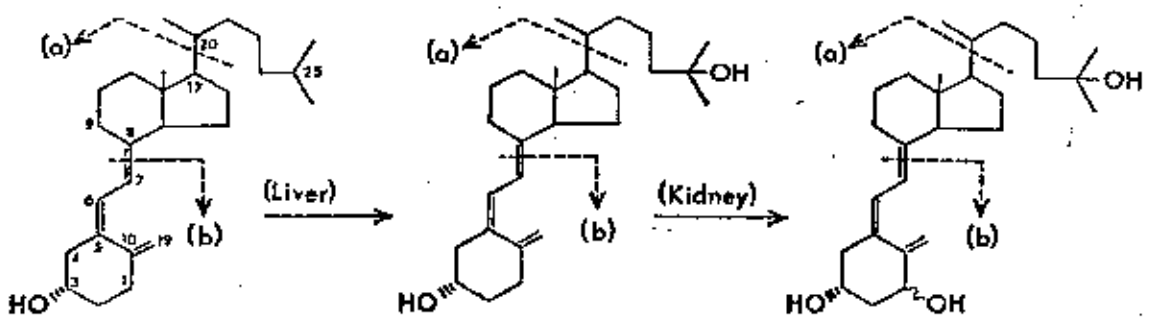
confirmed by high-resolution mass spectra determination of metabolite 4B. The molecular weight of metabolite 4B was found experimentally to be 416.32900, which is in good agreement the expected mass of 416.32903 $C_{27}H_{44}O_3$.

Since 25-OH-CC ($C_{27}H_{44}O_2$) had served as a substrate in the homogenate incubation for the production of metabolite 4B and since it was believed that metabolite 4B contained only one additional functional group which made it more polar than 25-OH-CC (*2, 7*), it seemed probable that two of the three oxygen atoms of metabolite 4B were still present at their original carbon-3 and carbon-25 positions (*15*). Furthermore we have reported (*7*) that the metabolism, in vivo, of mixed doses of [1,2-^{3}H]CC or [1-^{3}H]CC and [4-^{14}C]CC results in the stereospecific loss of tritium from the carbon-1 position in the formation of metabolite 4B. These results strongly suggest that the additional oxygen atom present in metabolite 4B is introduced at carbon-1. Since the molecular formula of metabolite 4B is $C_{27}H_{44}O_3$, this means that the additional oxygen atom had to be present at carbon-1 as a hydroxyl group rather than as a ketone. These suggestions were confirmed by comparison of the fragments of the three compounds.

The mass spectra of CC and 25-OH-CC both give fragments at *m/e* 271 which results from loss of the side chain due to cleavage of the bond between carbon 17 and 20, while metabolite 4B gives a fragment at *m/e* 287 (271 + 16). Thus the additional oxygen atom in metabolite 4B is not associated with the side chain. Both CC and 25-OH-CC give intense peaks at *m/e* 136. All molecules so far examined in this and other laboratories (*4, 13*) which have an intact triene structure with Δ 5-6, 7-8, 10-19 double bonds give this characteristic fragment which results from cleavage of the bonds between carbons 7 and 8. Both CC and 25-OH-CC gave a peak at *m/e* 118 (136—18) associated with the loss of water from this fragment containing ring A of the steroid nucleus. Metabolite 4B gave intense peaks at *m/e* of 152 (136 + 16) and 134 (152 − H_2O). Thus the additional oxygen atom known to be associated with metabolite 4B is unequivocably present in the ring A fragment, since the *m/e* 136 ion is shifted by 16 mass units to *m/e* 152. Also, the presence of the *m/e* 152 and 134 fragments is consistent with retention of the triene structure characteristic of CC (vitamin D) present in metabolite 4B (*4, 13*). Carbons 1, 2, and 4 are the only possible positions in the ring A fragment for the position of the additional hydroxyl. Consideration of the stereospecific loss of tritium from the α-position of carbon-1 in the production of metabolite 4B (*7*) indicates that the additional hydroxyl group of metabolite 4B is located at carbon-1. Thus the structure of metabolite 4B has been established to be 1,25-dihydroxycholecalciferol. This result confirms the previous prediction of Myrtle and Norman (*2, 7*).

Additional data of the mass spectrum of metabolite 4B which are consistent with this structure assignment include the ions at *m/e* of 398, 380, and 362 which correspond to the loss of one, two, and three molecules of H_2O from the molecular ion *m/e* 416 and the peaks at *m/e* of 269 and 251 resulting from the loss of one and two molecules of H_2O from the *m/e* 287 fragment. In a similar fashion CC had a fragment at *m/e* 366 (384 − H_2O), and 25-OH-CC had peaks at *m/e* 382 (400 − H_2O) and 364 (382 − H_2O).

The identification of 1,25-dihydroxycholecalciferol, the most biologically active form of vitamin D yet found, promises to provide many new avenues of experimentation. Myrtle and Norman have previously proposed that metabolite 4B (1,25-dihydroxycholecalciferol) is the biologically active form of vitamin D in the intestine. It now should be possible to synthesize this compound chemically and to examine directly its action in a number of systems. Several important problems that can now possibly be examined include determination of whether 1,25-dihydroxycholecalciferol (i) is further metabolized in the intestine, (ii) is biologically "active" as a result of its interaction with the genome of the mucosa or whether it mediates calcium transport directly via some membrane interaction, (iii) is biologically active in other CC-dependent systems, particularly bone deposition and resorption, (iv) is capable of acting in



	CHOLECALCIFEROL (VITAMIN D_3)	25-HYDROXYCHOLECALCIFEROL (25-OH-VITAMIN D_3)	1,25-DIHYDROXYCHOLECALCIFEROL (1,25-diOH-VITAMIN D_3)
Emp. Formula	$C_{27}H_{44}O$	$C_{27}H_{44}O_2$	$C_{27}H_{44}O_3$
ecular Wt.	384	400	416
(a)	271	271	287
(b)	136	136	152

Fig. 2. Structures of cholecalciferol (vitamin D_3) and its two metabolites with a tabular evaluation of their fragmentation patterns.

concert with the other known prime regulators of calcium homeostasis, parathyroid hormone and calcitonin, and (v) is effective in alleviating some of the pathological conditions of vitamin D–resistant rickets, osteomalacia, osteodosis, and uremia, all diseases which have been postulated to be due to abnormalities in CC metabolism.

It is possible that 1,25-dihydroxycholecalciferol should be reclassified as a steroid hormone. Certainly its secretion by the kidney followed by its selective accumulation in the target intestinal mucosa where it exerts its characteristic physiological effect on calcium metabolism satisfies the minimal definitions of a hormone. Norman has previously commented on the analogies in the mode of action of CC to that of several other steroid hormones, particularly aldosterone, testosterone, and estrogen (*16*).

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The lipid was applied in diethyl ether to a 50-g (1 by 80 cm) column of silicic acid. The column was batch eluted sequentially with 200 ml of 100 percent diethyl ether (25-hydroxycholecalciferol), 300 ml of 50 percent diethyl ether in dichloroethane (vol/vol) (metabolite 4B), and 200 ml of 100 percent methanol (other polar metabolites). The metabolite 4B fractions were concentrated under nitrogen and applied to a 1 by 80 cm column of Sephadex LH-20 eluted with chloroform-petroleum ether (65 : 35). Metabolite 4B emerged between 275 and 325 ml. These fractions were concentrated under nitrogen and applied to a 20-g (1 by 80 cm) Celite liquid-liquid partition column which was eluted with a mobile phase of 20 percent (vol/vol) 1,2-dichloroethane and 80 percent petroleum ether saturated with a stationary phase of methanol-water (90 : 10 by volume) as described previously (*2*). The metabolite 4B was eluted after 700 to 800 ml, concentrated under nitrogen, and subjected to silica gel thin-layer chromatography. The silica gel had been prewashed with 100 percent acetone, followed by 100 percent diethyl ether, and dried, and the plates were preeluted with 100 percent ethyl acetate to remove trace lipids. After application of the metabolite 4B sample to the plate, it was developed with ethyl acetate : petroleum ether (93 : 7). In this system cholecalciferol, 25-hydroxycholecalciferol, and metabolite 4B have R_f's of 0.89, 0.76, and 0.45, respectively. The metabolite 4B was eluted from the gel with redistilled 100 percent methanol, and concentrated under nitrogen prior to mass spectrometric analysis.
15. The kidney homogenate incubations employing 25-hydroxycholecalciferol (25-OH-CC) were found to be one-step metabolic transformations. That is, only one major compound more polar than 25-OH-CC was found in an extensive chromatographic analysis of the incubation media (*11*).
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Fertilization of Rabbit Ova in vitro by Sperm with Adsorbed Sendai Virus

Abstract. *Fertilization occurs when rabbit ova are cultured in vitro with epididymal sperm to which Sendai virus is adsorbed. These sperm do not require capacitation in vivo in order to fertilize. Evidence for fertilization is penetration of sperm, the appearance of two polar bodies and pronuclei, and cleavage through the eight-blastomere stage. The viruses attach almost exclusively to the sperm acrosome, with resultant head-to-head agglutination of the sperm.*

Chang (*1*) in 1959 submitted incontestable evidence for fertilization in vitro; live young were born after transplantation of cleaved rabbit ova to foster mothers. Others have confirmed and extended the technique (*2, 3*). Common to these studies was the use of sperm which have undergone a physiological change (capacitation) within the female tract. Several workers have attempted, with partial success, to capacitate rabbit sperm in vitro (*4*), but all successful studies of fertilization of rabbit ova in vitro have used sperm capacitated beforehand in vivo. We report here the fertilization of rabbit ova in vitro with sperm that have not been capacitated in vivo. Originally we mixed Sendai virus with washed ejaculated sperm for a short incubation with ova. Some of these ova underwent cleavage when transplanted 3 to 5 hours later to recipient does. In this work we used epididymal sperm, because more agglutinate with the same amount of virus; improved equipment permitted longer incubation of ova. Criteria for fertilization are penetration of sperm, production of the second polar body, formation of pronuclei, and cleavage (*5*). The experiments are reproducible with approximately 50 percent of ova cleaving (Table 1).

Dutch-belted and New Zealand does were bred to vasectomized males or injected intravenously with 125 I.U. of chorionic gonadotrophin (National Biochemical Company). Their freshly ovulated ova were recovered by flushing of the oviducts 12 hours later with culture medium TC-199 kept at 37°C (flushing was carried out at room temperature). Gametes were incubated at 37°C in a 35 by 10 mm petri dish with 1.5 to 2.0 ml of TC-199 (*p*H 7.6) and 100 units of penicillin and 100 μg

Table 1. In vitro fertilization of rabbit ova by epididymal sperm with adsorbed Sendai virus. From these and other experiments an additional 159 (sperm with virus), 22 (sperm control), and 26 (virus control) ova were observed before cleavage for evidence of fertilization 3 to 12 hours after culture. In the table, those ova which had cleaved had reached the two- to eight-cell stage.

Experiment	Sperm with virus		Sperm control	
	Ova cultured (No.)	Ova cleaved (No.)	Ova cultured (No.)	Ova cleaved (No.)
1	8	3		
2	31	8	12	0
3	15	4	12	0
4	22	16		
5	15	5		
6	38	28		
7	15	9	25	0
8	25	9		
9			36	0
10	16	9		

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430?

Actinomycin D and the Response to Vitamin D

Abstract. *The administration of low doses of actinomycin D to rachitic chicks inhibits the action of a subsequent dose of vitamin D_3 in promoting calcium absorption from the intestine.*

Many studies have demonstrated the essential role of vitamin D in mediating the absorption of calcium in the intact animal and in everted intestinal sacs or intestinal slices from the rat and chick (*1, 2*). But despite intensive efforts the exact biochemical mechanism and site of action of the vitamin remain unknown.

One of the characteristic features of the physiological expression of vitamin-D activity has been the time lag between the administration of vitamin D and the enhancement of calcium absorption across the intestinal mucosa. Although suboptimum enhancement of calcium absorption occurs in the rat in 3 to 5 hours after a massive dose [50,000 international units (I.U.)] of vitamin D, maximum expression of vitamin-D activity is not apparent until after 12 to 15 hours (*3*). In the chick, very little calcium absorption enhanced by vitamin D_3 occurs until 12 to 16 hours after either an oral, intracardial, or intraperitoneal injection of 100 I.U. of vitamin D_3 (*2, 4*).

It has been suggested (*5*) that the vitamin must be converted by the adrenals to a metabolically active form before the increased absorption of calcium can be observed. However, little direct evidence for this hypothesis has been found. Experiments with tritium-labeled vitamins D_2 and D_3 revealed that, in both the chick and rat, the adrenals accumulated an insignificant amount of vitamin D in any time interval after the administration of the ^{3}H-vitamin D. Further experiments with the chick revealed that the intestinal mucosa accumulated 70 percent of the radioactivity found at 30 hours within 3 to 5 hours after either an intracardial or intraperitoneal dose of 500 I.U. of ^{3}H-vitamin D (*6*). Although vitamin D was present at 5 hours, the manifestation of this vitamin did not become apparent until after 12 to 16 hours.

A possible hypothesis for the delay in biological response to vitamin D may be that delay reflects some as yet undefined induction process. Perhaps one or several enzymes necessary for the translation of calcium absorption are synthesized by the [illegible]

[illegible] the stimulus of the vitamin. Thus, [illegible]mycin D, which blocks DNA-[illegible]ected RNA synthesis and subsequent [illegible]ycin synthesis, would be expected to [illegible] any protein synthesis, induced by [illegible]amin D, essential to calcium absorp[illegible]n. There have been numerous reports [illegible]mentating the potent inhibitory ac[illegible] of actinomycin D on RNA and [illegible]ein synthesis (7).

The results (Fig. 1) record the effect [illegible] an oral dose of 100 I.U. of vitamin [illegible] upon the intestinal absorption of [illegible]cium in rachitic chicks at varying [illegible]rvals after administration of the vi[illegible]min. Intestinal absorption of calcium [illegible] measured by the appearance of [illegible]Ca in the serum prepared from whole blood obtained from the wing vein 60 minutes after oral intubation of 2.0 mg of [illegible]Ca-labeled calcium chloride. The concentration of [illegible]Ca in the serum increases approximately linearly with time from 15 minutes until 1½ hours after the oral dose. At this time the [illegible]Ca fall slowly, undoubtedly because of [illegible]libration with rapidly-exchangeable bo[illegible] [illegible]Ca.

No significant enhancement by vitamin D of calcium absorption can be observed before 12 to 16 hours. But, if actinomycin D (10 μg/110 g of body weight of chick) is injected intraperitoneally 2 hours before the oral administration of 100 I.U. of vitamin D_3, the characteristic response of increased calcium absorption is abolished.

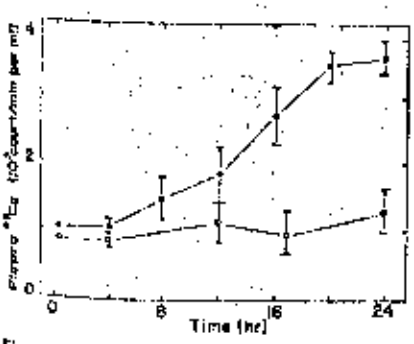

Fig. 1. The serum-calcium response of vitamin-D deficient chicks to an oral dose of 100 I.U. of vitamin D_3 in the absence (closed circles) and presence (open circles) of actinomycin D. The rachitic chicks received 10 μg actinomycin D per 110 grams of body weight in 0.20 ml of 0.9 percent NaCl intraperitoneally 2 hours before the dose of vitamin D_3 (in Wesson oil) was administered orally. At varying times after the vitamin D_3 treatment, 2.0 mg of [illegible]Ca + [illegible]Ca (1 to 3 × 10[illegible] count/min) in 0.20 ml was fed orally. Blood from wing veins was drawn with syringes containing heparin 30 and 60 minutes later. Each point represents the mean of the values obtained on the plasmas of 3 to 5 chicks (± s.d.).

Table 1. Inhibition by actinomycin D of calcium absorption mediated by vitamin D. Chicks were raised to the age of 4 to 5 weeks on a rachitogenic chick diet (commercial source) until the growth rate reached a plateau. Chicks in Experiment A, unless indicated otherwise, received 100 I.U. of vitamin D_3 2 hours after actinomycin D and 24 hours before [illegible]Ca. Chicks in Experiment B, unless indicated otherwise, received 20,000 I.U. of vitamin D_3 2 hours after actinomycin D and 24 hours before [illegible]Ca. Chicks in Experiment C received 4000 I.U. of vitamin D_3 daily for 4 days prior to actinomycin-D treatment, and [illegible]Ca was administered 26 hours after the actinomycin D. Actinomycin D was injected intraperitoneally in 0.20 ml 0.9 percent NaCl. Vitamin D_3 was injected intraperitoneally in 0.20 ml of 1,3-propanediol; 2.0 mg of [illegible]Ca + [illegible]Ca (1 to 3 × 10[illegible] count/min) in 0.20 ml was fed orally. Blood from wing veins was collected in syringes containing heparin 30 and 60 minutes after [illegible]Ca administration. Each number is the average of values from 7 to 12 chicks. One international unit (I.U.) of vitamin D_3 is equivalent to 0.025 μg.

Vitamin D_3 treatment (I.U.)	Actinomycin treatment (μg/110g chick)	[illegible]Ca incorporation (count/min) per ml 30 min	60 min
	Experiment A		
None	None	840 ± 230	1200 ± 240
100	10	780 ± 320*	1280 ± 360*
100	1	1290 ± 500†	1500 ± 430†
100	None	2440 ± 430*†	2750 ± 370*†
	Experiment B		
None	None	350 ± 70	600 ± 150
20,000	10	690 ± 420‡	750 ± 250*
20,000	None	1770 ± 390‡	2100 ± 490*
	Experiment C		
4000 24 hr before actinomycin D	None	2310 ± 320	2920 ± 290
4000 24 hr before actinomycin D	10	2500 ± 450	3100 ± 150

* Difference between ± actinomycin D treatment is significant $P < .001$. † Difference between ± actinomycin D (1 μg/110 g chick) is significant $P < .02$. ‡ Difference between ± actinomycin D (10 μg/110 g chick) treatment is significant $P < .01$.

A more detailed examination of the relation between vitamin D and actinomycin D is shown in Table 1. In these experiments the vitamin D_3 was administered intraperitoneally rather than orally to avoid any possible effect of actinomycin D upon intestinal absorption of the vitamin. In confirmation of the results of Fig. 1, actinomycin D (10 μg per 110 grams of body weight) effectively blocked a dose of 100 I.U. of vitamin D_3. Moreover, when as little as 1 μg of actinomycin D per 110 grams of body weight was administered, an approximate 50-percent inhibition of the calcium absorption response to a dose of 100 I.U. of vitamin D_3 was observed. This is one of the lowest effective dosages for actinomycin reported. But if chicks deficient in vitamin D were given an oral dose of 4000 I.U. of vitamin D_3 daily (Experiment C) for 4 days before the intraperitoneal injection of actinomycin D (10 μg/110 g chick), no impairment of calcium absorption was observed. This finding tends to exclude the likelihood that the inhibitory effects are due to blocking of parathyroid-hormone synthesis by actinomycin. If parathyroid hormone were obligatory for calcium absorption, then actinomycin D should have blocked calcium absorption in the chicks which received 4000 I.U. of vitamin D_3 4 days before actinomycin D.

Perhaps more significant is the actinomycin inhibition of an intraperitoneally injected dose of 20,000 I.U. of vitamin D_3 (Experiment B). If vitamin D functioned strictly as a coenzyme, this amount of the vitamin should be adequate to saturate any receptor sites crucial for calcium absorption that would be present. Yet apparently any manifestation of the vitamin activity is strictly dependent upon the sequence of actinomycin-sensitive events involved in the conversion from a deficient to a normal state.

Not all lags or latent metabolic events are *a priori* sensitive to actinomycin D. Ray *et al.* have reported (8) that glucocorticoids can effectively stimulate gluconeogenesis (9) in adrenalectomized or normal rats even though the rats have been treated with sufficient actinomycin D to block induced synthesis of enzymes. Also, Greengard and Gordon (10) observed a lag in the pyridoxine-induced rise of tyrosine transaminase, in rat liver, that was sensitive to puromycin but insensitive to actinomycin D. The possibility remains, however, that other vitamins may act on enzyme synthesis by way of DNA-directed RNA synthesis.

One cannot decide from the foregoing experiments whether the biochemical role of vitamin D is to promote the induction of the appropriate enzyme systems or the alteration of membrane structure necessary for calcium absorp-

tion, or whether vitamin D in addition to participating in such an induction process can also function as an obligatory catalyst in the induced system. However, the promotion of calcium absorption by vitamin D probably requires an unimpaired RNA-synthesizing system.

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THE MODE OF ACTION OF VITAMIN D

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I. DELINEATION OF THE PROBLEM

The objective of this review article is to re-evaluate the role of vitamin D in calcium metabolism from the viewpoint that vitamin D may be acting more as a steroid hormone than as a classical vitamin or cofactor.

Although a 'vitamin' is defined classically as an essential dietary trace constituent which is necessary for the normal nutritional and physiological development of the organism, there has been in recent years a second meaning superimposed upon the original. The implied meaning is that a vitamin is a co-factor for a particular enzymic reaction. This is, no doubt, in part due to the magnificent progress made in the field of water-soluble vitamins, where many of them have been shown to be intimately involved in a specific enzymic reaction.

Aside from the role of vitamin A aldehyde in the visual cycle, the mechanism of action of the fat-soluble vitamins is at the present time only poorly understood. This may be in part due to the fact that the implied concept that a vitamin is a cofactor has in a sense biased the approach to the study of their mechanism of action.

Two methods of approach have been applied in elucidating the mechanism of action of vitamin D. Firstly, experiments have been devised to examine the role of the vitamin in mineral metabolism, i.e. calcium and phosphate absorption and excretion, bone formation and solubilization. Secondly, studies have dealt with the role of the vitamin

in organic metabolism, i.e. citric acid metabolism, and also in membrane permeability and animal growth. It is quite difficult to integrate all the studies into an organized scheme that might adequately explain the biochemical mechanism of action of vitamin D. The overriding influence in the conduct of many of these studies has been that vitamin D was acting as a cofactor in some particular reaction. But in each of the instances cited, the observation being investigated was apparently several steps removed from the first fundamental action of vitamin D.

There are some recent findings with regard to vitamin D that make it appear that the biochemical mechanism of action of the vitamin may be explained not in terms of a cofactor involvement but in terms of an ability to activate or stimulate the biochemical expression of genetic information. It was found (Norman, 1965; Zull, Czarnowska-Misztal and DeLuca, 1965) that actinomycin D, an inhibitor of DNA-directed RNA synthesis (Reich, Franklin, Shatkin & Tatum, 1961), is capable of inhibiting the response of the vitamin D-deficient chick or rat to vitamin D. It was further observed (Norman, 1965) that actinomycin D was capable of inhibiting the response to a dose of vitamin D_3 that was 4000 times that of a physiological dose. If vitamin D were functioning as a cofactor, this amount of the vitamin would be more than that necessary to saturate any receptor sites on an enzyme. Apparently any manifestation of the vitamin's activity is strictly dependent upon the actinomycin D-sensitive events involving the conversion from the deficient to a normal state.

The concept that an understanding of the mechanism of action of hormones must be sought in terms of hormonal interactions with genetic information has become increasingly emphasized in recent years (Kirby & Kidson, 1964), particularly for hydrocortisone (Kenney & Albritton, 1965), oestrogen (Hamilton, 1964), aldosterone (Edelman, Bogoroch & Porter, 1963), and ecdysone hormones (Karlson, 1961).

It would appear appropriate at this time to re-evaluate and integrate the literature on vitamin D, using as a working hypothesis the concept that the vitamin may be acting analogously to a steroid hormone in terms of its ability to interact with genetic information and ultimately elicit a characteristic physiological response. The remainder of the review is a discussion of the biochemical and physiological observations which can directly be related to effects of vitamin D. It has not been necessary to consider in detail much of the older literature concerning nutritional aspects of vitamin D as pertains to the calcium and phosphorus content of the diet. For a discussion of the older literature, the involvement of other regulators such as parathyroid hormone and thyrocalcitonin and other viewpoints, the reader is referred to a number of other reviews (Nicolaysen & Eeg-Larson, 1953; Nicolaysen, Eeg-Larson & Malm, 1953; Deuel, 1957; Copp, 1957; Rasmussen, 1961; Rasmussen & DeLuca, 1963; Wasserman, 1963).

II. STRUCTURAL REQUIREMENTS OF VITAMIN D FOR BIOLOGICAL ACTIVITY

In order that the reader may place the comments appearing later in this review article in proper perspective, it is important to understand the structural requirements for vitamin D and the means available for assessing its biological activity.

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Vitamin D was first recognized as being an essential dietary trace constituent in 1919 when Edward Mellanby (1919) produced rickets in dogs and was able to alleviate this condition by the feeding of certain animal-fat preparations. Shortly thereafter McCollum, Simmons, Shipley & Park (1921) realized that the antirachitic activity, which had previously been identified as 'the fat-soluble vitamin', in reality was only one of two fat soluble vitamins present in the fat extract.

The biological demonstration of the existence of vitamin D prompted intensive efforts to isolate and chemically characterize the active component. These studies were culminated in the 1930s when Windaus, Linsert, Lüttringhaus & Weidlich (1932) and Askew *et al.* (1932) simultaneously identified vitamin D_2 and vitamin D_3 (Fieser & Fieser, 1959). Vitamin D_2 is derived from ergosterol and vitamin D_3 from 7-dehydrocholesterol by a complex series of photochemical reactions when the provitamin is irradiated with ultraviolet light. A number of excellent review articles on the detailed chemistry and structure of vitamin D are available (Bills, 1954; Inhoffen & Irmscher, 1959).

The structure of vitamin D and a number of related compounds without their side-chains is shown in Fig. 1. The characteristic feature of a provitamin is that it must have a $\Delta_{5,7}$ double bond in the B ring of the steroid nucleus. The conformation shown for vitamin D is that of its crystalline form as determined by X-ray crystallographic analysis (Crowfoot-Hodgkin, Webster & Dunitz, 1957).

Previtamin D and vitamin D are in thermal equilibrium with each other, the equilibrium being 89% in favour of vitamin D at 37° C. (Hanewald, Rappoldt & Roborgh, 1961). Tachysterol is another compound produced by the ultraviolet irradiation of the provitamin. The structures of tachysterol, dihydrotachysterol and dihydrovitamin D are presented for comparison with the structure of vitamin D.

It has been found that vitamin D cannot be altered by replacing the hydroxyl at position 3 with a sulfhydryl (Bernstein & Sax, 1951), keto (Windaus & Buckholz, 1938), or halogen group (Bernstein, Oleson, Ritter & Sax, 1949) and still retain biological activity. The location of the conjugated double bonds also markedly affects the biological activity of vitamin D. The preferred location is at the 10–19, 5–6, 7–8 positions. Tachysterol has a much lower 'antirachitic property' (Meunier & Thibaudet, 1946) than does vitamin D while dihydrotachysterol-3 has significant biological activity. The exact biological potency of dihydrotachysterol is subject to question. Bosmann & Chen (1966) claim that crystalline dihydrotachysterol-2 and -3 (these numbers relate their side-chain to vitamin D_2 and D_3) were equally as antirachitic in the chick as vitamin D_3; while McChesney (1943) found that dihydrotachysterol-2 was only one-fifth as active in the chick as vitamin D_3. Carlsson & Lindquist (1955*b*) found in the rat that vitamin D_2 was approximately thirty times more effective than dihydrotachysterol-2 in maintaining serum calcium. Dihydrovitamin D has unfortunately not been rigorously assayed, while previtamin D has no biological activity (Hanewald *et al.* 1961).

In Fig. 2 is shown how only a seemingly slight modification in the side-chain of vitamin D has a profound effect on the biological activity. When the side-chain is replaced by a hydroxyl group (Dimroth & Paland, 1939), or by a bile acid side-chain (Haslewood, 1939), the compounds are devoid of vitamin D activity. Unfortunately

the biological assays carried out on vitamin D_4 (McDonald, 1936) and the vitamins produced by irradiation of $\Delta_{5,7}$ stigmasterol (Rosenberg, 1945) and $\Delta_{5,7}$ sitosterol (Grab, 1936) were done only with the crude irradiation mixture and not with the crystalline compounds. Vitamin D_2 has equal activity in the chick and the rat (Chen & Bosmann, 1964). In comparison, vitamin D_2 has only one-tenth the activity of vitamin D_3 in the chick, but is equally as potent in the rat (Chen & Bosmann, 1964).

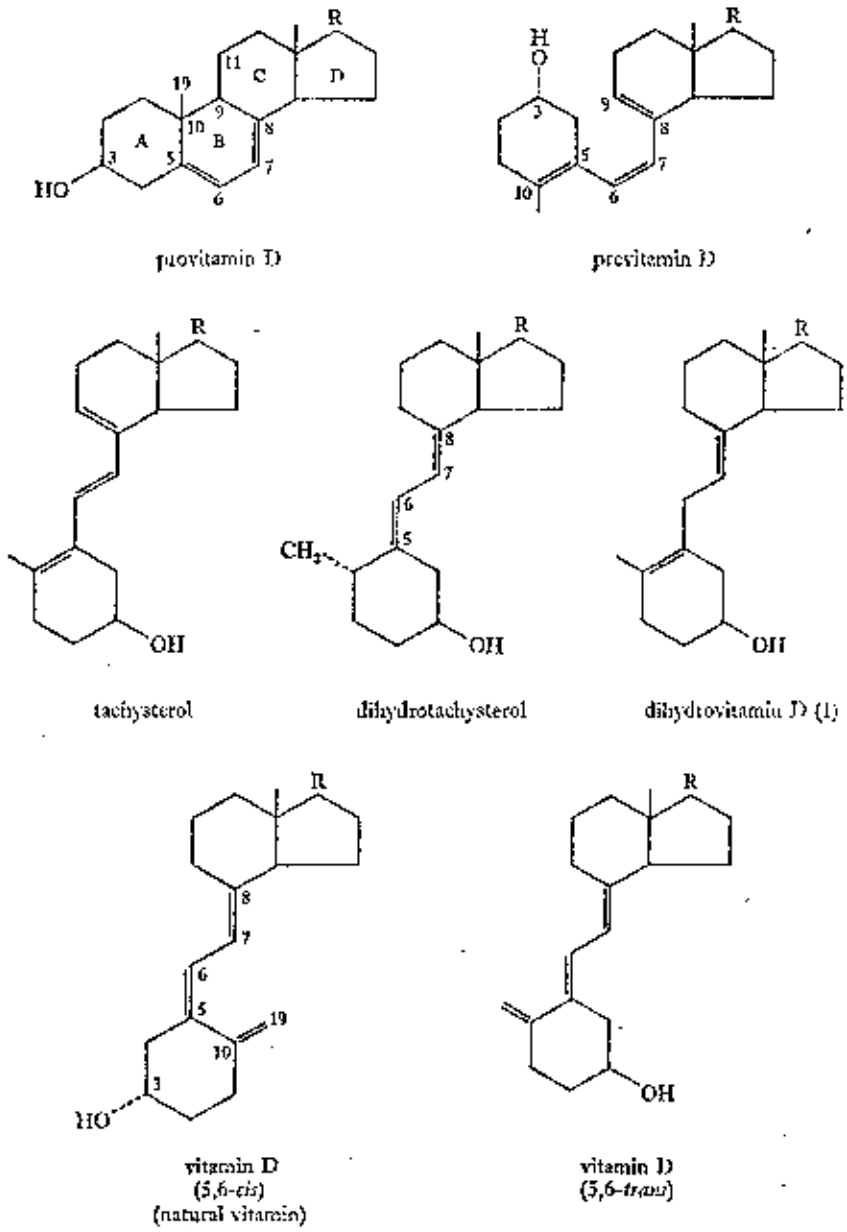


Fig. 1. Compounds of the vitamin D family.

It is apparent that there are a great variety of compounds which possess some vitamin D-like activity. It would appear that this would be a fruitful field for further investigation as a means of determining the role of a particular portion of the vitamin D molecule in eliciting a subsequent activity. Are the structural requirements the same for vitamin D in the different loci of its action, e.g. intestine, kidney, and skeletal system? Can a compound slightly different in structure from vitamin D, e.g. an octahydro-vitamin D, act as an antagonist or anti-vitamin by binding at the active site but not carrying out the subsequent biochemical action?

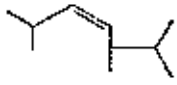
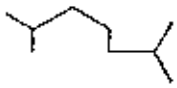
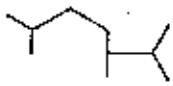
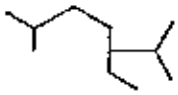
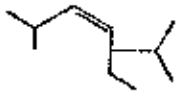
provitamin:	ergosterol	7-dehydro-cholesterol	22,23-dihydro-ergosterol
sidechain			
vitamin:	D_2	D_3	D_4
relative activity:			
rat	100%	100%	50–75%
chick	10%	100%	20%
provitamin:	Δ 5,7-androsta-diene-3,17-diol	7-dehydro-β-sitosterol	7-dehydro-stigmasterol
sidechain:	—OH		
vitamin:	—	D_5	D_4
relative activity			
rat	2%	10%	5%
chick	?	5–10%	?

Fig. 2. Effects of side-chain alteration on biological activity of vitamin D.

One of the most characteristic features of vitamin D activity is its extreme potency. One international unit (i.u.) has been defined as being equivalent to 0·025 μg. of pure crystalline vitamin D_2 (League of Nations, 1935). One normally assumes that 1·00 i.u. of vitamin D_2 is equivalent to 1·00 i.u. of vitamin D_3.

There is at the present time no simple means of directly measuring small physiological amounts of vitamin D in biological systems. The several chemical or physical assays available which can measure as little as 0·2 μg. (Nield, Russell & Zimmerli, 1940; Hanewald, 1959; Avioli & Lee, 1966) are not in general applicable to crude

mixtures of compounds such as one normally encounters in biological systems. Thus vitamin D is the only one of the vitamins for which chemical or physical means of measurement have not largely replaced biological methods.

The official methods of estimating vitamin D are either the 7-day curative test in the rat (United States Pharmacopoeia, 1950) or the A.O.A.C. (Association of Official Agricultural Chemists, 1940) 21-day prophylactic assay in the chick. Both assays will detect the presence of 1–10 i.u. (0·025–0·25 μg.), but the precision of the assays is only ± 20%, and they are laborious and time-consuming operations. It is now firmly established that the weekly requirement for the normal physiological development of a 100–150 g. chick (Chen & Bosmann, 1964) is 50–75 i.u. of vitamin D_3, while that for a 100–150 g. rat is 100–200 i.u. of vitamin D_2 or D_3 (Steenbock & Herting, 1955; Carlsson & Lindquist, 1955*a*). As little as 2·5–5 i.u. of vitamin D_3 (Norman, 1966*a*) will give a response in terms of stimulating intestinal calcium absorption in a vitamin D-deficient chick. An *in vitro* biological assay has been developed that is capable of detecting 0·50–2·0 i.u. of vitamin D which has been fed 2–3 days before the experiment (Schachter, Kimberg & Schenker, 1961). This assay measures binding of $^{45}Ca^{2+}$ to rat intestinal slices. It is very important when attempting to elucidate the normal biochemical effects of vitamin D to give physiological doses of the vitamin, i.e. 1–200 i.u.

III. PHYSIOLOGICAL INTERACTIONS AND EFFECTS OF VITAMIN D

Since the work of Mellanby, rickets has been recognized as being one of the primary physiological end-points of a vitamin D deficiency. Rickets has been defined classically as a lack of calcification of the hypertrophic cartilage zone and osteoid, with a consequent elongation and widening of the epiphyseal cartilage plate. Many studies have been carried out concerning the relationship between the ratio of calcium and phosphorus in the diet and the subsequent development of rickets in rats and chicks. In this respect there is a species difference. The classical skeletal abnormalities of rickets will develop in the rat only when the dietary phosphate is low or unavailable, perhaps by virtue of a high Ca/P ratio (Steenbock & Herting, 1955). The chick will develop the skeletal abnormalities of rickets regardless of the ratio of Ca/P in the diet (Migicovsky & Emslie, 1947). Thus the chick would appear to be the more suitable experimental species for studies concerning the relationship and interaction of vitamin D in calcium metabolism.

Steenbock & Herting (1955) have advanced the concept that in the rat, vitamin D performs a specific metabolic function necessary for growth and soft-tissue development in addition to its well-documented effects on intestinal absorption and the skeletal deposition of minerals. Migicovsky & Emslie (1947) have obtained evidence from chick studies that support this non-calcium effect of vitamin D. These effects on growth may reflect some complex interaction by vitamin D in metabolic control processes.

Other physiological disorders that can be related to vitamin D concern osteomalacia (Liu *et al.* 1940; Harrison, 1957), in a sense an adult form of rickets, idiopathic hypercalcemia (Fellers, 1959) and vitamin D-resistant rickets. It has been shown

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(Winters *et al.* 1958; Engfeldt, Zetterström, & Winberg, 1956; Villanueva, Hnicki, Frost & Arnstein, 1966; Williams, Winters & Burnett, 1960; Albright, Butler & Bloomberg, 1937; Harrison, 1958) that some humans who have rickets do not respond to treatment with non-pharmacological doses of vitamin D. These people are said to have vitamin D-resistant rickets. Some workers (Dent, 1952; Fanconi & Girardet, 1952; Fanconi, 1955) feel that this terminology is inappropriate because the disease is primarily characterized by hypophosphatemia and diminished renal tubular absorption of phosphate and only secondarily by a defect in intestinal absorption of calcium. However, evidence on this point is not yet conclusive.

Winters *et al.* (1958) have reviewed 65 documented cases of vitamin D-resistant rickets and have presented evidence that the disease is almost always inherited, and is usually congenital. In one instance the abnormality was found to follow a female sex-linked dominant pattern, wherein the females tended to have either mild deformities or no deformities, while the male normally had moderate to severe deformities. These results are in accord with the concept that vitamin D may act at the gene level either to derepress or initiate the utilization of DNA-coded information essential for the ultimate biochemical and physiological expression of vitamin D activity. These individuals may have undergone some non-lethal mutation, which has, however, had a profound influence on their ability to respond to vitamin D.

Another important involvement of vitamin D concerns its interaction with parathyroid hormone. Parathyroid hormone and vitamin D are known to act synergistically in regulating and maintaining a normal calcium metabolism via a homeostatic mechanism. This involves the control not only of the intake of calcium and excretion of phosphate, but also the delicate balance between serum calcium and phosphorus levels and calcification in the skeletal system. It has been shown that vitamin D is necessary for the effect of parathyroid hormone on calcium metabolism (Rasmussen *et al.* 1963; Harrison & Harrison, 1964). That is, in the absence of vitamin D or in a vitamin D deficiency, parathyroid hormone is unable to exert its characteristic action on calcium metabolism. However, in this situation parathyroid hormone is still able to exert its effect on phosphate metabolism, i.e. promoting the renal excretion of phosphate.

IV. ACTION OF VITAMIN D ON CALCIUM

(1) *Intestinal calcium absorption*

(*a*) *Background information*

Before the availability of radioactive calcium and phosphorus in the 1940s it was difficult to establish unequivocally that one of the primary actions of vitamin D was to promote or facilitate the intestinal absorption of calcium. Early workers utilized primarily balance studies where the intake and the faecal and urine levels of calcium and phosphorus were monitored. These studies were carried out in children (Findlay, Paton & Sharpe, 1920), in the rat (McClendon, 1922; Brown *et al.* 1932), the chick (Bethke, Kennard, Kik & Zinzalian, 1939) and in the dog (Jones, 1927). It was generally agreed that there was an increased loss of phosphorus and calcium in

vitamin D deficiency, primarily through an increased output of these elements in the faeces.

It was not until the thorough studies of Nicolaysen that the direct causal relationship between vitamin D and calcium absorption was established. In a notable series of experiments he showed the following. (*a*) When a Thiery Vella fistula was made of the entire colon in the rat, there was no alteration in the faecal amount of calcium or phosphorus of vitamin D-deficient or normal rats (Innes & Nicolaysen, 1937). (*b*) Intravenous injection of calcium was not followed by increased faecal calcium but was instead followed by an increased renal calcium excretion. These results were independent of the vitamin D status of the rats (Nicolaysen, 1937*a*). (*c*) Under dietary phosphorus-starvation conditions vitamin D markedly stimulated intestinal calcium absorption, while under dietary calcium-starvation conditions vitamin D had no enhancing or inhibitory effect on phosphorus absorption (Nicolaysen, 1937*b*). The latter was irrespective of whether the dietary phosphorus source was inorganic or organic phosphate. (*d*) In a pioneering experiment utilizing isolated, ligated jejunal loops of rachitic and normal rats *in vivo*, Nicolaysen (1937*c*) found that the rate of absorption of calcium was markedly increased in the normal rats as compared to the deficient rats. Under similar conditions the absorption of calcium from the abdominal cavity was identical in normal and vitamin D-deficient rats. This study demonstrated for the first time, in an unequivocal fashion, the stimulatory effect of vitamin D on intestinal calcium absorption.

Thus when radioactive calcium and phosphorus became available in the early 1940s the groundwork was laid for more sophisticated studies. Greenberg (1945) concluded from one of the early tracer experiments utilizing both orally and intraperitoneally administered $^{45}Ca^{2+}$ that the intestinal excretion of calcium was not affected by the vitamin D status of the animal. Other studies by Migicovsky & Nielson (1951) in the chick and by Carlsson (1951) and Lindquist (1952) in the rat, utilizing radioactive calcium, substantiated the earlier work of Nicolaysen that vitamin D had a significant effect on intestinal calcium absorption in the rat.

(*b*) *General considerations of the problem*

The physiological studies demonstrating the enhancing effect of vitamin D on the intestinal absorption of calcium have laid the groundwork for future rigorous biochemical examination of the detailed mechanism by which the metal ion is moved across the small intestine. The problem is basically one of determining whether the transport of calcium is an active transport, i.e. transport against an electrochemical gradient (Ussing, 1949) or passive transport*, and what the detailed role of vitamin D is in the process. Does the vitamin act as a catalyst in an active transport process, functioning essentially as a cofactor perhaps to couple the energy source to the transport mechanism or to 'carry' the calcium across the membrane? Does the vitamin act as an effector and bind to some crucial membrane and change the con-

* This specific problem is a part of the much larger field of ion transport, for which there are available a number of excellent reviews (Anderson & Ussing, 1960; Wilbrandt & Rosenberg, 1961; Christensen, 1962; Rosenberg & Wilbrandt, 1963; Wiseman, 1964).

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formation so that now the membrane has an increased permeability, thus in essence facilitating a passive transfer? A third and perhaps more likely possibility is that the vitamin may either induce the synthesis of appropriate enzyme systems involved in an active transport system or induce the synthesis of a modified, more permeable membrane (to calcium). In this circumstance, although the vitamin might not directly be involved in calcium transport, it would still be an intriguing question as to how the calcium actually crosses the cell membrane. If vitamin D induces the production of a transport system, either active or passive, it is pertinent to know whether the parameters of the induced system (vitamin D treated animal) are identical or different from the non-induced system (vitamin D-deficient animal). Does vitamin D induce more of the same system, alter an existing system, or initiate the production of a second calcium transport system? Unfortunately all of these questions are virtually unanswered at the present time.

(*c*) *Studies on calcium absorption in the rat*

Many workers have approached the problem of whether vitamin D-mediated calcium absorption is an active or passive process by either (*a*) utilizing everted intestinal sacs *in vitro* and studying the distribution of radioactive $^{45}Ca^{2+}$ into the mucosal and/or serosal compartments, or (*b*) studying $^{45}Ca^{2+}$ flux *in vivo* with ligated intestinal loops. The primary criticism of the everted-sac technique is that each sac can be sampled only once and that the total volume of serosal fluid is only 0·5–1·0 ml. while the mucosal compartment is 2·5–5·0 ml. Thus there is the grave possibility that a significant error may be introduced by membrane absorption and adsorption. Also these *in vitro* experiments require that the calcium be transported in an unphysiological fashion across the serosal membrane.

Schachter and associates, utilizing primarily the everted intestinal sac technique, have concluded that the transport of calcium from mucosa to serosa of the rabbit, rat, and guinea-pig is an active process dependent upon dietary vitamin D (Schachter & Rosen, 1959; Dowdle, Schachter & Schenker, 1960). The active transport process was primarily limited to calcium (Schachter, Dowdle & Schenker, 1960*a*); no significant transfer of Sr^{2+}, Ba^{2+} or K^{+} from mucosa to serosa could be demonstrated. Dowdle *et al.* (1960) claimed that the active transport process was increased within 1 h. of orally administered vitamin D_2. However, they administered 50,000 i.u. of the vitamin and noted only a slight increase in the calcium transport. The active transport mechanism was maximal in the duodenum, less in the ileum and lowest in the jejunum (Kimberg, Schachter & Schenker, 1961; Schachter *et al.* 1961). It was also noted (Schachter, Dowdle & Schenker, 1960*b*) that respiring slices of rat intestine carried out an oxygen and vitamin D-dependent accumulation of $^{45}Ca^{2+}$ *in vitro*. A most interesting observation was that the small intestine of the rat is able to respond facultatively to a diet low in calcium by increasing its active transport mechanism for this ion (Kimberg *et al.* 1961). This could be accomplished only in the presence of vitamin D and is most suggestive of a dual control mechanism involving both vitamin D and calcium which enables the organism to adapt to varying dietary circumstances. Perhaps this control is effected at the genome level.

The only direct test reported of the energy-dependent nature of the active transport process (Schachter *et al.* 1960*b*; Schachter *et al.* 1961) is the effects of incubating either inverted intestinal sacs or intestinal slices under nitrogen. The average percentage change in the active transport mechanism along the entire small intestine, due to vitamin D, was +74% in the presence of oxygen and +7% in the presence of nitrogen. The only end-point in all these studies was the establishment of a concentration gradient between the serosal and mucosal compartments of the everted intestinal sac. No attempts were made to study other possible types of calcium transfer, e.g. simple diffusion or facilitated diffusion in the presence and absence of vitamin D.

In contrast, Harrison & Harrison (1960) have concluded that vitamin D influences calcium transport by an action on the permeability of cell surfaces. Although they observed that everted intestinal sacs prepared from vitamin D-treated rats had an active transport mechanism (by the criterion of Schachter and colleagues), it was also apparent that the vitamin increased the rate of downhill diffusion of $^{45}Ca^{2+}$ across the intestinal membrane. This effect was specific for Ca^{2+}, as no vitamin D effect was apparent on $^{24}Na^{+}$ transfer. Also they found that incubation of everted intestinal sacs under (*a*) nitrogen, (*b*) at reduced temperatures (10° C), or (*c*) in the presence of 5×10^{-4} M cyanide, all conditions which should inhibit oxidative metabolism, did not abolish the effect of vitamin D. As a consequence they suggested that vitamin D may affect the diffusibility of calcium into the cell. If this were the rate-limiting step, then it is conceivable that the vitamin could elicit an apparent effect on the energy-dependent active process by limiting the availability of Ca^{2+} to the transporting mechanism. It is possible that the vitamin has multiple sites of action, both on permeability and active transport.

Harrison & Harrison (1965) have obtained further support for the concept that vitamin D acts to increase the membrane permeability to calcium. They incubated both everted intestinal sacs, from which the mucosal epithelium had been removed, and normal sacs, in the presence and absence of *N*-ethyl maleimide, at 37° or 5° C. All the results obtained were in accordance with the concept that vitamin D increases the uptake step of calcium by the mucosal cell by reducing the permeability barrier.

(*d*) *Studies on calcium absorption in the chick*

Wasserman & Kallfelz (1962) have concluded from measurements on the unidirectional flux of calcium across the chick duodenum *in vivo* that vitamin D does not operate through a unidirectionally oriented, active calcium transport system, but that the vitamin may affect the bidirectional permeability of the mucosal cell to calcium. They observed (Wasserman & Kallfelz, 1962; Wasserman, Taylor & Kallfelz, 1966) that vitamin D_3 caused an increased influx (lumen to plasma) and an increased efflux (plasma to lumen) of calcium. Sallis & Holdsworth (1962*a*) in further studies utilizing ligated intestinal loops and everted intestinal sacs concluded on the basis of inhibitor studies that vitamin D-mediated calcium transport is energy-dependent and that this energy can come from glycolysis. They also observed that in the chick, maximal transport was obtained in sacs prepared from the ileum. This contrasts with the rat, where the duodenum is most active.

More recently Holdsworth (1965) has developed a model system for calcium transport. This model considers a sheet of mucosal cells as having two faces, one adjacent to the lumen of the intestine and the other adjacent to the serosal surface. Each face was assumed to have two properties: (*a*) a bi-directional calcium pump operated by metabolic energy; (*b*) a unidirectional diffusion leak out of the cell. After examining the effects of cold and inhibitors, Holdsworth proposed that vitamin D inhibits a metabolically operated pump that returns calcium from the mucosal cell to the intestinal lumen. This is the most successful report in the literature to date from the point of view of recognizing that there may be more than one calcium transport system in a cell and of attempting to dissect the various calcium transport systems in an effort to study and assess the role of vitamin D in individual steps. These results are not in agreement with the suggestion of a bi-directional calcium-oriented effect of vitamin D on membrane permeability (Wasserman *et al.* 1966). Obviously much more detailed work of this type must be carried out to resolve effectively the question of active versus passive transport and the nature of the vitamin D interaction. Only then can a detailed picture of the biochemical mechanism of calcium transport be elucidated.

(*e*) *Effect of adrenal hormones on calcium absorption*

Another aspect of vitamin D-mediated intestinal calcium absorption concerns the effects of adrenal steroids on this process. It was originally observed by Harrison, Harrison & Park (1957) that when rachitic rats were given 1000 i.u. of vitamin D_2 7 days before, and 2 mg/100 g. rat of cortisol daily for 3 days before the experiment, that the cortisol treatment reduced the rate of $^{45}Ca^{2+}$ transfer in both the downhill diffusion and active accumulation experiments. That is, cortisol treatment antagonized or partially inhibited the action of vitamin D. This result has been confirmed by Williams, Bowser, Henderson & Uzgiris (1961). Sallis & Holdsworth (1962*b*) found in the chick that 3-day administration of cortisol or deoxycorticosterone interfered with the enhancement of vitamin D-mediated calcium transport, but had no effect on calcium absorption by rachitic chicks. They also observed that these same compounds when injected intracardially 1 h. before the test enhanced the vitamin D_3 effect. Kimberg *et al.* (1961) found that although hypophysectomy and adrenalectomy did not abolish the facultative response of rats to calcium deprivation, they did markedly decrease their *in vitro* active transport system. Although at first these results may appear contradictory it is possible that they may be varying manifestations of the same fundamental involvement of the adrenal hormones in one or other of the many steps involved in the activation or stimulation of the biochemical expression of genetic information. Hydrocortisone has been shown to be intimately involved in the control of synthesis and repression of several enzymes (Kenney & Albritton, 1965). These compounds may interact in some complex fashion with vitamin D or its presumed receptor to alter the control of factors associated with vitamin D-mediated calcium absorption.

(2) *Skeletal effects*

The question of what, if any, are the exact biochemical details of the interaction of vitamin D in the skeletal system is exceedingly difficult to resolve. The problem is severely complicated by the complex nature of the skeletal system itself. The mineral content of bone can be divided into two compartments: the readily exchangeable fraction which is in rapid or instantaneous physico-chemical equilibrium with bathing fluids, and the non-exchangeable fraction which is in a slow, metabolically regulated equilibrium with bathing fluids (McLean & Urist, 1961). The concept has been advanced that parathyroid hormone may interact on the non-exchangeable bone fraction, perhaps by a stimulating effect on osteoclastic bone cells (bone resorbing cells) or a depressing effect on osteoblastic bone cells (bone forming cells) (Rasmussen, 1961). Thus it is important when studying movements of radioactive calcium in bone to determine which of the two compartments is being affected. A complete understanding of this complex system also is dependent upon the elucidation of the biochemical details of bone formation.

There are two primary questions with regard to vitamin D and the skeletal system. (*a*) Is there a direct effect of vitamin D on bone tissue, and if so is the effect on bone formation or bone dissolution, or both? (*b*) Is the effect of vitamin D on bone formation an indirect one and a consequence of the vitamin's net effect upon intestinal calcium absorption and perhaps renal phosphate re-absorption?

Much of the evidence obtained to date suggests that vitamin D increases bone resorption or solubilization, although the results are not unequivocal. This hypothesis has been supported indirectly by noting no difference in the rate and amount of deposition of $^{45}Ca^{2+}$ in rachitic and normal chick tibia after oral and intramuscular doses of $^{45}Ca^{2+}$ (Migicovsky & Emslie, 1950; Migicovsky & Jamieson, 1955). Also the lack of effect of vitamin D on the retention of orally administered $^{45}Ca^{2+}$ in the femur and incisors of rats fed low or high calcium diets (Carlsson, 1951; Harrison & Harrison, 1950) is further indirect evidence in support of this hypothesis. The most conclusive direct evidence is that of Carlsson (1952, 1954*b*), who reported that vitamin D administration to a rachitic rat on a very low calcium diet led to bone dissolution as measured by an increased serum calcium level. But before concluding that there is a direct action of vitamin D in a bone resorption process, it is important to establish that this is not a permissive action of the vitamin by allowing parathyroid hormone to act. It has been reported on the basis of calcium excretion studies (Migicovsky & Emslie, 1949) and indirect measurements of rates of bone accretion and resorption (Migicovsky, 1957) that vitamin D was not primarily or directly associated with bone resorption. These latter results are subject to reinterpretation because of their complex nature.

Other evidence in support of an effect of vitamin D on bone resorption are the results obtained after massive dosing of vitamin D. The dominant characteristic of hypervitaminosis D is the dissolution of preformed bone (Shelling, 1935; Freeman, Rhoads & Yeager, 1946; Lindquist, 1952). It does not seem unreasonable that high doses of vitamin D could accentuate the normal physiological effect of the vitamin.

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Significant in this respect is the fact that the structurally closely related dihydrotachysterol is often clinically administered in large quantities to alleviate hypoparathyroidism.

Nicolaysen & Jansen (1939) and Bélanger & Migicovsky (1958) have presented histological evidence that vitamin D treatment increases the density of bone trabeculae or matrix. Dikshit & Patwardhan (1952) suggested that vitamin D influenced the metabolism or structure of epiphyseal cartilage cells, osteoblasts and bone matrix, rendering them capable of bone accretion. However, Carlsson (1954*c*) concluded on the basis that vitamin D had no action on the uptake of $^{35}SO_3^{2-}$ in bones that the vitamin had no direct influence on matrix formation. Other workers have noted that a vitamin D deficiency alters the crystallographic appearance of the bone crystal (Reed & Reed, 1945; Muller, Posner & Firschein, 1966). It is not clear whether this is a primary or secondary effect of vitamin D.

(3) *Subcellular calcium metabolism*

Primarily as a result of reports that vitamin D was involved in both mediating the transfer of calcium across intestinal mucosal cell membranes and in determining the morphological structure of isolated rat kidney mitochondria, as visualized with an electron microscope (DeLuca, Reiser, Steenbock & Kaesburg, 1960), experiments were initiated by DeLuca and co-workers to examine the relationship of vitamin D and parathyroid hormone to mitochondrial calcium and phosphorus metabolism. They felt that mitochondrial membranes would be a convenient and valid model system for approach to the mechanism of action of these regulators.

Isolated mitochondria have been shown by many workers to be capable of carrying out an energy-dependent accumulation of calcium, strontium, barium, manganese, magnesium, potassium, phosphate, and arsenate ions followed in some instances by an energy-dependent ejection of hydrogen ions. For a detailed discussion of the complexities of oxidative phosphorylation and mitochondrial ion transport the reader is referred to the following excellent reviews (Lehninger, 1964; Mitchell, 1966). It has not been conclusively established whether the ion transport system is anion- or cation-oriented. However, recent evidence demonstrating that low amounts of calcium can be transported into the mitochondria in the absence of inorganic phosphate is suggestive of a cation orientation (Rossi & Lehninger, 1964).

No effects of vitamin D or parathyroid hormone have been observed on either the rate of mitochondrial calcium accumulation or on the absolute amounts of calcium accumulated (DeLuca & Engstrom, 1961; DeLuca, Engstrom & Rasmussen, 1962). This was irrespective of whether vitamin D-deficient or parathyroidectomized animals were used as a source of mitochondria, or whether high levels of purified parathyroid hormone or high levels of vitamin D were added *in vitro* to the mitochondrial incubation medium. These results with a protein hormone and a hormone-like steroid contrast with the results of Kimberg & Goldstein (1966), who studied the *in vitro* binding of calcium by liver mitochondria isolated from rats which had been previously treated with a variety of steroid hormones. They found that cortisol, prednisone and cortisone decreased, deoxycorticosterone, aldosterone and 17-β-

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oestradiol enhanced, while testosterone and progesterone had no effect upon calcium binding. It is not known whether these observations are related to the previously mentioned alterations by either glucocorticoid administration or adrenalectomy on calcium transport by everted intestinal sacs (Harrison & Harrison, 1960; Kimberg *et al.* 1961).

Both vitamin D and parathyroid hormone have a dramatic effect on the release of calcium ions which have previously been accumulated by the mitochondria (Engstrom & DeLuca, 1962, 1964; DeLuca *et al.* 1962). The effects of parathyroid hormone could be obtained only in the presence of vitamin D. The vitamin could be administered either *in vivo* or *in vitro*, but was absolutely required for the hormone's effect. The effect of vitamin D on the release of the cation was (*a*) limited primarily to calcium and not observed with barium and strontium, (*b*) noted regardless of the calcium and phosphorus content of the diet, (*c*) noted *in vitro* by only large doses (2000 i.u.) of vitamin D, (*d*) was not elicited with other vitamin D-related steroids such as ergosterol, 7-dehydrocholesterol, cholesterol, Δ-7-cholesterol or dihydrotachysterol, and (*e*) obtained in rat kidney and liver but not heart mitochondria.

A point of concern relates to the high levels of vitamin D required of the *in vitro* effect in comparison to the low daily physiological requirement of only 5–10 i.u. Also the ineffectiveness of dihydrotachysterol is puzzling in view of its known hypercalcaemic and vitamin D-like activities. Normally when vitamin D is administered *in vitro* to a deficient animal there is a lag of 5–15 hr. before an increase in intestinal calcium absorption is noted. When vitamin D was added *in vitro* to the mitochondrial incubation the release of calcium was instantaneous. Also no convincing evidence has been presented that liver or heart are target organs for vitamin D or that the mitochondria contain the sub-cellular receptor sites. It is quite significant that Zull, Czarnowska-Misztal & DeLuca (1966) reported that prior treatment of vitamin D-deficient rats with actinomycin D, while inhibiting an *in vivo* vitamin D-induced serum calcium rise, did not inhibit the vitamin D-stimulated release of calcium bound *in vitro* by mitochondria. Similar results were obtained by Tashjian, Ontjes & Goodfriend (1964). Evidently the *in vitro* actions of vitamin D are actinomycin D-insensitive, while the *in vivo* actions are actinomycin D-sensitive.

The effect of vitamin D on mitochondrial membranes may be to alter their permeability to calcium and in this sense may reflect possible effects on intestinal mucosal membrane permeability. Quite clearly further rigorous studies are necessary to define precisely whether the *in vitro* effects of vitamin D on mitochondrial metabolism are a valid measure of its *in vivo* physiological activity. It may be that these effects on the mitochondrial membrane are non-specific chemical interactions rather than physiologically significant biochemical interactions.

Subcellular membranes, other than mitochondria, have been implicated in vitamin D-calcium interrelationships. Snellgrove & DeLuca (1965) have described a microsomal fraction from chick intestine which bound calcium by an adenosine triphosphate-dependent process. The amount of calcium bound by this membrane preparation was somewhat decreased by addition of 100–200 i.u. of vitamin D_3/ml. incubation volume but not by similar amounts of vitamin D_2, ergosterol or 7-dehydrocholesterol.

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Wasserman & Taylor (1963; Taylor & Wasserman, 1965) have described a vitamin D_3-dependent factor which influences calcium binding by homogenates of chick intestinal mucosa. This vitamin D-induced factor, which had the ability to bind calcium, was subsequently found to be a protein present in the supernatant fluid after removal of the heavy microsomes (Wasserman & Taylor, 1966). The lag in appearance of this protein factor in the supernatant correlated closely with the lag in vitamin D-enhanced intestinal calcium absorption. This very interesting, but preliminary, work is suggestive of a possible vitamin D-induced synthesis of a protein factor essential to calcium absorption. Conclusive evidence is needed demonstrating that the appearance of this factor represents vitamin D-mediated *de novo* protein synthesis. Such evidence is crucial to the hypothesis that vitamin D interacts at the genome to initiate RNA and protein synthesis.

V. ACTION OF VITAMIN D ON INORGANIC PHOSPHATE

(1) *Intestinal phosphate absorption*

There has been no conclusive evidence obtained which demonstrates an unequivocal effect of vitamin D on the transport of inorganic phosphate from the lumen of the small intestine. One of the first studies, conducted by Nicolaysen (1937*d*) utilizing ligated intestinal loops *in vivo*, found that inorganic phosphate and sodium glycerophosphate are absorbed equally well in normal and vitamin D-deficient rats. Later studies of Dols, Jansen, Sizoo & de Vries (1938) in the rachitic chick and Cohn & Greenberg (1939) and Carlsson (1954*a*) in the rat utilizing orally administered radioactive inorganic phosphorus noted no effect of vitamin D on phosphorus absorption. Harrison & Harrison (1961) carried out *in vitro* studies with everted intestinal sacs and found that the active transport of phosphate was totally dependent upon the presence of calcium and potassium and independent of the vitamin D status of the rats. Thus all available evidence indicates that any effects of vitamin D on intestinal phosphate absorption are secondary to its effects on calcium absorption.

(2) *Renal phosphate reabsorption*

Harrison & Harrison (1941) were the first to present data which suggested that vitamin D administration increased the renal tubular absorption of inorganic phosphate in rachitic animals. However, this potentially very important observation has not received a direct confirmation by later work of the original investigators (Harrison & Harrison, 1964) or other workers (Rasmussen *et al.* 1963). In contrast, Gran (1960) claimed, on the basis of parenteral administration of calcium to a rachitic and vitamin D-treated puppy, that the effect of the vitamin was to promote renal calcium reabsorption. A similar conclusion was reached by Wolf & Ball (1949).

The problem of the exact nature, if any, of the interaction of vitamin D on renal phosphate absorption is severely complicated by the established effects of parathyroid hormone on promoting renal phosphate excretion and the requirement of vitamin D for parathyroid action on calcium metabolism (Rasmussen *et al.* 1963; Harrison & Harrison, 1964). The basic question is whether the effects on phosphate

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reabsorption observed (Harrison & Harrison, 1941) are direct, or secondarily a consequence of hyperparathyroidism. Thus in a vitamin D deficiency there is an over-production of parathyroid hormone in an effort to maintain normal serum calcium levels. This results in an apparent increased urinary excretion of phosphate in a vitamin D-deficient state. When vitamin D is administered parathyroid hormone production is lowered, with a consequent action on reducing urinary phosphate levels which produces an apparent effect of vitamin D on renal phosphate reabsorption

Thus it is not clear whether the kidney is one of the primary target organs of vitamin D. If the vitamin D effect on renal phosphate excretion is real, then this probably means that there are two basic mechanisms of action of vitamin D: one for calcium and one for inorganic phosphate. The concept has been advanced by Rasmussen & DeLuca (1963) that vitamin D and parathyroid hormone have synergistic actions on the movement of calcium and phosphate across the cellular membranes of the intestine, bone and kidney in an effort to maintain a constant serum calcium and phosphate ion product. Clearly much more work should be done to validate the role of vitamin D on phosphate metabolism in this scheme.

VI. ACTION OF VITAMIN D ON CITRIC ACID METABOLISM

Dickens (1941) first observed that the citrate content of bones from rachitic kittens was 50% less than that of normal kittens. This finding was confirmed by Nicolaysen & Nordbo (1943) and Waasjö & Eeg-Larson (1951). Although the feeding of citrates had been known to cure rickets in rats (Hathaway & Meyer, 1939), injections of citrates did not cure rickets (Nicolaysen & Nordbo, 1943). Subsequently it was shown that dietary citrate cures rickets in rats fed cereal diets by complexing the intestinal calcium, thereby making the phytic acid phosphorus in the diet available (Pileggi, DeLuca, Cramer & Steenbock, 1956). Vitamin D also increases (*a*) the urinary excretion of citrate by rats (Bellin & Steenbock, 1952), (*b*) the serum citrate of dogs and rats (Freeman & Chang, 1950; Carlsson & Hollunger, 1954; Harrison, Harrison & Park, 1958), and (*c*) the citrate content of kidney, heart, and small intestine (Steenbock & Bellin, 1953).

There are two alternative explanations for the vitamin D-mediated increase of citrate. The vitamin may stimulate the synthesis or prevent the destruction of citric acid. Patwardhan and co-workers (Ramalingaswami *et al.* 1954; Dikshit, Joshi & Patwardhan, 1956; Joshi, Dikshit & Patwardhan, 1957) and later Meyer, Bolen & Antin (1959) have suggested that vitamin D induces an increase in citrogenase activity, particularly in bone cartilage. That is, in vitamin D-deficient cartilage the orderly progression of glycogen metabolism is arrested at or before the citrate stage due to a lack of citrogenase. Under this scheme the administration of vitamin D removes this block and permits the normal metabolic scheme to go forward. However, these workers have not demonstrated increases in lactic, pyruvic or acetic acids in the vitamin D-deficient animal, and unless there are new metabolic pathways present in rachitic cartilage or unless the deficient tissue has a lower rate of glycolysis they have not shown at what point of the glycolytic pathway the end-products accumulate.

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Results obtained by the author (Norman & DeLuca, 1964*b*) and later confirmed by Kunin & Krane (1965) from slices of epiphysis, cartilage, spongiosa or bone shaft, utilizing ^{14}C-acetate, demonstrated that administration of vitamin D to rachitic rats led to (*a*) an increase in the amount of radioactive citrate, (*b*) a decrease in the amount of radioactivity in the other organic acids of the tricarboxylic acid cycle, and (*c*) a decrease in the amount of radioactive carbon dioxide produced. These results demonstrate that vitamin D does not increase the synthesis of citric acid in bone, but rather decreases its rate of conversion into subsequent intermediates in the tricarboxylic acid cycle.

It has also been shown that vitamin administration to rachitic rats specifically reduced citrate and isocitrate oxidations by kidney homogenate (DeLuca, Gran & Steenbock, 1957*a*) and isolated kidney mitochondrial preparations but not by liver homogenate or mitochondria (DeLuca, Gran, Steenbock & Reiser, 1957*b*). The effect could also be obtained instantaneously by the *in vitro* addition of 5000 i.u. of vitamin D_2 to kidney mitochondria from deficient rats (DeLuca & Steenbock, 1957), while equal quantities of ergosterol or Δ-7-cholesterol were inactive. Electron microscopy of isolated rat kidney mitochondria from vitamin D-deficient and normal rats revealed that dietary vitamin D had a marked protective effect on the structural integrity of the organelles (DeLuca *et al.* 1960). The mitochondria from the kidneys of vitamin D-deficient rats showed gross morphological alteration, primarily manifested as damaged and swollen cristae. The diminished oxidation of citrate after vitamin D administration could then be explained most simply in terms of a physical inhibition of citrate penetration into the mitochondria.

Again it should be pointed out that the normal physiological response to vitamin D is not instantaneous and that a physiological dose of the vitamin is only 5–10 i.u. Unfortunately no electron micrographs were prepared of the mitochondria *in situ*, so it is not known whether the morphological alterations actually exist *in vivo* or are a consequence of a membrane fragility which results in alteration in appearance due to the isolation techniques. Also no evidence is available demonstrating these effects in the intestinal mucosa, which is definitely a target organ of vitamin D action.

The exact role of citrate in the action of vitamin D on mineral metabolism is not yet elucidated. Carlsson & Hollunger (1954) and Neuman & Neuman (1958) have postulated that local concentrations of citric acid induced by vitamin D and/or parathyroid hormone may be responsible for bone mobilization. However, it has been shown (Harrison *et al.* 1957, 1958; Guroff, DeLuca & Steenbock, 1963) that the elevation of extracellular citrate by vitamin D can be prevented by pantothenic acid or pyridoxine deficiency, or by cortisol treatment without interfering with the action of the vitamin on the elevation of serum calcium. It therefore appears that the action of vitamin D on citrate is not a primary action, but may instead be a consequence of the action of the vitamin on membrane permeability or function. In this respect it would be valuable to learn the effect of actinomycin D upon vitamin D-mediated increase in citric acid. This would perhaps help assess the specificity of action of vitamin D on membrane permeability and divorce direct chemical effects of the vitamin on the membrane from induced protein synthetic effects.

VII. ACTION OF VITAMIN D ON LIPID METABOLISM

Both semiphysiological and pharmacological levels of vitamin D have been shown by direct analysis to increase the lipid content of bone. Cipera, Migicovsky & Bélanger (1960) found that 48 hr pretreatment of rachitic chicks with 4000 i.u. of vitamin D_3 approximately doubled the acetone-extractable contents of epiphyseal cartilage. Similar results were obtained by Cruess & Clark (1965) in rats that were fed toxic amounts of vitamin D. Rats which received 30,000 i.u. of vitamin D for 44 days had twice the amounts of bone cholesterol, triglyceride, free fatty acids and particularly phospholipid as did normal rats.

Thompson & DeLuca (1964) carried out extensive metabolic studies which demonstrated that dietary vitamin D caused a threefold increase of incorporation of radioactive inorganic phosphate into the major phospholipids of intestinal mucosa cells and kidney slices, but not liver slices during *in vitro* incubation. No effect on non-lipid organic phosphate was noted. This incorporation of $^{32}P_i$ into the phospholipids was totally dependent upon oxidative metabolism but was independent of the presence or absence of calcium in the incubation medium. However, the level of endogenous calcium in the intact cells was not determined. They further claimed that the incorporation of ^{14}C-glycerol and ^{14}C-serine into intestinal mucosal phospholipids was not stimulated by vitamin D. These results are at variance with the presently accepted schemes of phospholipid synthesis. Essentially similar results have been obtained by Hosoya, Watanabe & Fujimori (1964). They have also found that Ca^{2+} would stimulate the incorporation of DL-3-^{14}C-serine into the phospholipid fraction during *in vitro* incubation of liver mitochondria obtained from vitamin D-deficient rats. Vitamin D_3 (2000 i.u.) added *in vitro* would further stimulate this Ca^{2+}-stimulated phospholipid synthesis.

In this latter work, which did not utilize mitochondria from a primary target organ of vitamin D, it is not clear whether the primary effect of vitamin D is on the phospholipid synthesis or upon calcium metabolism. It has been shown previously, in subcellular studies not concerned with vitamin D, that the incorporation of ^{14}C-serine into lipid fractions was greatly stimulated by the presence of calcium ions (Hübscher, 1962); whereas the enzyme phosphoryl choline-glyceride kinase is completely inhibited by low concentrations of calcium (Weiss, Smith & Kennedy, 1958).

In spite of these complications it may be that vitamin D has a fundamental action on the metabolism of phosphorus in the intestinal mucosa which is in some fashion coupled with the translocation of calcium. Significant in this respect is the report (Zull *et al.* 1966) that actinomycin D will inhibit the stimulatory effect of vitamin D on $^{32}P_i$ incorporation into phospholipids. This may reflect (*a*) a blockage of the synthesis of specific enzymes essential for normal phospholipid metabolism which has the end-result of an altered membrane permeability; or (*b*) a specific inhibition of the formation of a calcium 'carrier' system with which phosphate or phospholipids may be integrated as a prosthetic group. There have been proposals that phospholipids might function in the movement of cations across various cellular membranes (Solomon, Lionetti & Curran, 1956).

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VIII. LAG IN ACTION OF VITAMIN D

One of the characteristic yet puzzling features of the physiological expression of vitamin D activity is an apparent time-lag between the administration of vitamin D and the initiation of the response under study. This facet of vitamin D action was first appreciated by Irving (1944), who stated that the vitamin requires a definite interval of time to produce an effect on the skeleton. However, at that time the latency in action of vitamin D was not universally recognized because of the necessity for biological assay to determine the effects of the vitamin. The end-points used to indicate the presence of the vitamin were gross morphological, histological or roentgenographic observations which of necessity required a finite period of time to develop.

It was not until the advent of radio-isotopes that the investigator was potentially capable of measuring instantaneous metabolic changes or fluxes. Lindquist (1952), who studied the effect of vitamin D on radioactive calcium metabolism, observed a maximal effect of 15 i.u. of vitamin D_3 on rat intestinal calcium absorption in 4 h., on elevation of serum calcium levels in 6 h., and upon deposition of $^{45}Ca^{2+}$ in the skeleton in 36–48 hr. Since this report, a lag in the action of dietary vitamin D has been found in the following responses to the vitamin: (*a*) skeletal deposition of calcium after an oral dose of $^{45}Ca^{2+}$ to chicks (Patrick & Schweitzer, 1955; Migicovsky, 1957); (*b*) intestinal absorption of calcium as measured by appearance of $^{45}Ca^{2+}$ in the serum of chicks and rats (Migicovsky & Nielson, 1951; Coates & Holdsworth, 1961; Saffis & Holdsworth, 1962*a*; Norman, 1966*a*); (*c*) increased level of serum calcium as measured by total serum calcium (Carlsson & Hollunger, 1954; Zull *et al.* 1966); (*d*) apparent energy-dependent accumulation of $^{45}Ca^{2+}$ into rat intestinal slices (Schachter *et al.* 1961) or across everted intestinal sacs (Dowdle *et al.* 1960); (*e*) apparent increased permeability of intestinal mucosa as determined by $^{45}Ca^{2+}$ transfer with everted rat intestinal sacs (Harrison & Harrison, 1965); (*f*) bone resorption or solubilization (Carlsson, 1952); (*g*) increased levels of citric acid in serum bones and incisors (Carlsson & Hollunger, 1954); (*h*) the increased incorporation of $^{32}P_i$ into rat intestinal mucosal phospholipids (Thompson & DeLuca, 1964).

A direct comparison of the length of the lag in each observation is severely complicated by widely varying dosage levels of the vitamin and the assay techniques utilized. It is very important to note that there is a lag in the action of vitamin D for every physiological response to the vitamin that is known. As a consequence, any *in vitro* effects of vitamin D must be carefully assessed for a meaningful relationship to the normal physiological effects of the vitamin.

It was found (Norman, 1966*a*) that the duration of the lag and the magnitude of response is proportional to the size of dose of vitamin D. With oral, intraperitoneal, or intracardial doses of 20,000, 100 or 10 i.u. of vitamin D_3, the lag in response to chick intestinal calcium absorption was 5–8, 10–15 or 15–25 hr. respectively. This suggests that there may be a competition among specific and non-specific receptor sites in the intestinal mucosa, bone and perhaps kidney. Only at higher dosage levels can all the receptor sites in all target organs become rapidly saturated.

There are several possible explanations for the lag in action of vitamin D. (*a*) It

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may be due to necessity for a metabolic conversion to an 'active' form before it can perform its biochemical role as has been suggested by Raoul & Gounelle (1958) and Sallis & Holdsworth (1962*b*). (*b*) It may be due to a slow absorption and transportation of the vitamin to the target organ and subsequent association with its receptor site(s). (*c*) It may reflect some as yet undefined induction process involving the expression of genetic information which is ultimately utilized to elicit the expected physiological effect. It is the contention of the author that vitamin D and/or its metabolites is acting to facilitate the expression of genetic information.

IX. LOCALIZATION OF VITAMIN D

(1) *Tissue distribution*

Before the recent advent of high specific activity, radioactive vitamin D, no thorough investigation had been made of the absorption and tissue distribution of vitamin D. The extreme biological potency of vitamin D, coupled with the lack of physical or chemical means for direct assay, meant that investigators had to rely upon slow and imprecise biological assays. Biological assays by Morgan & Shimotori (1943) provided evidence that vitamin D activity may be found in the kidney, heart, large intestine, brain, lung, spleen, muscle, fat, skin and hair. But no more than 10% of the administered dose of 40,000 i.u. was accounted for. More recently, Cruickshank, Kodicek & Armitage (1953, 1954) found after administration of massive doses of vitamin D_2 (40,000 i.u.) to a single rat that vitamin D activity could be found by biological assay in the liver, bones, intestines, and faeces. However, only 25% of the administered dose could be accounted for. It is likely that the remainder was metabolized to biologically inactive compounds.

An important question that cannot be answered through reliance on biological assays concerns the possible metabolism of vitamin D to a biochemically active form. Radioactive vitamin D is required for metabolism studies. Initially, several syntheses of radioactive vitamin D were carried out (Havinga & Botts, 1954; Kodicek, 1955, 1956; Schaltegger, 1960) but the specific activities were too low to allow adequate detection in the tissues following physiological doses of the vitamin (Kodicek, 1956; Blumberg, Schönholzer & Aebi, 1959; Blumberg, Aebi, Hurni & Schönholzer, 1960).

DeLuca and I (Norman & DeLuca, 1963*b*) were the first to develop a relatively simple method for the preparation of ^{3}H-vitamins D_2 and D_3. These preparations were of high enough specific activity to detect the tissue distribution resulting from administration of physiological doses of 70–500 i.u.

Using these preparations the tissue distribution of a dose of 500 i.u. of ^{3}H-vitamin D_3 as a function of time has been carried out in the rat (Norman & DeLuca, 1963*b*) and in the chick (Norman, 1966*a*; Haussler & Norman, 1967). The liver rapidly accumulated the largest amount of radioactivity, whether the dose was administered orally or intracardially, but by 24 h. after the dose there was a generalized distribution of radioactivity in the kidneys, heart, liver, blood, brain, lung, small intestine, muscle, skeleton and faeces (Norman & DeLuca, 1963*b*). Ninety-four per cent of the administered dose was found. Disappointingly, no preferential localization of vitamin D

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was noted in presumed target organs, i.e. intestine, skeleton or possibly kidney, after these doses of 500 i.u. However, no radioactivity was found in the adrenals.

Subsequently to these first studies there have been further reports on the tissue distribution of doses of 1100 i.u. of ^{14}C-vitamin D_3 in chicks (Bosmann & Chen, 1965), the association of vitamin D with serum proteins (Chalk & Kodicek, 1961) and the role of serum proteins in the *in vivo* transport of the vitamin (Chen & Lane, 1965). More recent work by Kodicek's group (Fraser & Kodicek, 1966*a*, *b*) has indicated the presence of palmitate, linoleate, stearate and oleate esters of vitamin D in the intestine, liver and kidney of rats.

Evidence was also obtained for the involvement of the ileum in the intestinal absorption of the vitamin (Norman & DeLuca, 1963*b*). This result confirmed earlier work by Kodicek (1960) who used 40,000 i.u. of vitamin D_2. More recent work by Schachter, Finkelstein & Kowarski (1964) suggests that two steps are involved in absorption of the vitamin; a rapid uptake at the mucosal surface and a slow transfer across the mucosal cell to the lymph.

The availability of radioactive vitamin D also facilitated radioautographic studies. Using this technique, Kodicek (1960; 1965) and Kodicek & Thompson (1965) have observed a localization of the vitamin in (*a*) the liver and Kupffer cells, (*b*) in the proximal convoluted tubules of the kidney, and (*c*) in the proliferating chondrocytes of the metaphysial plate of bone (a region concerned with the synthesis of organic matrix), but not bone osteoblasts or osteoclasts. The significance of these potentially very important observations is clouded by the use of pharmacological amounts (20,000–40,000 i.u.) of vitamin D.

The chemical synthetic techniques for production of radioactive vitamin D have been further refined by Neville & DeLuca (1966) and the techniques of extracting and processing tissue samples containing radioactive vitamin D have been modified and extensively refined (Haussler & Norman, 1967) so that now the tissue and subcellular distribution of as little as 1–10 i.u. (0·025–0·25 μg.) of radioactive vitamin D may be conveniently and accurately determined. Neville & DeLuca (1966) have found that 24 hr. after a dose of 10 i.u. of 1,2-^{3}H-vitamin D_3 to vitamin D-deficient rats the skeleton, muscle and intestinal contents contain most of the radioactivity; but when expressed as amount of radioactivity per unit weight of cells, the skeleton and intestinal mucosa contain the highest concentration of radioactivity. It was found (Haussler & Norman, 1967) that 24 hr. after an intracardial administration of a dose of only 5 i.u. of ^{3}H-vitamin D_3 to rachitic chicks that the skeleton accumulated 0·40 i.u., the small intestine 0·12 i.u., the liver 0·16 i.u. and the kidneys 0·06 i.u. These first reports of the tissue distribution of a physiological dose of vitamin D emphasize the exceedingly small quantities of the vitamin that are bound and may provide insight into the nature of the primary target organs for the vitamin.

(2) *Subcellular distribution*

The subcellular distribution in kidney, liver, and intestinal mucosa 24 hr. after an oral dose of 500 i.u. of ^{3}H-vitamin D_3 to deficient rats has been investigated (Norman & DeLuca, 1964*a*). In each tissue approximately equal amounts of the vitamin were

found in the nuclear, mitochondrial and microsomal fractions, while very little radioactivity was found in the cytoplasmic fraction. When the radioactivity was expressed on a weight basis (disintegrations per min./100 mg. dry weight of fraction), the microsomal fractions from the intestinal mucosa and kidney, but not that of the liver, were slightly higher than the mitochondrial and nuclear fractions.

Nair & Bucana (1966) utilizing a new sensitive gas chromatographic technique and doses of 8000 or 80,000 i.u. of non-radioactive vitamin D_2 and D_3, found that the supernatant fraction of liver accumulated the bulk of the vitamin. Interestingly, more total vitamin D_2 than vitamin D_3 was accumulated, but unfortunately only the liver was examined.

Both of these reports suffer from the use of unphysiological doses (500 i.u. and 8000 i.u.). When the dose of the vitamin far exceeds the physiological requirement of the animal, it is likely that the vitamin will be non-specifically associated with 'neutral', non-target organs and that the resulting subcellular distribution will also probably be blurred by non-specific binding of excess vitamin D to all the subcellular membranes.

Recently an extensive time-course study of the subcellular distribution of doses of 15, 50 and 500 i.u. of ^{3}H-vitamin D_3 in the liver, intestine and kidney of rachitic chicks was carried out (Haussler & Norman, 1967). The results indicate that when the unphysiological dose of 500 i.u. of ^{3}H-vitamin D_3 is administered, there is a generalized distribution of the radioactivity into the nuclei, mitochondria and microsomes of the liver, kidney and intestinal mucosa. In contrast, when the physiological dose of 15–50 i.u. of H-vitamin D_3 is administered, 60–75% of the radioactivity isolated from the intestinal mucosa is found in the crude nuclear fraction. This selective nuclear localization is not found in the liver or kidney cells and may be a reflexion that these are not primary target organs for vitamin D action in the chick.

The dominant intestinal mucosa nuclear localization of vitamin D_3 was obtained after oral, intracardial or intraperitoneal administration of the dose. These results have been obtained with randomly labelled ^{3}H-vitamin D_3, 1,2-^{3}H-vitamin D_3 and 3-^{14}C-vitamin D_3. Maximal amounts of radioactivity were found in the nuclei within 3 hr. of an intracardial injection of 15 or 50 i.u. Thus the lag in action of vitamin D cannot be due to a slow transportation to the target intestinal mucosa. When 15 i.u. of ^{3}H-vitamin D_3 are administered intracardially, only about 0·4 i.u. are accumulated by the intestinal mucosa. Seventy-five per cent of this is located within the nucleus, giving a value of about 0·30 i.u. (0·0075 μg.)/chick intestine (which contains 3–4 g. of mucosal cells), or $4{\cdot}8$–$6{\cdot}5 \times 10^{-12}$ moles of vitamin D_3/g. of wet cells. This gives a cellular vitamin D concentration of $6{\cdot}2 \times 10^{-9}$M. If we assume approximately 0·75 cells/mg. fresh weight of intestinal mucosa (Marcus & Wasserman, 1966), then there are approximately 3800–5100 molecules of vitamin D and/or metabolites per mucosal cell assumed to be specifically engaged in initiating an actinomycin D-sensitive event. However, further studies with subphysiological doses of vitamin D or subfractioning the nucleus may show that even a part of this vitamin is non-specifically bound.

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(3) *Metabolism*

A number of laboratories have postulated that the reason for a delay in biological response to vitamin D was the necessity for its metabolism to an 'active' compound. The adrenal gland has been suggested by both Raoul & Gounelle (1958) and Sallis & Holdsworth (1962*b*) as the site of this conversion. The former workers claimed that 87% of an intravenously injected dose of 1600 i.u. of non-radioactive vitamin D_2 was accumulated in the rat adrenals within 30 min. The latter workers implicated the adrenal glands by use of a presumed specific inhibitor of adrenal action. Administration of this compound, 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (o, p, DDD), for 3 days to rachitic chicks abolished both adrenal function and the ability to respond to a dose of 100 i.u. of vitamin D_3.

However, the adrenal hypothesis can be refuted for the following reasons. (*a*) Whereas Quarterman (1965) found, as had Raoul & Gounelle (1958), that there was an increase in the concentration of a component in the rat adrenal shortly after intracardial injection of vitamin D, this component had quite different chromatographic properties from vitamin D and most importantly had no antirachitic activity. (*b*) Radioactive vitamin D shows no tendency to localize in the adrenals (Blumberg *et al.* 1960; Norman & DeLuca, 1963*b*; Norman, 1966; Haussler & Norman, 1967). (*c*) The compound o, p, DDD is not a specific inhibitor of adrenal function. It also inhibits (Norman, 1966*b*) the labelling of intestinal mucosa ribonucleic acid by ^{3}H-uridine. (*d*) Schachter, Kowarski & Finkelstein (1963) concluded that vitamin D acted directly upon the intestinal mucosa without prior activation in another organ. When vitamin D_3 was placed directly, *in vivo*, in loops of rat duodenum in deficient rats, the subsequent rate of *in vitro* transport of $^{45}Ca^{2+}$ by slices of duodenum was much greater than when the vitamin was given intravenously or placed in jejunal loops. (*e*) Czarnowska-Misztal, Zull & DeLuca (1966) have shown that vitamin D-deficient adrenalectomized rats can still respond to vitamin D administration.

Sahashi and co-workers (Sahashi *et al.* 1962; Tanaka *et al.* 1963; Higaki, Takahashi, Suzuki & Sahashi, 1965*a*, *b*) have postulated that there is a relationship between vitamin D and sulphate metabolism. This hypothesis was based on the finding of higher amounts of both non-radioactive and radioactive sulphate in tibiae of rachitic chicks which had received vitamin D_3. They have proposed that chick liver has the requisite enzymes to form vitamin D-sulphate. They further claim to have isolated such a compound from the urine of rabbits which received 200,000 i.u. of vitamin D_2. The significance of these observations is unknown at the present time.

Callow, Kodicek & Thompson (1966) have carried out an extensive study of the distribution and metabolism of [3α-^{3}H] vitamin D_2 and [24,25,26,27-^{3}H], [7-^{3}H], [6-^{3}H] and [1α-^{3}H] vitamin D_3 in the rat and noted a number of metabolites which are probably breakdown products. They concluded that tritiated vitamin D is not suitable for metabolic studies of the vitamin because much of the label is lost into the body water. These results are not in agreement with earlier reports (Norman & DeLuca, 1964*a*; Norman, Lund & DeLuca, 1964) where significant amounts of lipid extractable tritium were found 48 hours after administration of the ^{3}H-vitamin D_3. The former

workers used specifically labelled vitamin D whereas ours was randomly labelled. It is conceivable that particular positions of the vitamin D molecule are more labile than others. Certainly the use of randomly labelled vitamin D is more desirable in initial metabolic studies so that the existence of any and all fragments will be detected.

A detailed study of the metabolism of ^{3}H-vitamin D was first carried out by Norman, Lund & DeLuca (1964) and then extended by Lund & DeLuca (1966). Chromatography of the chloroform extracts by two systems which will separate mixtures of vitamin D, ergosterol and tachysterol (Norman & DeLuca, 1963*a*) revealed that after an oral dose of 500 i.u. of ^{3}H-vitamin D_3 a total of 70% of the kidney and 42% of the intestinal mucosa radioactivity was present in a material which migrated identically with crystalline vitamin D and which possessed biological potency equivalent to standard vitamin D when assayed in the rat. Also, three other chloroform-soluble radioactive compounds were detected in the kidney, liver, intestinal mucosa, serum and skeletal system, all of which showed considerable amounts of biological activity.

Quite significantly it was found (Lund & DeLuca, 1966) that as the dosage level of ^{3}H-vitamin D_3 was decreased from 500 i.u. to 100 i.u. and 10 i.u. that the amount of one of the metabolites increased respectively from 21% to 62% and then 83% of the total chloroform soluble radioactivity. This metabolite, which is more polar than vitamin D and which has approximately equivalent biological activity, was found in all the tissues examined. It is highly possible that these results are indicative of the metabolism of vitamin D to a new 'active' compound.

Conclusive substantiation of this suggestion must await isolation of the receptor molecule(s) for vitamin D and/or its metabolites and positive chemical identification of the active form of this compound in the basic biochemical systems dependent upon the vitamin.

X. VITAMIN D–ACTINOMYCIN D INTERACTION

There are three key processes—replication, transcription and translation—involved in the biochemical activities related to production and eventual use of genetic information for the synthesis of new protein or enzyme molecules. For a detailed discussion of these matters consult the reference by Jacob & Monod (1961).

In order to establish that vitamin D is acting analogously to a hormone to initiate the synthesis of new proteins or enzymes via utilization of stored genetic information it is essential to establish that the vitamin has the expected effects, in the proper location, and in the proper chronological order, which are consistent with the details of the particular hypothesized mechanism. Some preliminary attempts in this direction are described below.

A number of laboratories have reported that puromycin (Kunin & Krane, 1965) or actinomycin (Tashjian, Ontjes & Goodfriend, 1964; Milhaud, Moukhtar, Cherian & Pérault, 1964; Rasmussen, Arnaud, & Hawker, 1964; Tashjian, 1965; Kunin & Krane, 1965) treatment *in vivo* will inhibit the subsequent action of parathyroid hormone on parathyroidectomized rats, as measured by serum calcium elevation, without altering

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the hormone's stimulatory effect on renal phosphate excretion. But with the reports (Norman, 1965; Zull *et al.* 1965) that actinomycin D could inhibit the action of vitamin D on mediating intestinal calcium absorption and elevating serum calcium levels, the precise interpretation of the earlier work which indicated an inhibitory effect of the antibiotic on the hypercalcaemic and bone mobilization actions of parathyroid hormone are subject to question and reinterpretation. The former workers may, in reality, have been observing an actinomycin D inhibition of vitamin D action. It should be recalled that Harrison & Harrison (1964) and Rasmussen *et al.* (1963) have shown that vitamin D is obligatorily required for the hypercalcaemic action of parathyroid hormone. Thus if actinomycin D can inhibit the action of vitamin D, then it could possibly inhibit the permissive action of the vitamin for parathyroid hormone action.

Further detailed evidence for the interaction of vitamin D in transcriptional events has been obtained. Zull *et al.* (1966) reported that not only actinomycin D, but also 5-fluoro-orotic acid and puromycin inhibit the action of vitamin D. Furthermore they established that the effect of actinomycin D was not due to its toxic nature. There was no histological difference between the intestinal mucosa of normal rats and actinomycin D-treated rats. Both Zull *et al.* (1966) and I (Norman, 1966*a*) have found that actinomycin D treatment does not alter the tissue distribution of doses of 2000 i.u. or 350 i.u. of ^{3}H-vitamin D_3 in deficient rats and chicks respectively. However, these studies should be repeated with a dose of 1–5 i.u. of ^{3}H-vitamin D_3 as with the larger dose there is the likelihood of non-specific distribution.

Eisenstein & Passavoy (1964) noted that actinomycin D also could inhibit the hypercalcaemia induced by pharmacological doses of vitamin D. This is noteworthy because the hypercalcaemic effects are due to an effect of the vitamin D on bone resorption. Thus probably both the intestinal and osseous effects of vitamin D are actinomycin D-sensitive. This may reflect a fundamental similarity in mode of action of the vitamin in the two target organs.

An extensive study of the time-course interactions of actinomycin D on the lag in action of vitamin D in rachitic chicks has been carried out (Norman, 1966*a*). Evidence was obtained that biochemical events pertinent to the vitamin's effect on intestinal calcium absorption were occurring throughout the lag period. Actinomycin D could block the action of vitamin D only when given before or within 2 hr. of 100 i.u. of vitamin D_3. When actinomycin D was given 5–8 hr. after 100 i.u. of vitamin D_3, although at the specific time of antibiotic treatment the chick had not demonstrated an increased calcium absorption, the latent effect of the vitamin was still fully manifest 24 hr. later. This time period of actinomycin D-insensitiveness, but of no measureable stimulation of calcium absorption, could be the period after messenger RNA synthesis but before completion of protein synthesis.

Also it was found (Norman, 1966*a*) that actinomycin D could convert rachitic chicks, which had been treated 24 hr. earlier with 20,000 i.u. of vitamin D_3 and which were now normal chicks, back to an apparent vitamin D-deficient chick. This conversion process took approximately 48 hr. and is presumably a reflexion of the rate of turnover of any enzymes or membrane systems crucially involved in vitamin

D_3-mediated calcium absorption in the chick. The decay cannot be due to a lack of the vitamin to act as a cofactor, since 20,000 i.u. were originally administered. Some support for the concept of turnover times of endogenous factors related to calcium metabolism can be obtained from other recent reports (Harrison, 1966; Harrison & Harrison, 1966).

It should be emphasized that antagonism of the action of vitamin D by actinomycin D does not unequivocally establish that the vitamin acts primarily to initiate the utilization of genetic information stored in DNA. Such antagonism merely indicates that genetic activity must be operative in the early time periods of the vitamin's action or that a short-lived product of gene activity, perhaps m-RNA, is an essential mediator of the vitamin's action. A direct confirmation of the latter possibility has been obtained (Norman, 1966*b*). Half an hour after administration of 5000 i.u. of vitamin D_3 to rachitic chicks there is a threefold increase in the 20 min. pulse-labelling of intestinal mucosa RNA by ^{3}H-uridine. This effect, which can be inhibited by pretreatment by actinomycin D or o,p,DDD (see Section IX, 3), represents the most rapid metabolic effect of vitamin D that has been reported to date. These results strongly suggest that the primary biochemical response of intestinal mucosal cells to vitamin D is the initiation of stimulation of RNA synthesis.

Although vitamin D is localized in the nucleus of the target organ intestinal mucosa apparently to mediate a transcriptional event, i.e. stimulation of the synthesis of m-RNA, no direct evidence is available implicating an effect of the vitamin on transitional events, i.e. protein synthesis.

Wasserman & Taylor (1966) have reported the presence of a vitamin D_3-induced calcium binding protein in chick intestinal mucosa (see Section IV, *c*). This evidence is highly suggestive of vitamin D effects at the translational level, but not conclusive because no direct measurements of protein synthesis *per se* were carried out. Thus as yet no unequivocal demonstration of an effect of vitamin D on *de novo* protein synthesis has been established. This may well be a difficult task to demonstrate experimentally. If vitamin D induces the synthesis of only one or two proteins or enzymes essential to calcium absorption, it may be difficult to detect their existence in the presence of the multitude of other proteins and enzymes normally synthesized by the cell. This difficulty is even less amenable to solution because as yet the biochemical properties of this enzyme/protein are unknown.

XI. CONCLUSIONS

The hallmark of vitamin D activity is an interaction in calcium metabolism in both the intestine and the skeletal system. Secondarily there is the possibility of vitamin D interaction in the renal reabsorption of phosphate. The end-result of these activities is to maintain the serum levels of calcium and perhaps phosphate at some relatively constant level. When the physiological activities of vitamin D are integrated with those of parathyroid hormone on bone resorption and renal phosphate excretion, the organism is provided with the sophisticated machinery for a precise and delicate homeostatic control of the level of calcium and phosphorus in its internal environment.

Thus it will be able to adapt to and cope with wide fluctuations in the availability of calcium and phosphorus in its external environment.

Homeostatic mechanisms inherently imply the concept of biological control; that is, the material being controlled can in some fashion communicate with a controlling centre so that a desirable alteration in the steady-state metabolic balance of the cell or organism can be achieved. This communication can be either or both a positive and negative feedback. The concept that vitamin D may interact on or with the genome to intervene in metabolic events related to calcium, plus the ability of the organism to control this intervention, constitute a classic example of homeostatic control. It is difficult to envisage how a biological system where vitamin D was acting solely as a cofactor for one enzymic reaction could effect such a striking and efficient control of calcium metabolism.

The concept of adaptability on the part of the organism to dietary calcium levels has been established (Kimberg *et al* 1961). The small intestine of the rat facultatively responds to a diet low in calcium by increasing the vitamin D-dependent absorption of calcium. The intestinal mucosa is particularly amenable to adaptation as it has been estimated that in the rat (Leblond & Messier, 1958) and chick (Imondi & Bird, 1966) the mucosa cells are completely renewed every 48–72 hr. Also in times of extreme calcium deprivation the organism may draw upon his extensive bone stores of calcium in some vitamin D-dependent process (Carlsson, 1952).

In a sense the rat and chick represent opposite ends of the adaptive spectrum. Under normal dietary circumstances, i.e. not a high Ca/P ratio, the rat can absorb adequate amounts of calcium from his diet in the absence of vitamin D. Furthermore, he can respond equally well to both vitamin D_2 and vitamin D_3. The chick, on the other hand, cannot overcome the handicap of a vitamin D deficiency and always requires the presence of vitamin D for adequate intestinal calcium absorption. The chick also has a high structural specificity for vitamin D_3 over vitamin D_2.

This latter point also argues against a cofactor role for vitamin D. The concept of a cofactor implies a dynamic intervention or participation in some reversible, cyclic process. It is difficult to envisage how the addition of a methyl group and a double bond, as in going from vitamin D_3 to vitamin D_2, could have such a weakening effect on the vitamin's activity if it were as a cofactor. This could not even markedly affect its lipid or lipoprotein solubility. Yet in the chick vitamin D_3 is ten times more active than vitamin D_2.

It is very difficult to establish when vitamin D may have become an integral component of the calcium homeostatic mechanism. Virtually nothing is known about calcium transport in lower organisms and the possible role of vitamin D in this process. Also no systematic study of the phylogenic distribution of vitamin D has been made other than that of Bills *et al.* (1935), who made a taxonomic study of the distribution of vitamins A and D in 100 species of fish. They noted no correlation between vitamin D content and degree of skeletal development. It may be that the exceedingly high levels of vitamin D in some species of fish are a reflexion of an aberrant vitamin D metabolic pathway. One can infer from the requirement of vitamin D for parathyroid hormone activity that vitamin D was a component of the

calcium homeostatic control mechanism prior to the evolutionary production of parathyroid hormone. Parathyroid glands did not appear until the amphibia (Fleischman, 1951); just when before the amphibia vitamin D intervenes is not known. As long as the organism had adequate levels of calcium in his environment, i.e. sea water,* there was possibly no need to have a specific calcium-oriented absorption system. His needs would be met via simple diffusion. However, if this organism had any kind of exo- or endoskeletal system he may well have had to have a control system operative at this locus to effect efficient control of calcium movements between the reservoir and the blood. These controls would have to be much more stringent for an endoskeleton which is in intimate contact and dynamic equilibrium with the blood than for an exoskeleton. Perhaps vitamin D intervened to prevent overcalcification via an action in bone resorption. An endoskeleton first was produced in the teleost. Thus vitamin D may play a role in homeostatic control of calcium in organisms as primitive as teleosts.

Possibly the intestinal action of vitamin D on calcium absorption did not arise until the organism left his sea-water environment with its abundant supply of calcium. This would be somewhere at the amphibian stage. Now the organism would have to be much more conservative in his calcium metabolism, and might respond facultatively by developing a vitamin D-dependent intestinal calcium mechanism and perhaps a vitamin D-dependent renal phosphate reabsorption mechansim. It is significant in this respect that the one site of calcium control in which vitamin D and parathyroid do not both interact is the intestinal mucosa. No convincing effects of parathyroid hormone on intestinal calcium absorption have been established. Perhaps parathyroid hormone intervened in the evolving calcium homeostatic control mechanism after the development of the vitamin D-dependent skeletal system but before the development of the vitamin D-dependent intestinal system to effect a more refined control over calcium metabolism.

The possibility that the action of vitamin D on its two prime target organs, the skeletal system and the intestinal mucosa, arose at two evolutionarily distinct periods raises a fundamental question. Is the biochemical mechanism of action of vitamin D identical, similar or quite different in these two organs? Teleologically speaking, the mechanisms are probably similar but not necessarily identical. In lieu of a detailed understanding of these mechanisms, one can ask whether the structural requirements of the vitamin D molecule are identical for both target organs. Again no careful assessment has been made comparing activities of related compounds on intestinal calcium absorption versus activities on bone resorption in the same animal at the same time.

Carlsson & Lindquist (1955*a*, *b*) found that with physiological doses of vitamin D or dihydrotachysterol there was little difference with respect to their relative effects on intestinal calcium absorption and on bone resorption. But with increasing dosage the effect of both compounds on the bone resorption in comparison to the intestinal process becomes more dominant. The important point here is that the effect was

* The calcium concentration of sea water is 10 mM. The serum calcium concentration of salt water fish or amphibia is approximately 2·5 mM (Prosser, 1952).

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more marked for dihydrotachysterol than for vitamin D. This implies that perhaps the fundamental mechanism of action of vitamin D is identical in both the intestinal mucosa and bone, but that the control mechanism for regulating the effects of large amounts of the compounds was more specific in the skeletal system for vitamin D over dihydrotachysterol. This may explain the partial effectiveness of the clinical administration of large amounts of dihydrotachysterol rather than vitamin D to patients with vitamin D-resistant rickets.

It is difficult to rationalize inclusion of the kidney as a target organ for vitamin D. As discussed earlier this is not supported firmly either by evidence of a renal localization of the vitamin after physiological doses of ^{3}H-vitamin D_3 or by repeated measurements of a biochemical or physiological effect. The claim is that vitamin D mediates phosphate reabsorption. If this is the case, then the biochemical consequences of vitamin D action are anion-oriented in the kidney and cation-oriented in the intestinal mucosa and skeletal systems. Under these circumstances it is difficult to envisage an identical mode of action in all three target organs.

It is impossible at the present time to ascertain the source of vitamin D for the first homeostatic control mechanisms present in lower organisms. It could have been either the photochemical activation of the provitamin, 7-dehydrocholesterol, known to be present in the skin, or it could have been enzymically synthesized, perhaps in one organ. Certainly high amounts of vitamin D may be produced via the photochemical reaction and most organisms rely upon this mechanism for their source of vitamin D. It was only after civilization became established that in some species (man) what was once a quasi-hormone became instead a vitamin. Many species do not require a dietary source of vitamin D.

Many of the topics of this review may be integrated into the proposed mechanism of action of vitamin D. The existence of the genetically related vitamin D-resistant rickets and the disorder idiopathic hypercalcaemia both may reflect aberrations in the homeostatic control mechanism. In the one instance the control mechanism has lost the ability to recognize vitamin D, and as a consequence large doses of the vitamin or, preferably, related compounds such as dihydrotachysterol are administered. In the other instance, idiopathic hypercalcaemia, the organism may be overreacting to the presence of normal levels of vitamin D. Apparently the feedback control mechanism is inoperative. Although a detailed biochemical description of the parameters of the calcium transport system is lacking, i.e. simple diffusion versus passive carrier-mediated transport versus active transport, it is known that this system is adaptive. The limits of this adaptation may relate to the flexibility of the homeostatic control system to respond to new conditions. Also the inhibiting effects of adrenal hormones, such as cortisone, on this system may reflect interactions of these compounds in some phase of the control mechanism.

All of the established, primary physiological effects of vitamin D—increased intestinal calcium absorption, increased bone resorption, increased levels of citric acid, increased labelling of phospholipids by $^{32}P_i$—have a lag in the manifestation of their appearance after vitamin D administration. This lag in action is not due to a paucity of vitamin D in the target tissues. All of these actions, except the rise in citric

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acid (which has not been tested), have been shown to be actinomycin D-sensitive, i.e. dependent in some fashion on genomic activity. Furthermore it has been shown that the level of vitamin D and/or metabolites present in target tissues is so low as to question its ability to function as a cofactor. The hypothesis that vitamin D may act analogously to some other steroid hormones and initiate the utilization of genetic information permits the explanation of many of the anomalies of vitamin D action, particularly the time lag, the exceedingly low daily dose requirements, the lack of *in vitro* effects to physiological quantities of vitamin D, and possible instances of genetically related vitamin D-resistant rickets.

The exact biochemical details of how vitamin D is able to elicit its hormone-like action on the homeostatic control of calcium metabolism remains to be elucidated. It seems most likely that this control is effected through an interaction of vitamin D and/or metabolites on either gene activation or repression or one of the other biochemical steps involved in the regulation of this process. Jacob & Monod (1961) have discussed metabolic control at the transcriptional level, whereas Cline & Bock (1966) have suggested that in some instances metabolic control can be effected at the translational level. The rationale of the structural specificities of an effector are not clear. Certainly the characteristic feature of the vitamin D molecule is its opened B ring with three conjugated double bonds. Any alteration of these features, as in tachysterol, dihydrotachysterol or 7-dehydrocholesterol, either substantially alters or abolishes its biological activity. Apparently the hydroxyl group and side-chain are also essential to a strong interaction with the receptor molecule. These facts are all suggestive of vitamin D fitting into a specifically designed receptor slot. This may either be directly on the DNA molecule, to perhaps initiate transcription, or it may be on an inducer or a repressor where it acts in an allosteric fashion to induce or repress. This receptor site could be on the nuclear membrane, in the nucleolus, associated with the chromatin (a complex of DNA and histone), on the DNA, or at some other unknown location in the nucleus. The end-result of vitamin D's action here is to stimulate at the translational level, outside of the nucleus, the synthesis of a protein(s) or enzyme(s) which is capable of interacting in the calcium absorption process. This interaction could be direct, i.e. the newly synthesized protein could act directly as a 'carrier' or 'coupler', or it could act as a modifier to change the tertiary structure of the cell membrane in a fashion compatible with increased calcium absorption.

Somewhere in this maze of possibilities has to be a mechanism for the homeostatic control process; that is, for calcium to communicate with the transducer. This could take place in a number of ways. (*a*) Calcium ions could directly act as an inhibitor at some specific transcriptional or translational enzymic step by changing the ionic environment. (*b*) Calcium ions could directly alter the permeability of the nucleus to vitamin D. (*c*) Calcium ions could allosterically alter the affinity of the receptor molecule for vitamin D. Both of the possibilities (*b*) and (*c*) would inhibit the binding of vitamin D to its receptor molecule. (*d*) Calcium ions could bind to an apoprotein product resulting from the original stimulus of vitamin D. This feedback transducer could then act as a repressor at the transcriptional level by either directly blocking vitamin D directed m-RNA synthesis or by destroying existing vitamin D-

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related m-RNA, or at the translational level by blocking calcium oriented protein synthesis. The proper integration of vitamin D and calcium activities both at the genome level and at the calcium absorption site in both the intestinal mucosa and the bone cells would constitute a functional homeostatic mechanism for calcium metabolism. The substantiation of these speculations must await further experimentation.

XII. SUMMARY

1. The purpose of this review article is to re-evaluate and integrate many of the observations related to the physiological effects of vitamin D, using as a working hypothesis the concept that the vitamin may be acting analogously to a steroid hormone in terms of its ability to interact with genetic information and ultimately elicit a physiological response. Prior to this time the problem of the mechanism of action of vitamin D has primarily been approached from the point of view that the vitamin was acting as a cofactor for some specific enzymic reaction.

2. The physiological activities of vitamin D are integrated with those of parathyroid hormone to provide a homeostatic control for the regulation of primarily calcium and secondarily phosphate metabolism. It is proposed that the role of vitamin D in this homeostatic control mechanism is older and more fundamental than that of parathyroid hormone. The interaction of vitamin D on skeletal calcium metabolism may have evolved before the effects of the vitamin on intestinal calcium absorption.

3. There are several physiological defects of calcium metabolism—rickets, osteomalacia, vitamin D-resistant rickets and idiopathic hypercalcaemia—all of which may be a consequence of an aberration in one or another of the interlocking steps of the vitamin D-dependent and calcium-dependent homeostatic control mechanism.

4. The most thoroughly established action of vitamin D *in vivo* is to promote or facilitate the intestinal absorption of calcium. Although the exact biochemical details of this process are not available, this may involve vitamin D-mediated synthesis of the appropriate enzyme systems or the alteration of membrane structure necessary for calcium absorption. It is not yet unequivocally established whether calcium absorption is an energy-dependent active transport process or is a passive carrier-mediated or simple diffusion process.

5. The exact action of vitamin D on bone metabolism is not as well established, but the primary effect of the vitamin is likely to mediate bone resorption. The vitamin D-dependent activities of the cell in both the intestine and bone are to absorb calcium and transfer it to the blood.

6. No direct effects of vitamin D on intestinal absorption of phosphate have been found. Furthermore the validity of a vitamin D-mediated renal reabsorption of phosphate is questioned, for the major effects of vitamin D are cation oriented. If the renal effects of vitamin D are true, it is postulated that the mechanism of action of the vitamin here on the anion, phosphate, is fundamentally different from its cation oriented mechanism.

7. There is a lag in the action of vitamin D on the vitamin mediated: (*a*) transport of calcium both *in vivo* in rats and chicks, and *in vitro* with everted intestinal slices;

(*b*) the apparent ... ility of intestinal mucosa; (*c*) in ... levels of citric acid in ser... the increased incorporation of radi... inorganic phosphorus into ... phospholipids. As shown by t... of radio-active vitamin D, ... to a lack of the vitamin in the t... gans.

8. Whereas ... doses of radioactive vitamin ... e in all tissues and all sub... small physiological doses of radio... amin D localize predomin... ucleus of the intestinal mucosa. ... ount of vitamin D localize... is would appear to be too low fo... min to function as a co... ure indicative of an interaction on ... deoxy-ribonucleic acid.

9. Actinomycin ... of DNA-directed RNA synthe... bits the action of vitamin D ... intestinal calcium absorption and ... orption. Vitamin D also ... ger-RNA synthesis in intestinal ... within ½ hr. of vitamin ... in D may play a crucial role, along ... thyroid hormone and cal... , gene-dependent, homeostatic ... hanism for calcium ... system the vitamin D molecule ... in very specific structural ... which are probably a reflection of ... icity of its receptor mole... an structural requirements for a ... enzyme relationship.

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XIV. ADDENDUM

Several publications concerning the metabolism and subcellular distribution of physiological doses of radioactive vitamin D have recently appeared. Mori, Lund, Neville & DeLuca (1967) have assessed the biological activity of a metabolite of vitamin D which was found previously by Lund & DeLuca (1966) to be the major radioactive component after a physiological dose of 10 i.u. of ^{3}H-vitamin D_3. The metabolite was as effective as vitamin D in curing rickets, in stimulating calcium transport by everted intestinal sacs, and in the elevation of serum calcium concentration. Significantly, the metabolite was able to stimulate calcium transport within 8–10 hr. of administration, whereas vitamin D required 20 hr. for equivalent stimulation. It is quite possible that these results are indicative of the metabolism of vitamin D to a new 'active' form which then participates in the actinomycin D-sensitive induction of a calcium transport system. Avioli *et al.* (1967) have also detected this same metabolite in the serum of humans after a dose of 10,000 i.u. This metabolite also has biological activity equivalent to the parent vitamin. Lund, DeLuca & Horsting (1967) have identified the presence of a palmitate ester of vitamin D in rat liver after varying doses of ^{3}H-vitamin D_3. Maximal production of the ester occurs 8 hr. after vitamin administration; however, its concentration remains quite low regardless of the amount of vitamin D administered. Thus it does not appear to be involved either in the physiological activities of vitamin D nor does it represent a storage form of the vitamin.

Imrie, Neville, Snellgrove & DeLuca (1967) have carried out a preliminary study of the comparative tissue distribution in the rachitic chick of ^{3}H-vitamin D_3 and ^{14}C-vitamin D_2. It will be recalled that in the chick, vitamin D_2 is only one-tenth as active as vitamin D_3. They found that after a dose of 10 i.u., the tritium and carbon-14 were accumulated equally well by the intestinal mucosa, skeletal system, kidney and liver. In contrast in the serum and muscle, the carbon-14 from the vitamin D_2 was lost rapidly by 6 hr. The loss was accompanied by an appearance of carbon-14 in the gall bladder and faeces. The exact significance of these results remains to be established. Essentially similar results were obtained by Hurwitz, Harrison, Bull & Harrison (1967) who biologically assayed the vitamin D activity present in various tissues after varying doses of non-radioactive vitamin D_2 or D_3.

Wilson, Lawson & Kodicek (1967) examined the subcellular distribution of ^{3}H-vitamin D_3 in the intestine of vitamin D-deficient and supplemented rats after a dose of 100 i.u. Their results confirmed the work of Haussler & Norman (1967) that the nuclear fraction of the cell contains significant quantities of the vitamin and/or its metabolites after a physiological dose.

Bell (1966) has compared the intestinal absorption and esterification of 4-^{14}C-vitamin D_3 and 4-^{14}C-cholesterol. Approximately equal quantities of radioactivity were recovered in the lymph 24 hr. after an oral dose of vitamin D or cholesterol. However, far less of the vitamin was recovered in the esterified form indicating that perhaps vitamin D did not obligatorily need to be esterified before absorption.

Blondin, Kulkarni & Nes (1967) have studied the possible origin of vitamin D_3 from 7-dehydrocholesterol in fish. They found that a homogenate of liver was capable of metabolically producing very small quantities of the vitamin. These results suggest that fish have the ability to produce vitamin D by a nonphoto-chemical process. Sahashi, Suzuki, Higaki & Asano (1967*a*) and Sahashi *et al.* (1967*b*) have continued their studies on the physiological activities of vitamin D sulfate. They find that it has biological activity equivalent to the unesterified vitamin D.

Several papers have also appeared concerning some of the details of vitamin D-mediated calcium absorption. Hurwitz, Harrison & Harrison (1967) have found that the exact concentration of sodium present in an *in vitro* incubation plays an important role in determining whether a concentrative transport of calcium will occur. In low sodium media, concentrative transport of calcium by the ileum was greatly enhanced by vitamin D_3 treatment of the chick, whereas in high sodium media, the effect of vitamin D was minimal. These findings may help to rationalize apparent differences in the properties of calcium transport in the duodenum and ileum of the rat and chick. Taylor & Wasserman (1967) have reported a more extensive characterization of their calcium-binding protein. The protein has been purified approximately 30-fold. It was detected in all segments of the small intestine and kidney of a vitamin D_3-treated rachitic chick, but not in the colon, liver or muscle. Unfortunately no information was presented, indicating whether the formation of the calcium-binding protein represented *de novo* protein synthesis in response to vitamin D or as to whether its production was actinomycin D-sensitive.

Au & Bartter (1966) have presented evidence of a direct effect of vitamin D on bone metabolism. Vitamin D treatment *in vivo* resulted in an increased rate of bone resorption as determined by an *in vitro* assay of surviving mouse calvaria. The effect of the vitamin was observed in both parathyroidectomized rats and rats in which a low serum calcium level had been induced by a low-calcium diet.

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Nose, O.: EFFECT OF VITAMIN D ON THE METABOLISM OF THIAMINE. I. THE THIAMINE CONTENTS OF THE INDUCED RACHITIC AND NORMAL RATS. Department of Pediatrics, Kyoto Prefectural University School of Medicine, Kyoto.

It has long been known that vitamin D is an antirachitic vitamin, but, since no chemical procedure for determining vitamin D has yet been established, the mechanism of its direct action on living organisms is still undetermined. In view of the fact that vitamin D is necessary for the metabolism of inorganic salts, particularly Ca and P, and its relationship with serum al-Pase*1 and organ al-Pase, and its effects on citric acid and pyruvic acid, vitamin D has been associated with the enzyme systems. Up to the present, little has been written on its relationship with other vitamins, especially water-soluble vitamins. Riihä-Forsander (1951) (1-3) measured the CoC*2 concentration in infant blood, and found that rachitic infancts contained less CoC and supplementary ingestion of vitamin D increased its level. It was also noted that, in infants, the CoC level varied from before the administration of vitamin D to after its ingestion. Furthermore, they experimentally demonstrated a difference by approximately 3γ% in the blood CoC level between vitamin D-deprived white rats and normal rats, and suggested that there is a close relationship between vitamin D and the metabolism of thiamine, particularly the mechanism of its transformation into phosphoric acid.

In an attempt to clarify these points, the author studied the distribution of thiamine in the blood, liver, and kidneys in vitamin D-deprived group and normal group of white rats.

EXPERIMENTAL PROCEDURES

(1) EXPERIMENTAL ANIMALS

Purebred male and female white rats from the same mother were used. No

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*1. Al-Pase = Alkaline phosphatase. *2. CoC = Cocarboxylase

difference in thiamine concentration and the manifestation of vitamin D deficiency was noted between male and female, but the difference was considerable between the rats born from different mothers, even when they were raised under the same conditions. Thus, it was found that the animals must be uniformly distributed with respect to litter for the experiments of the similar nature.

(2) MAINTENANCE OF THE ANIMALS

Vitamin D deficiency was produced according to Sakurai's method (4). White rats weighing approximately 30 g, about 1 month after weaning, were first raised on a prepared diet (Table 1) until they weighed about 40 g. Then, the animals were kept individually, and given the vitamin D deficient feed shown in Table 2. They were kept in dark place, by avoiding direct sunlight, and special cages, with wooden top and side walls, and metal-mesh floor, were prepared. No straw was spread on the floor in order to prevent their ingestion of feces. The room temperature was controlled within the range of 15 - 25°C, and the room was ventilated during the summer. The feed in an amount equivalent to 1/10 - 1/8 the body weight was diluted with 2 parts of water, and kneaded. Water was given upon demand.

TABLE 1. PREPARED FEED

a 小麦澱粉	58	h 無機塩類組成	(%)
b グラニュ糖	20	KCl	20
c 牛乳カゼイン	18	$MgSO_4 \cdot aq.$	7
d 無機塩類	4	Fe-citrate	3
e A (1匹当り)	200 i.u.	Na_2CO_3	10
f 水溶性ビタミン類	g (別表)	Ca-lactate	40
		$CaCO_3$	20

KEY: a, wheat starch; b, granulated sugar; c, milk casein; d, inorganic salts; e, A (per rat); f, water soluble vitamins; h, composition of inorganic salts; g, (Table 3)

TABLE 2. VITAMIN D-DEFICIENT FEED

イ 小麦澱粉	33	ム 無機塩類	4
ロ グラニュ糖	40	メ A（1匹当り）	300 I.U.
ハ 脱脂卵白	18	モ 水溶性ビタミン類	

KEY: a, wheat starch; b, granulated sugar; c, defat egg white; d, inorganic salts; e, vitamin A (per rat); f, water soluble vitamins

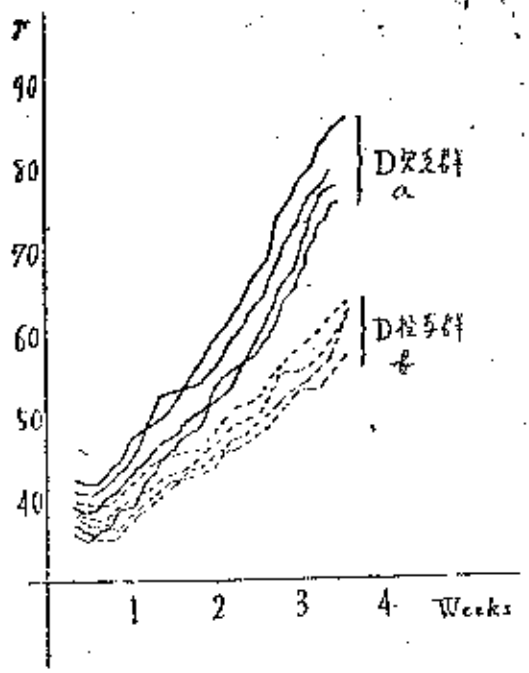
TABLE 3. WATER SOLUBLE VITAMINS
(Daily dose per rat in γ)

KEY: a, pantothenic acid; b, 30 or 80; c, nicotinic acid; d, folic acid; e, choline chloride

The water-soluble vitamins shown in Table 3 were given once daily at 0.1 cc per rat by the oral route with a tuberculin injector. Efforts were made to prevent fluctuation of vitamin B_1 intake as might have been caused by an increase or decrease in appetite. The control group comprised rats from the same mother and were given the same feed. They received 1000 I.U. of vitamin D_2 orally at 1 week intervals. These two groups were weighed at 1 day intervals. As shown in Figure 1, the growth in body weight exhibited the same pattern. As Sakurai (5) stated, the vitamin D deprived group gained weight at a higher rate than the control when the feed contained inorganic salts as prescribed by the authors.

(3) CONFIRMATION OF VITAMIN D DEFICIENCY

The knee joints were X-rayed 25 days after the beginning of the vitamin D-deficient diet, and the upper extremities of the tibial bones were carefully examined with regard to any change due to vitamin D deficiency. When a change was noted, the animal was regarded as vitamin D deprived (6), and those which indicated severe malnutrition, as determined by the condition of growth, appetite, and movement, were excluded from experiments.



KEY: a, vitamin D deprived group; b, vitamin D group

Figure 1. Growth Curves of the White Rats

TABLE 4. GROUPING OF EXPERIMENTAL ANIMALS

No.	a 群	非经口负荷 (皮下注射)		
		b	D_2	
I	b D 欠 乏	−	−	
II	c 対 照	−	−	
III	d D 欠 乏	+	−	負荷時より
IV	e D 欠 乏	+	+	48時間後に
V	c 対 照	+	−	屠殺測定

KEY: a, group; b, vitamin D deprived; c, control; d, non-oral administration (hypodermic injection); e, subjected to measurement after slaughtered 48 hours from the time of D_2 administration.

(4) EXPERIMENTAL PROCEDURE

According to Räihä et al., the white rats were divided into 5 groups, as shown in Table 5, and the thiamine concentrations in the blood, liver, and kidneys were determined. Group I consisted of vitamin D deprived rats, group II, control, group III, vitamin D-deprived rats to which thiamine was administered by a non-oral route (hypodermic), group IV, vitamin D deprived rats to which 10,000 I.U.

of D_2 was given by a non-oral route along with vitamin B_1, and group V, control rats to which only thiamine was given. The thiamine solution used for injection was a 0.5 cc portion of an aqueous crystalline thiamine solution containing the vitamin at the prescribed dose. Vitamin D_2, donated from Ezai Co., was added to sterile olive oil at 5000 I.U. per 0.1 cc.

The rats given thiamine and vitamin D (groups III, IV, and V) were killed precisely 48 hours after the injection. On the day when they were killed, the rats received no feed and were decapitated without anesthesia, the blood was collected, and the liver and kidneys were promptly removed for thiamine assay. In groups III, IV, and V, the oral administration of thiamine was withdrawn from the time of non-oral injection of thiamine to the time of sacrifice.

(5) THIAMINE ASSAY

The thiamine concentrations in the liver, blood, and kidneys were determined according to Fujita's permutit-thiochrome fluorescence method (7), facilitated by a Farrand fluorometer (8).

EXPERIMENTAL RESULTS

Each group was divided into 2 subgroups by daily thiamine intake (oral administration), the 30 γ group (intermediate daily dose) and the 80 γ group (relatively large daily dose). The former were given 500 γ of thiamine by hypodermic injection and the latter, 5 mg. The amount of D_2 was 10,000 I.U. for both subgroups.

(1) THIAMINE DISTRIBUTION IN THE 30 γ DAILY THIAMINE ORAL INTAKE GROUP

The free and total thiamine concentrations in the blood, liver, and kidneys of groups I - V are shown in Table 5. The total thiamine concentrations in the liver and blood of the vitamin D deprived group (group I) averaged 620 and 21.9 γ,

respectively, lower than 725 and 25.5 γ of the control (group II). However, the concentrations in the kidneys were 321 and 322 γ%, both values being nearly equal. The vitamin D deprived group to which only vitamin B_1 was given (group III) showed lower total thiamine concentrations in the liver and blood than group IV which received thiamine and D or group V which received thiamine, just as group I showed lower values than group II. In other words, the liver of group III contained approximately 100 γ% less thiamine than group V, and the blood, about 3 γ% less thiamine than group V. However, the total thiamine concentrations in the kidneys showed no variation among the groups.

All groups showed extremely low concentrations of free thiamine in the liver and blood, as compared to the bound thiamine, indicating that the increase or decrease in thiamine concentration is derived from the fluctuation of bound thiamine. The rate of bound thiamine and total thiamine ranged from 87 to 92% in all groups, with no significant difference between the groups.

(2) THIAMINE DISTRIBUTION IN THE 80 γ THIAMINE DAILY ORAL INTAKE GROUP

It has already been reported by Shibata (9,10) and Murakami (11) that the thiamine concentrations in various organs and blood exhibit gradual increases when a large dose of thiamine is administered orally or non-orally for more than 3 weeks consecutively. The 80 γ groups indicated higher concentrations of thiamine in the blood, liver and kidneys than the 30 γ groups. In the liver, the difference in concentration was more than 1000 γ%. The average organ concentrations of groups I to V are given in Table 6.

The blood concentrations of groups I and II were 26.5 and 31.4 γ%, respectively, the difference being about 5 γ%, and the liver concentrations, 1043 and 1110 γ%, respectively, the difference being about 70 γ%, but the kidney concentrations showed a difference of only 30 γ%. Between Group III to which thiamine was given, and groups IV and V, the difference in blood concentration was approximately 5 γ%,

whereas the kidney and liver concentrations showed differences between 30 and 40 γ%. From these results, it was assumed that the large thiamine intake brought the thiamine concentrations in the liver and kidneys to their tolerance levels.

TABLE 5. THIAMINE CONCENTRATIONS IN THE BLOOD, LIVER, AND KIDNEYS OF THE 30 γ DAILY THIAMINE INTAKE GROUP (UNIT : γ%)

a 群	ネズミ No.	c 血液 F	c 血液 T	d 肝 F	d 肝 T	e 腎 F	e 腎 T
f D欠乏 I	1	2.7	22.5	60	600	24.7	247.0
	2	2.2	21.7	63	570	26.3	312.5
	3	2.3	20.3	54	600	20.0	400.0
	4	2.8	23.3	63	710	37.5	325.0
	g 平均	2.5	21.9	60	620	27.1	321.1
	E/T		88.6		90.3		91.6
h 対照 II	1	3.1	27.0	75	720	26.0	343.0
	2	2.0	22.0	66	780	36.0	275.0
	3	3.6	26.0	54	720	31.3	317.8
	4	2.5	27.0	75	680	42.8	328.6
	g 平均	2.8	25.5	67.5	725	34.8	322.4
	E/T		89.0		99.6		89.1
i D欠乏 B1負荷 III	1	2.2	21.0	96	750	33.8	350.6
	2	3.1	22.5	93	650	30.0	311.3
	3	2.9	21.0	78	580	32.3	353.8
	4	2.7	21.7	75	600	30.0	342.8
	5	3.4	26.2	72	840	40.0	312.5
	g 平均	2.8	23.1	82.8	686	33.8	335.4
	E/T		87.9		87.9		89.9
j D欠乏 B1 D負荷 IV	1	2.0	24.0	66	670	31.7	352.9
	2	3.0	27.3	69	740	46.7	310.0
	3	2.7	26.2	93	850	21.4	335.7
	4	2.7	26.2	93	930	38.6	428.6
	5	3.4	28.5	60	730	40.8	381.6
	g 平均	2.7	26.5	77.4	788	36.2	378.2
	E/T		89.8		90.2		90.5
k 対照 B1負荷 V	1	3.0	27.0	60	750	41.3	425.0
	2	2.6	24.0	96	760	36.3	352.8
	3	2.5	26.3	90	730	30.0	400.0
	g 平均	2.7	25.8	82.0	747	36.0	392.6
	E/T		89.5		89.0		90.5

F: free B_1. T: total B_1. E/T: bound B_1/total (%)

KEY: a, group; b, rat No.; c, blood; d, liver; e, kidneys; f, vitamin D deprived; g, average; h, control; i, vitamin D deprived and B_1 loaded; j, vitamin D deprived and B_1 and D loaded; k, control with B_1 injection

DISCUSSION

It is evident that the organ level of thiamine or other water soluble vitamins is influenced by the amount of intake, varying over a wide range. The necessary amount of thiamine for a white rat is estimated at 20 - 40 γ,

TABLE 6. THIAMINE CONCENTRATIONS IN THE BLOOD, LIVER, AND KIDNEYS OF THE 80 γ DAILY THIAMINE INTAKE GROUP (UNIT: γ%)

a 群	b ネズミ No.	c 血液 F	c 血液 T	d 肝 F	d 肝 T	e 腎 F	e 腎 T
I f D 欠乏	1	2.5	25.0	142	925	–	–
	2	3.1	25.6	247	1087	73.2	579.2
	3	3.1	28.8	139	1118	40.2	555.5
	k 平均	2.9	26.5	176	1043	56.7	567.4
	F/T		89.0		83.1		89.8
II g 対照	1	2.0	30.8	150	1169	–	–
	2	1.0	30.5	121	1070	–	–
	3	2.8	30.5	118	1050	30.5	579.2
	4	2.5	33.8	200	1140	79.0	625.0
	k 平均	2.1	31.4	147	1110	54.8	602.0
	F/T		62.7		85.8		90.8
III h D 欠乏 B_1 負荷	1	2.5	24.6	192	937	–	–
	2	2.5	28.5	275	1067	–	–
	3	2.0	27.0	174	1162	79.0	625.0
	4	2.0	26.0	113	1101	60.8	540.6
	k 平均	2.3	26.6	181	1079	69.9	582.8
	F/T		91.4		83.0		88.0
IV i D 欠乏 D, B_1 負荷	1	2.0	32.3	248	1293	–	–
	2	2.0	30.0	200	1073	–	–
	3	1.0	30.4	141	1083	60.8	608.0
	4	1.5	30.5	151	1228	83.3	660.0
	5	2.8	32.5	111	1011	98.9	659.0
	k 平均	1.9	31.9	171	1125	81.0	622.5
	F/T		94.0		84.8		87.0
V j 対照 D, B_1 負荷	1	2.3	32.8	216	1063	79.0	625.0
	2	2.5	33.8	200	1140	73.2	579.2
	3	2.0	30.0	118	1150	40.0	555.5
	k 平均	2.3	31.5	178	1123	63.7	569.9
	F/T		92.7		84.1		89.4

KEY: a, group; b, rat No.; c, blood; d, liver; e, kidneys; f, D deprived; g, control; h, D deprived and B_1 loaded; i, D deprived and D and B_1 loaded; j, control with B_1 injection; k, average

although the value varies according to the investigator. The 30 γ/daily intake group showed nearly normal thiamine concentrations in the blood and the organs, but the 80 γ group gave values in excess of normal level, indicating that the dose is excessive. The amount of thiamine intake through natural food in individual living organisms naturally varies greatly according to the thiamine content of the food and the amount of food intake. In the preparation of a vitamin D deficient diet, defat yeast is occasionally used for water soluble vitamins, but the result is greatly influenced by the considerable variation in the content of vitamins in the yeast and individual variation in food intake. Zhmeido et al. (12) reported that an adequate amount of water soluble vitamins

given to a case of excessive vitamin D intake delays the manifestation of the symptoms caused by the excessive intake. Thus, the amount of vitamins in a feed must be determined with caution. The author adopted the following method in order to eliminate any effect of external factors, such as the loss of appetite due to D deficiency, on thiamine intake. First of all, the feed was carefully checked with regard to its complete absence of thiamine, so that the animals always consume a prescribed amount of thiamine regardless of the amount of feed intake. Subsequently, a solution of water soluble vitamins was accurately administered with a tuberculin injector.

Räihä-Forsander determined the blood CoC level in infants by the pressure method and found that the CoC levels in vitamin D deprived infants were lower than those in normal infants. They further confirmed experimentally that the administration of vitamin D along with vitamin B_1 elevated the blood CoC level. Since the difference was minimal among the infants and adults to which a sufficient amount of vitamin D had been administered in advance, they assumed that vitamin D exerted some action on thiamine metabolism, and carried out an experiment using white rats.

Reviewing the thiamine concentrations in the blood, liver, and kidneys of white rats in the present experiment, there were differences of 5 γ% and approximately 100 γ% in the blood and liver, respectively, between the vitamin D deprived group and the control group. Furthermore, the administration of vitamin D to the vitamin D deprived rats produced a notable difference in value from those without supplementary vitamin D within 48 hours, and the values were at the control levels. Thus, it is evident that vitamin D in some way increases the thiamine concentrations in the blood and tissues, particularly the liver, and plays a direct or indirect role in thiamine metabolism. Thus, what Räihä et al. found with regard to the blood concentration could be confirmed with regard to the liver concentration as well. In terms of the bound thiamine concentration/total thiamine concentration

as an indicator of the condition in which the vitamin is bonded to phosphate, the pronounced disturbance in the conversion of the vitamin into phosphoric acid as noted in alloxan diabetes (13,14) and other forms of liver disturbance could not be observed in vitamin D deprived white rats. The decrease in thiamine concentration as a result of vitamin D deficiency is derived from that of the bound type, and, since the amount of free thiamine is extremely minimal, the bound thiamine concentration/total thiamine concentration indicated no appreciable difference, the two factors changing apparently in a parallel fashion.

Generally speaking, the white rats on vitamin D deficient diet becomes less active, with dysbasia, loss of appetite, and diarrhea. The severer the symptoms, the lower the thiamine concentrations. The possibility in reduced thiamine intake due to diarrhea seems to be a logical consequence. In view of a report stating that intestinal bacterias in vitamin D deprived white rats show a smaller percentage of Bifidus bacteria and a large proportion of B. coli Mig. (15), the difference in the amount of synthetic thiamine due to intestinal bacteria may need to be taken into consideration. Kikuchi (16) measured the muscular creatine concentrations of rachitic rats and normal rats. Since the concentration was lower in the former, he maintained that vitamin D was related to creatine metabolism to some degree. The important role played by vitamin D in P and Ca metabolism is well known. In view of this knowledge, it is deemed possible that vitamin D deficiency causes nutritional disturbance, deteriorates the activity of a living organism, and influences metabolic mechanism. Although vitamin D does not seem to exert any action specifically to thiamine metabolism,it influences the bonding of thiamine with tissues, particularly with liver cells, as one of its many activities.

CONCLUSION

1. The thiamine concentrations in the blood, liver, and kidneys in vitamin D deprived white rats were determined, and compared with those of the control.

2. At a daily intake of 30 r, the vitamin D deprived group showed approximately 5 r% and 100 r% lower concentrations of thiamine in the blood and liver, respectively.

3. When vitamin D was given to vitamin D deprived animals, the thiamine concentrations as of 48 hours later approached the control levels.

4. At a daily dose of 80 r%, the tendency was similar to that of the 30 r% group, but since the liver concentration was significantly increased, the difference was not as notable. No appreciable difference in kidney concentration between the groups was noted.

5. Vitamin D seems to influence thiamine metabolism in some way, but there was no difference in the thiamine-phosphorus bonding rate between the vitamin D deprived group and the normal group. Thus, its effect on thiamine metabolism is assumed to be confined within the areas of intestinal absorption and tissue assimilation.

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し濾過後精製する方が労力，資材ともに有利と思われる。

Bu吸着活性炭よりのBuの溶出には10%アセトン・10%メタノールまたは10%酢酸エチル・20%n-プロパノール混合溶液で加熱抽出するか，あるいは常温溶出には酢酸とジメチルアミンまたは重曹のような塩類を添加すれば50%アセトン水溶液に匹敵する効果がえられ工業的に応用できるものと考えられる。

終りにのべみ教授ご指導を賜わつた菊田教授，清水・岡田の諸先生に深く感謝いたします。とくに直接ご指導，ご教示いただいた上久保先生に心から感謝いたします。またご指導いただいた松田博士，石田技官ならびに名古屋大泡発酵研究所の方々のご協力に深く感謝いたします。　(昭34. 6. 6. 受理)

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ビタミンB_1代謝に及ぼすビタミンDの影響について

(1) ビタミンD欠乏シロネズミと正常シロネズミのビタミンB_1含有量について

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能　勢　　修

EFFECT OF VITAMIN D ON THE METABOLISM OF THIAMINE

(1) THE THIAMINE CONTENTS OF THE INDUCED RACHITIC AND NORMAL RATS

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It was observed that in the induced rachitic rats the thiamine contents in liver and blood were considerably lower than those of the normal rats. When the rachitic rats were given intramuscularly thiamine and vitamin D, two days later the thiamine contents of the rat tissues, especially liver, developed equally those of the normal cases. But when they were given thiamine only, the increase of thiamine concentration was not obvious. This result suggests that the administration of vitamin D has caused the accumulation of thiamine in liver tissue.

Dが抗クル病性ビタミンであることは古くから明らかなところであるが，生体に及ぼす直接的な作用機序に関してはDの化学的定義さえ確定されていない今日，いまだほとんど解明せられていない。しかしDが無機塩類の代謝，ことにCaとPの代謝に必要なことはすでに明らかであり，また血清al-Pase*1)，臓器al-

*1) al-Pase = Alkaline phosphatase　　*2) CoC = Cocarboxylase

表1 準 備 食

小麦澱粉	58	無機塩類組成	(%)
グラニュ糖	20	KCl	20
牛乳カゼイン	18	$MgSO_4 \cdot aq.$	7
無機塩類	4	Fe-citrate	3
A（1匹当り）	200 i.u.	Na_2CO_3	10
水溶性ビタミン類	(別表)	Ca-lactate	40
		$CaCO_3$	20

表2 D 欠 乏 飼 料

小麦澱粉	38	無機塩類	4
グラニュ糖	40	A（1匹当り）	200 i.u.
脱脂卵白	18	水溶性ビタミン類	

Paseとの関係や，クエン酸，ピルビン酸に及ぼす影響などからDが間接的に酵素系になんらかの作用を及ぼすものと考えられて来た．しかしDの他のビタミンとくに水溶性ビタミンとの関係については，ほとんどその報告をみない．Hählä・Forsander (1951)[10][11]は乳児血液のCoC[60]を測定し，クル病活児にCoC含有量の少ないこと，Dの投与によりCoC量の増加すること，また乳児ではDの投与前後にCoC量の差のあることを確かめた．さらにシロネズミについてD欠乏群と正常群との間の血液CoC含有量に約3%の差があることを実験的に証明し，このことよりDがB[12]の代謝とくにその腸吸収機序になんらかの関係を有することを推論した．

著者はこれらの諸点をさらに明瞭にしたいと考え，シロネズミについてD欠乏群と正常群との血液，肝臓，腎臓等のB分布状態をしらべたのでその結果を報告する．

実 験 方 法

(1) 実験動物

純系同腹シロネズミを雌雄の区別なく用いた．本実験においてB[12]含有量，D欠乏の起り方については雌雄間には大差を認めなかつたが，同腹でない場合は同一条件で飼育しても総計B[12]含有量や発育状態にかなりの差があり，したがつて同一系統の実験には同腹のシロネズミを適当に配分する必要のあることを知つた．

(2) 飼育法および飼料

D欠乏を起きせるには桜井の方法[9]によつた．すなわち離乳1ヶ月後，30g前後のシロネズミを最初準備食(表1)で飼育し体重40g前後になるを1匹飼いとして表2に示すD欠乏食を投与した．飼育にあたり直射日光にさけなるべく暗い場所を選んで飼育し飼育かごは上面，両側は木製，下面は金網を用いた特製のものを作り，敷わらは用いず糞食をさけた．飼育室の温度はなるべく15～25℃の範囲になるようにし，とくに夏期には適当な通風の行なわれるように努めた．飼料投与量はだいたい体重の1/10～1/8の量を約2倍の水でよくねり水は自由に与えた．

水溶性ビタミン類は表3のように調製し1匹当りの投与量が0.1ccに含まれるようにし，ツベルクリン注射器で毎日1回直接経口的に正確に投与した．これによつて食餌の摂取による B[12] 吸収量が変動しないように努めた．対照群は前述のように同腹のシロネズミを無差別に選んで同一飼料で飼育し，1週間ごとにD 1000 i.u.

表3 水溶性ビタミン類
（1日1匹当りγ）

B_1	30または60	ニコチン酸	40
B_2	60	葉酸	10
B_6	40	塩化コリン	500
パントテン酸Ca	40		

を経口的に投与した．これら2群とも毎日に体重を測定したが，その一例の体重増加曲線は図1の如くだいたい同様の増加を示した．桜井[9]の述べているように著者の用いた無機塩類組成ではD欠乏群が対照群にくらべて体重増加が大きい傾向を示した．

(3) D欠乏の決定法

D欠乏食投与後25日目にX線によつて脛骨を撮影し脛骨上端の骨の変化でD欠乏の有無を確かめ骨変化のあるものをD欠乏[9]と定め発育状態，食餌の良否，運動のようすなどを参考にし著しく栄養障害を起しているものはこの実験から除外した．

図1　シロネズミ体重増加曲線

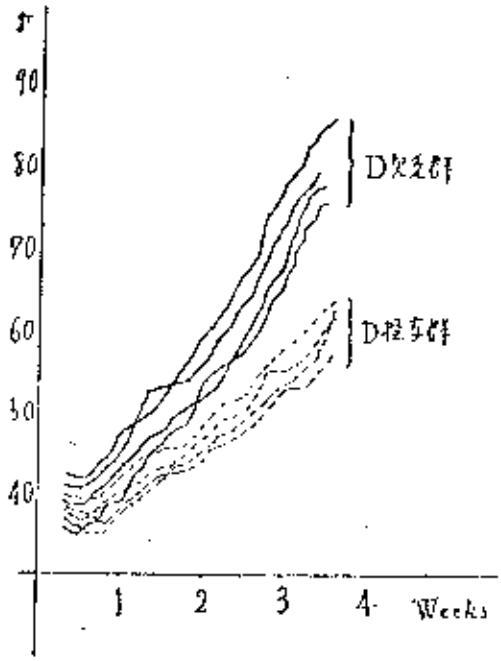

表4　実験動物配分方法

No.	群	非経口負荷（皮下注射） B₁	D₂	
I	D欠乏	−	−	
II	対照	−	−	
III	D欠乏	+	−	負荷時より
IV	D欠乏	+	+	48時間後
V	対照	+	−	屠殺測定

(4) 実験配分法

Rabinoらの方法に従つて表4のシロネズミを5群に分ち血液，肝臓，腎臓のB₁量を測定した．すなわちI群はD欠乏，II群は対照，III群はD欠乏にB₁を非経口的(皮下)に負荷したもの，IV群はD欠乏にB₁負荷と同時にD₂ 10000 i.u.を非経口的に投与した群，V群は対照にB₁のみを負荷した群である．注射に用いたB₁液は結晶B₁を水溶液とし，1定量が0.5ccに含まれるようにした．D₂はエーザイ株式会社より分譲されたD₂を綿実リーブ油0.1ccに5000 i.u.含まれるように作つた．

B₁およびD負荷群(III，IV，V群)は負荷より正確に48時間後に屠殺した．屠殺当日は絶食を与えず絶対安静の下に絶血殺血し即座ちなるべく速やかに肝臓，腎臓を取り出しB₁の定量に使した．なおII，IV，V群の負荷群はB₁負荷より屠殺までB₁の経口投与を中止した．

(5) B₁定量法

血液，肝臓，腎臓ともに藤田のパームチット・チオクロム螢光法[7]により行ない螢光測定はFarrand螢光度計を用いた[8]．

実験結果

実験は各群につき摂取B₁量（経口投与）を1日30γ群（中等量）と80γ群（比較的大量）の2群に分け，B₁負荷（皮下注射）を前者は500γ，後者は5mgとしD₂負荷量はいずれの群も10,000 i.u.とした．

1）B₁ 30γ/日経口的摂取群のB₁分布

I～V群の血液，肝臓，腎臓の遊離型，結合型B₁含有量は表5の通りである．D欠乏群(I群)は肝，血液の総B₁の平均値がそれぞれ620および621.9γ%で対照群(II群)の725および735.5γ%にくらべて少ない傾向を認めた．しかし腎では1321および1327γ%で両者はほとんど同等の値を示した．ついでD欠乏群B₁のみを負荷した群(III群)はB₁，D₂を負荷したIV群や対照群にB₁を負荷したV群にくらべて肝，血液の総B₁含有量はI群がII群より低い値を示したと同様に少ない傾向が認められた．すなわちIII群の肝はIV，V群にくらべて約100γ%，血液は約30γ%少なかつた．しかし腎臓ではいずれの群にも大差を認めなかつた．

肝臓，血液のB₁をみると各群とも遊離の含有量はいずれも結合型に比しひじように少なくB₁含有量の増減は主として結合型によることを知つた．しかし結合型B₁と総B₁との割合はいずれの群も87～92%の間にあり有意義な差を認めなかつた．

2）B₁ 80γ/日経口的摂取群のB₁分布

B₁の大量を継続的に3週間以上与えると経口的でも非経口的でも血液および諸臓器のB₁含有量がしだいに増加することは実用[9][10]，対上[11]の報告にみるとおりである．この群でも30γ投与群にくらべ血液，肝，腎いずれもB₁量が多く，ことに肝では各群ともに1000γ%以上の値を示したことは当然の結果であろう．I～V群の各群の平均値は表6のとおりである．

I群とII群の血液ではそれぞれ26.5および31.4γ%で約5γ%の差があり，肝では1043および1110γ%で約70γ%の差があつたが腎ではその差は30γ%であつた．B₁負荷量とIII，IVにはD₂投与してない群にくらべI群とII群の場合と同様に約5γ%の差が認め

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表5 B_1 30γ/1日 摂取群の血液，肝，腎B_1含有量（単位：γ%）

群	ネズミ No.	血液 F	血液 T	肝 F	肝 T	腎 F	腎 T
I D欠乏	1	2.7	22.5	60	600	24.7	247.0
	2	2.2	21.7	63	570	26.3	312.5
	3	2.3	20.3	51	600	20.0	400.0
	4	2.8	23.3	63	710	37.5	325.0
	平均	**2.5**	**21.9**	**60**	**620**	**27.1**	**321.1**
	E/T		83.6		90.3		91.6
II 対照	1	3.1	27.0	75	720	26.0	343.0
	2	2.0	22.0	66	780	36.0	275.0
	3	3.6	26.0	51	720	34.3	342.8
	4	2.5	27.0	75	680	42.8	328.6
	平均	**2.8**	**25.5**	**67.5**	**725**	**34.8**	**322.4**
	E/T		89.0		90.6		89.1
III D欠乏 B_1負荷	1	2.2	24.0	96	750	36.8	353.8
	2	3.1	22.5	93	660	30.0	314.3
	3	2.9	21.0	78	580	32.3	353.8
	4	2.7	21.7	75	600	30.0	312.8
	5	3.4	26.2	77	840	40.0	312.5
	平均	**2.8**	**23.1**	**82.8**	**686**	**33.8**	**335.4**
	E/T		87.9		87.9		89.9
IV D欠乏 B, D負荷	1	2.0	24.0	66	670	31.7	352.9
	2	3.0	27.8	69	740	48.7	340.0
	3	2.7	26.2	93	850	21.4	385.7
	4	2.7	26.2	93	950	38.6	428.6
	5	3.4	28.5	66	730	40.8	381.6
	平均	**2.7**	**26.5**	**77.4**	**788**	**36.2**	**378.2**
	E/T		89.8		90.2		90.5
V 対照 B_1負荷	1	3.0	27.0	60	750	41.3	425.0
	2	2.6	24.0	96	760	36.3	352.8
	3	2.5	26.3	90	730	30.0	400.0
	平均	**2.7**	**25.8**	**82.0**	**747**	**36.0**	**392.6**
	E/T		89.5		89.0		90.5

F：遊離型　T：総B_1　E/T：結合型/総%

られたが肝，腎では30～40γ%しか差がなかつた．これらのことから肝，腎ではB_1摂取が大量であつたのでその臓器の含有許容量に近づいたためと考えられる．

考　　察

B_1やそのほかの水溶性ビタミン類の各臓器含有量はその摂取量に影響をうけ，いろいろな値を示すことは明らかなところである．シロネズミのB_1必要量は諸家によつていろいろの値が報告されているがだいたい20～40γがもつともよいといわれている．30γ/日摂取群の臓器および血液のB_1測定値はいままで報告されている正常値に近い値を示したが80γ/日摂取群になるといずれも正常値を超え過剰投与であることがわかる．また自然食では食物のB_1含有量，摂取飼料の多少によつてその個体のB_1摂取量が大いに変動してくるのは当然であり，またD欠乏合成飼料として，水溶性ビタミン類を与えるのに脱脂酵母を用いる場合もあるが酵母のビタミン含有量に相当の差異があるうえ食物摂取の多少で著しい影響を受けることになる．Zhineidoら[12]はD過剰投与のとき水溶性ビタミン類を適当に投与するとD過剰症の症状の発現がおくれると報告していることから，飼料中のビタミン量の配合には充分の注意が必要となつてくる．したがつて著者は飼料摂取の多少に関係なくいつも一定量のB_1を摂取するように合成飼料には全くB_1を含有しないことを確かめ前述のように水溶性ビタミンの溶液を正確にツベルクリン注射器で投与しD欠乏による食慾減退などの外的試下がB_1摂取になるべく影響のないように努めた．

Räihä-Forsander は乳児の血中CoC量を検圧法を用いて測定しD欠乏児では正常児にくらべ含有量の低いことを指摘しB_1投与時にDを同時に与えた場合，血中CoC量の高くなることを実験的に確かめた．そして，

表6　B₁ 80γ/日 摂取群の血液，肝，腎のB₁含有量（単位：γ%）

群	ネズミ No.	血液 F	血液 T	肝 F	肝 T	腎 F	腎 T
I D欠乏	1	2.5	25.0	142	925	–	–
	2	3.1	25.8	247	1087	73.2	579.2
	3	3.1	28.8	139	1118	40.2	555.5
	平均	**2.9**	**26.5**	**176**	**1043**	**56.7**	**567.4**
	E/T		89.0		83.1		89.8
II 対照	1	2.0	30.8	150	1169	–	–
	2	1.0	30.5	121	1070	–	–
	3	**2.8**	30.5	118	1060	30.5	579.2
	4	2.5	33.8	200	1140	79.0	625.0
	平均	**2.1**	**31.4**	**147**	**1110**	**54.8**	**602.0**
	E/T		92.7		86.8		90.8
III D欠乏 B₁負荷	1	2.5	24.8	192	987	–	–
	2	2.5	28.5	225	1067	–	–
	3	2.0	27.0	171	1162	79.0	625.0
	4	**2.0**	26.0	143	1101	60.8	540.5
	平均	**2.3**	**26.6**	**184**	**1079**	**69.9**	**582.8**
	E/T		91.4		83.0		88.0
IV D欠乏 D, B₁負荷	1	2.0	32.3	248	1293	–	–
	2	2.0	30.0	200	1013	–	–
	3	1.0	30.4	144	1083	60.8	608.0
	4	1.5	30.5	151	1228	83.3	600.0
	5	2.8	32.5	111	1011	98.9	659.0
	平均	**1.9**	**31.9**	**171**	**1125**	**81.0**	**622.3**
	E/T		91.0		84.8		87.0
V 対照 B₁負荷	1	2.3	32.8	216	1080	79.0	625.0
	2	2.5	33.8	200	1140	73.2	579.2
	3	**2.0**	31.0	118	1150	40.0	595.5
	平均	**2.3**	**31.5**	**178**	**1123**	**63.7**	**599.9**
	E/T		92.7		84.1		89.4

前もつて充分D投与した乳児や成人ではその差の少ないことからDがB₁の代謝になんらかの影響を与えるものと考えてシロネズミの実験を行ない同様の傾向を見出している。

著者の行なつたシロネズミの血液，肝臓，腎臓の成績をみるとD欠乏群と対照群との間に血液で5γ%，肝で約100γ%の差がみられた。しかもD欠乏群にDを投与すると48時間後には投与しない群にくらべて差を生じB₁含有量が対照群と同じ値を示すようになる。したがつてDが血液や組織ことに肝臓のB₁含有量を増加せしめるのになんらかの影響を与えていることが考えられこれが直接的でないにしてもB₁代謝にある役割を演じていることが推定される。すなわちRaiba らの血液について述べたことが肝においても同様に認められることが確かめられたわけである。しかし前述のように結合B₁量／総B₁量をもつて消費状態を考えるならばアロキサン糖尿病[10]そのほか種々の肝臓病時などにみられる著明な燐酸化阻害はD欠乏シロネズミにはみられずD欠乏によるB₁含有量の減少はほとんど結合型によるものであるが遊離型がきわめて少ないために結合型B₁量/総B₁量は大差なく両者は平行して増減していることがわかる。

一般にD欠乏食を投与するとシロネズミはしだいに運動不活発となり歩行困難，食慾不振，下痢などを認める。このような症状の著しいものほどB₁濃度は低くことに下痢によるB₁摂取量の減少は当然考えられる。またD欠乏シロネズミの腸内細菌には*Bifidus* 菌が少なく大腸菌が多いという報告[15]から腸内細菌による合成B₁量の差異も考慮する必要がある。一方菊地[16]はクル病シロネズミと対照群との筋肉クレアチン量を測定して前者にその量の少ないことからDがクレアチン代謝にある程度関係していると報告している。またDがP，Caの代謝にたいし重要な調節の因子であることはよく知られているところである。これらのことからD欠乏が栄養障害を惹起し，生体の活動を低下せしめ多くの代謝機構に影響を与えることが当然考えられ

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る．したがつてDがB_1の代謝のみに特異的に作用を及ぼすとは考えられないがDの作用のひとつとしてB_1の組織固定とくに肝細胞への固定になんらかの効果を及ぼすものと考えられる．

結　論

(1) シロネズミにD欠乏を起させ血液，肝臓，腎臓のB_1含有量を測定して対照群と比較した．

(2) 30γ/日摂取群ではD欠乏群は血液では約5γ%，肝臓では約100γ%B_1濃度の少ないことを明らかにした．

(3) D欠乏動物にDを投与して18時間後にB_1量を測定するとB_1含有量は対照群に近づき回復することがみられた．

(4) 80γ%摂取群でも30γ%摂取群と同様の傾向を認めたが肝臓ではその含有量が大量となつて来るのでその差があまり著しくなかつた．腎臓の場合はいずれの群にも有意義な差を認めなかつた．

(5) DがB_1代謝になんらかの作用を及ぼすものと推定されるが，附着率からみるとD欠乏群と正常群の間に差が認められず，したがつてDのB_1代謝に及ぼす影響はその腸管吸収と組織内固定にあるものと考えられる．

終りにのぞみ終始懇篤なご指導ならびにご校閲を賜わつた恩師中村恒男教授に厚くお礼申し上げます．

本論文の要旨は第10回ビタミン学会大会において発表した．

(昭34. 6. 3. 受理)

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ビタミンB_1代謝に及ぼすビタミンDの影響について

(II) ビタミンD欠乏シロネズミのビタミンD投与時における ビタミンB_1含有量の時間的推移

(附) 幼児の血中ビタミンB_1量に及ぼすビタミンDの影響

京都府立医科大学小児科学教室（主任　中村恒男教授）

能　勢　修

EFFECT OF VITAMIN D ON THE METABOLISM OF THIAMINE

(II) THE HOURLY CHANGE OF THIAMINE CONTENT OF THE INDUCED RACHITIC RATS ON VITAMIN D LOADING

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The hourly change of thiamine content of the induced rachitic rats was investigated by dividing into two groups. In one group, thiamine contents in liver and blood were measured after administration of thiamine and vitamin D, in the other group thiamine

THE USE OF RADIOACTIVE PHOSPHORUS IN THE ASSAY OF VITAMIN D

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THREE FIGURES

(Received for publication August 2, 1954)

Numerous attempts have been made in recent years to improve the assay of vitamin D. Many of these methods have been chemical or physical in nature, but have not gained wide acceptance because of the serious limitation of questionable specificity. The inherent difficulties in vitamin D determination by physico-chemical or biological means have been reviewed by Bills ('47). Bourdillon et al. ('31) have estimated that nearly one-third of the total error in a vitamin D biological assay is due to the errors in assessing the various degrees of healing.

Biological methods, because of the specific nature of the responses, have been preferred, and considerable effort has been expended in attempts to improve the precision of the technique. One such approach has been described by Shue, Friedman and Tolle ('52) who used photographic methods for determining the extent and area of calcification in the line test, thus minimizing the customary subjective nature of this assay. Another approach, one which seemed to offer several advantages, was the description by Snyder, Eisner and Steenbock ('51) of the determination of vitamin D with radiophosphorus. This method, which measures the amount of radioactive phosphorus incorporated into bone as a function

of the amount of vitamin D administered, appeared to have the elements of an assay suitable for routine use. This paper describes our experience with the radiophosphorus procedure over a period of 18 months in which commercial multivitamin preparations containing vitamin D were tested. In addition, comparisons of the radiophosphorus and the U.S.P. "line test" methods were carried out simultaneously in several of these assays.

EXPERIMENTAL

Preparation and handling of the rats

Twenty-one-day-old rats, of the Sprague-Dawley Strain, 45 to 60 gm in weight, were used in these studies. This strain gave dose-responses which were considerably steeper and less variable than those obtained with another commercial strain. The question of strain variation should be examined if animals of other strains are used. On the day of receipt of the animals, the rats were randomized (Bliss, '52) and fed the following modified U.S.P. No. 2 vitamin D-deficient diet: Yellow corn, 76%; wheat gluten, 20%; calcium carbonate, 3%; sodium chloride, 1%; lysine monohydrochloride, 0.5%; and the following vitamins in milligrams per kilo of diet: Thiamine hydrochloride, 2; riboflavin, 4; niacin, 10; and calcium pantothenate, 4. Supplementing the diet with lysine and the vitamins listed reduced the time required for development of rickets by approximately 30%. The animals were fed this diet ad libitum during the 16 day depletion period. To insure ourselves that rickets developed in this period of time, 4 rats were examined by the line test technique (U. S. Pharmacopeia, Vol. XIV).

After rachitogenesis had been established, the rats were given orally, with a syringe fitted with a blunt edge No. 18 needle, the various vitamin D doses in 0.2 ml of corn oil. Forty-eight hours later each rat received, by intraperitoneal injection, 0.5 ml of physiological saline containing 20 to 25 μc of radioactive phosphorus. This amount of radioactivity is sufficient to give about 3000 counts/minute over the wrist of the rat at the time the experiment is terminated. During the

test period the rats were fed 10 gm of the diet daily for a total of 9 calendar days after the administration of vitamin D.

Determination of radioactivity

Equipment used in the determination of the radioactivity in the wrist of the rats is shown in figure 1. The Geiger-Muller counting tube (window thickness about 2 mg/cm²) is held in place on an ordinary ring stand with burette clamps.

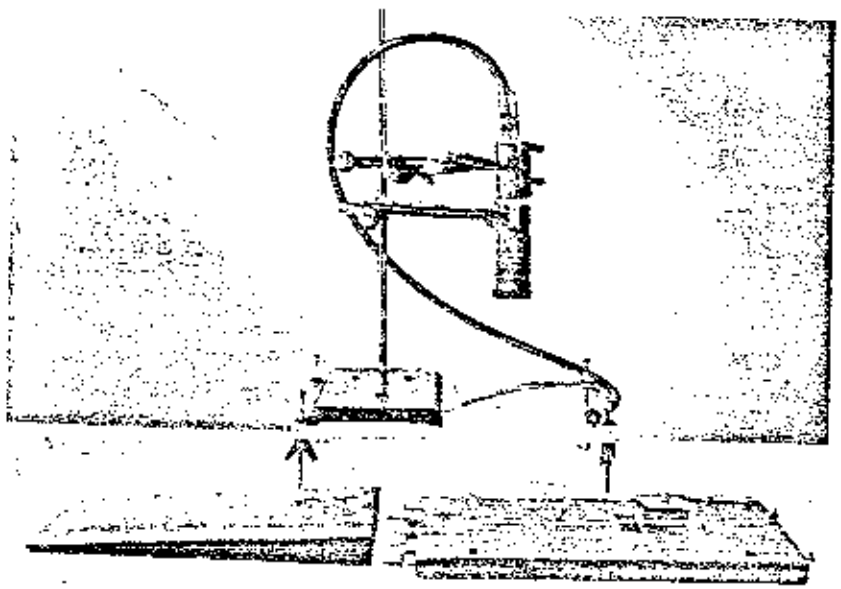

Fig. 1 Apparatus for measurements of radioactivity in the paw of rats.

The ring stand itself is fastened with screws to a piece of lucite 8 × 13 × 9/16 inches. A hole one-half inch in diameter is drilled through the plastic and the Geiger-Muller tube is centered over it. The hole acts as a collimator and eliminates all radiation other than that in the rat's wrist directly under the hole. A lucite thickness of 9/16 inch is sufficient to absorb completely the 1.7 Mev beta particle emitted by P^{32}.

The animal holder consists of a block of wood with a recess one-half inch wide and one-eighth inch deep mounted on a flat piece of board as shown in figure 1. In operation, the

animal, under anesthesia, is placed in position such that the right front paw rests in the recess on the raised block of wood. The paw is then held in position with a piece of cellophane tape; clean new tape is used for each animal. The position of the bone under the tape is then outlined with a red crayon. Care should be taken so that the cellophane tape covers all of the paw which will appear under the collimating hole. The animal is then placed under the tube stand in such a position that the red crayon mark appears in the center of the hole. The animal holder is held firmly against the collimating hole by pushing the wedge-shaped piece of wood under the animal holder far enough to raise two legs of the tube stand off the table. Under these conditions the distance between the Geiger-Muller tube and the wrist of the animal is kept constant. The total number of counts for two minutes is recorded.

U.S.P. line test

The "line test" was made directly on the bones of rats which had received P^{32} and had been assayed by the radioactive method. Separate groups of rats were used in assaying the samples by the "line test" only. At the higher dosages of vitamin D (20 to 48 units) the degree of healing was so great that the readings were indefinite. As experience was gained it was noted that the readings could be extended to a dosage range of three to 14 units.

RESULTS

Fourteen experiments have so far been performed. The results have been uniform in showing a definite log-dose response relationship between the amount of vitamin D injected and the C/M (counts per minute) measured. This relationship is substantially linear and similar from assay to assay as is illustrated by the data in figure 2. The standard deviation of individual readings about the average at any dose is consistently proportional to the average overall count for the assay.

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When the bones of the same animals are read by the line test method, curves similar to the radiophosphorus curves are obtained. These results are shown in figure 2. The short dotted lines show the results of a standard U.S.P. line test assay run simultaneously with the radiophosphorus assay.

There is a relationship between the C/M reading obtained in the radiophosphorus assay and line test reading on the

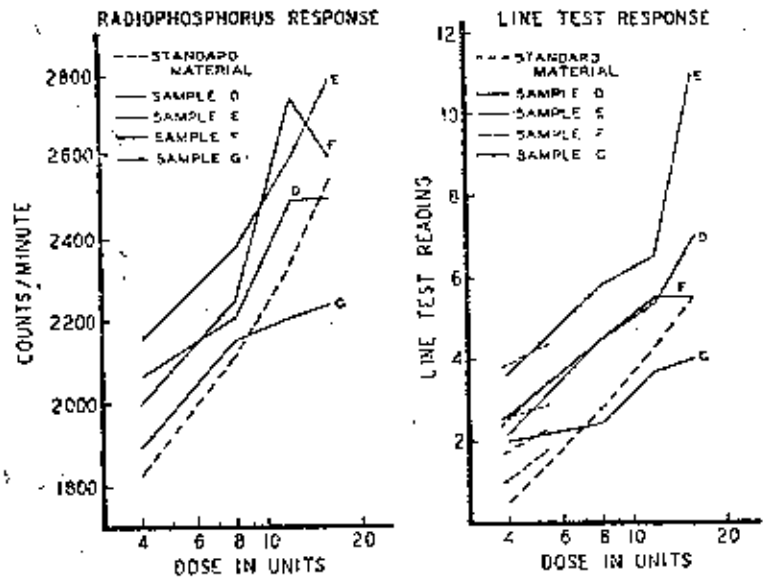

Fig. 2 Comparison of radiophosphorus and line test results. The curves on the right are line test readings on the radiophosphorus-treated animals shown at the left. The short dotted lines in the right figure are line test readings from a concurrent U.S.P. assay.

same animal. This is evident from the scatter diagram at the left in figure 3. The portion of figure 3 on the right shows the relationship between the average reading at each dose level as obtained by the two different methods. These lines connect points referring to the same sample.

Although Snyder et al. ('51) have reported a usable range of activity of 0.5 to 50 units in the radiophosphorus assay, we have limited our use to the range 4 to 16 units. Below two units and above 25 or 30 units less reliable results and a

flatter response curve were obtained. The reasons for this difference between our results and those of Snyder et al. are not apparent, but the differences themselves are of little consequence when compared to the usual rather restricted range of three to 8 units for the line test (fig. 2).

Tests were carried out to study the variability introduced into the results by different operators and by the placement

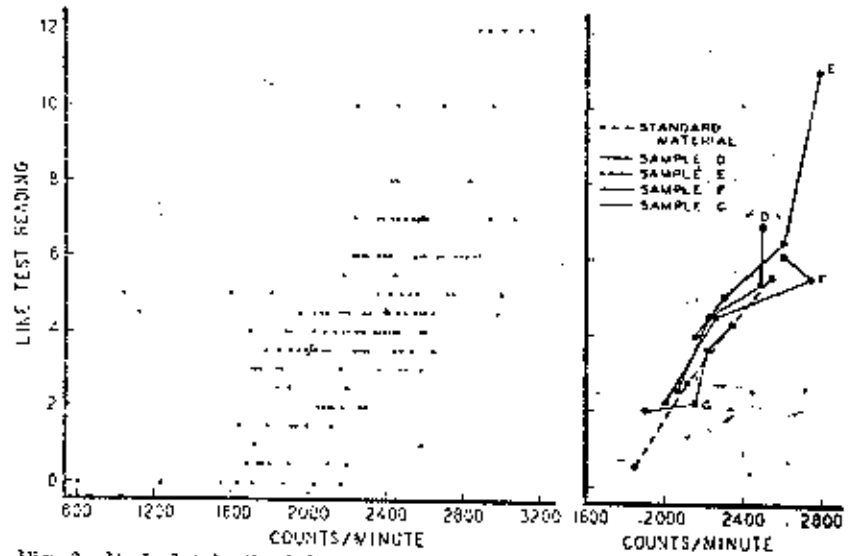

Fig. 3 Each dot in the left chart represents the c/m reading and the corresponding line test reading on an individual rat. In the chart on the right, each point represents an average c/m reading versus the average line test reading for a given dose. The lines connect, for each unknown tested, the points for successive doses of 4, 8, 12 and 16 units.

of the animal. The variability introduced by either of these factors is negligible compared to the inherent animal variability.

When the data of an assay are analyzed to obtain potencies of the unknowns tested, results are obtained similar to those in table 1. In order to obtain the maximum information about the response curve we have used a minimum of 4 dose levels so that a few more rats have been used than is usual in the standard line test assay. This resulted in a lower standard

error of the potency as obtained by the radiophosphorus method than that by the line test method. Preliminary investigations indicate that changing to a three-dose-level assay with the two-fold dose intervals will reduce the number of rats below that commonly used for the line test assays without increasing the standard error significantly above that of the line test assay. Indications are that further economies

TABLE 1

Results of simultaneous line test and radiophosphorus vitamin D assays

ASSAY METHOD	ASSUMED POTENCY	CALCULATED POTENCY	95% CONFIDENCE LIMITS	σ[1] POTENCY EXPRESSED AS % OF POTENCY	σ[1] SLOPE EXPRESSED AS % OF SLOPE	λ[2]
		Sample D				
Radio phosphorus	330 units	427	330–554	14	12	4.6
U.S.P. "line test"	per capsule	496	321–764	24	40	5.0
		Sample E				
Radio phosphorus	267 units	479	344–665	18	14	4.0
U.S.P. "line test"	per capsule	890	214–3773	105	54	3.4
		Sample F				
Radio phosphorus	267 units	394	286–537	17	14	3.8
U.S.P. "line test"	per capsule	443	205–956	47	62	3.1
		Sample G				
Radio phosphorus	384 units	312	226–428	17	15	3.7
U.S.P. "line test"	per gram	289	191–436	24	53	4.2

[1] σ = Standard deviation.
[2] λ = Slope/standard deviation.

in the number of rats injected at each dose level may be possible without a serious raising of the standard error. Higher values of λ are sometimes obtained with the line test method but these are based on values of slopes with much higher standard deviations than the slopes obtained by the radiophosphorus method. Thus the λ value for the line test method cannot itself be considered very reliable.

Agreement in final potencies, as obtained by the two methods, is good with the exception of Material E (table 1) where

the difference between the assumed potency and the actual potency is obviously very large. When a complete replicate of an assay is carried out results are obtained as shown in table 2.

TABLE 2

Comparison of potencies obtained in two different assays

ASSAY METHOD	ASSUMED POTENCY	FIRST ASSAY: Calculated potency	FIRST ASSAY: σ[1] Potency expressed as % of Potency	SECOND ASSAY: Calculated potency	SECOND ASSAY: σ[1] Potency expressed as % of Potency
		Sample H			
Radio phosphorus	1600 units per ml	1680	13	2487	24
"Line test" on radiophosphorus rats		1804	15	2023	16
U.S.P. "line test"		2800	18	2250	31
		Sample I			
Radio phosphorus	1000 units per capsule	639	13	777	22
"Line test" on radiophosphorus rats		668	19	668	19
U.S.P. "line test"		880	10	649	24
		Sample J			
Radio phosphorus	200 units per gram	126	21	155	29
"Line test" on radiophosphorus rats		181	21	204	16
U.S.P. "line test"		204	10	213	13

[1] σ = Standard deviation.

SUMMARY

The validity of the radiophosphorus procedure for vitamin D assay reported by Snyder, Eisner and Steenbock has been confirmed. The method has been compared against the line test in concurrent assays and has given potencies agreeing with those obtained with the U.S.P. standard line test. The radiophosphorus assay has been shown to possess several advantages over the U.S.P. "line test." The prime advantage

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is that the subjective nature of the line test assay is completely eliminated without any loss in accuracy. Furthermore, complications inherent in the line test, such as calcification evidenced as a diffuse accretion contiguous to the diaphysis, decreased food consumption and insufficient weight gain, do not appear to affect the results when the radiophosphorus method is used.

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Female Steroid Hormones and Target Cell Nuclei

The effects of steroid hormones on target cell nuclei are of major importance in the induction of new cell functions.

Bert W. O'Malley and Anthony R. Means

Studies designed to elucidate the sequence of events responsible for steroid hormone effects in endocrine target cells have led many investigators to consider the nucleus as the primary site of hormone action. Numerous experiments have supported the suggestion that steroid hormones regulate cell function by influencing the synthesis of proteins in the target tissue (*1–5*). In most instances, the stimulation of such protein synthesis by steroid hormone is preceded by quantitative and often qualitative changes in the synthesis cellular RNA. Stimulation of nuclear, rapidly labeled heterogeneous RNA followed by increased production of ribosomal RNA and often transfer RNA are frequently observed effects on RNA metabolism (*6–7*). It is likely, but has not been proved, that the messenger RNA (mRNA) of animal cells is a component of the giant heterogeneous nuclear RNA. Additional support for a primary effect of steroids on nuclear gene transcription is provided by the ability of actinomycin D and other inhibitors of RNA synthesis to block most steroid hormone–mediated cell responses. General theories in which stimulation of mRNA is regarded as the primary event should not be overestimated, however, since some evidence suggests other possibilities (*8*). On the other hand, recent experiments have conclusively demonstrated that steroid hormones are capable of inducting a net increase in specific mRNA molecules in target cells (*9–12*).

If steroids do in fact regulate nuclear gene transcription, certain considerations should be compatible with such a theory. There must be a mechanism for limiting the steroid-induced response to target tissues. There should be a defined sequence of events which results in the transport of a steroid molecule to its presumed nuclear site of action following penetration of the target cell membrane. The existence of mediators or "second messengers" must be delineated. The steroid hormone itself or an intracellular mediator should be capable of interacting at certain predetermined sites in the nucleus prior to alterations in DNA transcription. Changes in nuclear RNA synthesis should finally result in a net increase in the amounts of specific mRNA's, which should, in turn, be limited to steroid hormone target tissue and inducible by only the steroid in question. Increases in the intracellular concentrations of these mRNA molecules should precede fluctuations in the rate of synthesis of the corresponding specific proteins. Over the past decade many experimental data relating to these theoretical considerations have accumulated. In this article we summarize the evidence favoring our prejudice that the target cell nucleus is a major determinant in steroid hormone induction of new cell functions. Our discussions are limited primarily to the activity of estrogen in the rat uterus and chick oviduct and the activity of progesterone in the chick oviduct because most experimental data on mechanisms of female sex steroid action emanate from these model systems. However, the generality of these observations as applied to mechanisms of action of all steroid hormones has been recently reviewed (*13–15*).

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Steroid Hormone Receptors

The concept of steroid hormone "receptors" initially resulted from studies by Jensen and Jacobson (*16*) in which physiologic amounts of radioactive estradiol were injected into immature rats. It was noted that target tissue alone was capable of retaining 17β-[^{3}H]estradiol against a marked concentration gradient with blood. These observations were confirmed by both biochemical and autoradiographic methods (*17*), and were eventually extended to numerous vertebrate species (*14*). Upon homogenization of uterine target tissue, a soluble protein capable of binding [^{3}H]estradiol was shown to exist in the cytoplasm (obtained in the supernatant fraction after centrifugation at 105,000*g*) by Toft and Gorski (*18*). This binding protein was considered to be a "receptor" because of its limitation to estrogen target tissue, its high binding affinity for estrogens (dissociation constant, $K_d \sim 10^{-10}$), and its specific attraction for only biologically active estrogens, either naturally occurring or synthetic. The estrogen binding molecule is heat labile, nondialyzable, and precipitable with ammonium sulfate. When homogenates of uterus were incubated with various enzymes, the molecular binding was destroyed by proteolytic enzymes but not by ribonuclease or deoxyribonuclease. This result suggested that at least the active molecular binding site might be protein in nature.

Upon ultracentrifugation in a sucrose gradient, this cytoplasmic hormone-protein complex sedimented as a discrete band at about 8*S* (*18, 19*). Addition of 0.3*M* potassium chloride to these gradients resulted in the reversible transformation of this 8*S* complex to a more slowly sedimenting 4*S* form (*20*). Thus the cytoplasmic receptor appeared to contain a 4*S* estradiol binding unit which under conditions of low ionic strength underwent association with other binding entities to form an 8*S* complex. Recent experiments have indicated that in the presence of ionic conditions in the presumed physiological range, an intermediate 6*S* form may predominate (*21*). In any event, it appears that the sedimentation behavior can vary in relation to concentration and ionic conditions and, although probably artifactual, it is a useful and reliable method for identifying steroid binding proteins (*22*). At present there is no way to determine the exact size or configuration of a receptor in its natural state in the unbroken cell.

The generality of the estrogen receptor has been demonstrated by a variety of studies in which vagina, mammary gland, pituitary, and hypothalamus have been used, such tissues being obtained from the rat, mouse, human being, and other species (*14*).

The chick oviduct, a specific target tissue for progesterone, exemplifies a similar type of interaction with progestational steroids. When a chick treated with estrogen was subsequently injected with [^{3}H]progesterone, the major fraction of labeled steroid was detected in the cytoplasm and nucleus of the oviduct (*23*). Within this tissue, the cytoplasmic radioactivity appeared to be present in a macromolecular complex. The cytosol progesterone binding macromolecule had a sedimentation coefficient of 3.8*S* during sucrose gradient centrifugation in the presence of 0.3*M* KCl and aggregated to 5*S* and 8*S* when the ion concentration was low (no KCl) (*24*). The oviduct binding component showed a striking affinity for progesterone ($K_d \sim 8 \times 10^{-10}$ at 4°C) and appeared to comprise only 0.02 percent of the cytosol protein. The cytosol progesterone binding macromolecule was unequivocally distinguished from plasma transcortin by agarose gel chromatography, discontinuous polyacrylamide electrophoresis, isoelectric gradient chromatography, and protamine sulfate precipitation.

Indirect physical-chemical calculations suggested that the molecule was a protein which existed in the shape of a prolate ellipsoid with a monomeric molecular weight of approximately 90,000. The calculated axial ratio showed that the protein was a macromolecule with a length 14 to 18 times greater than its width (*24*). This explained an apparent discrepancy of molecular weight upon analysis by different techniques.

The binding protein in the cytosol was specific for oviduct and showed very little affinity for estrogens (estradiol, estrone), mineralocorticoids (aldosterone), glucocorticoids (cortisol), or progesterone precursors and inactive metabolites. The tissue concentration of progesterone binding protein was increased tenfold by prior estrogen treatment (*25*). This estrogen-mediated increase in progesterone binding protein correlated quite closely with the estrogen-induced quantitative enhancement of the oviduct-progesterone response (avidin synthesis). For these reasons, it was thought that the combined evidence was compatible with the concept that this progesterone binding molecule was in fact a physiologic receptor for the hormone and that the formation of this steroid hormone-receptor complex is an obligatory initial step in steroid hormone action.

Progesterone binding components have been partially characterized in the uterine cytosol fraction of the guinea pig (*26*), rabbit (*27–31*), rat (*27, 29–33*), mouse (*33*), and human being (*28*). Studies performed in several species have provided evidence for an estrogen-responsive, progesterone binding system in the uterus (*34, 35*) and vagina (*35, 36*). In the guinea pig, fluctuations in the progesterone binding capacity of the uterine cytosol fraction have been measured during the estrous cycle (*37*), but the factors responsible for the variation in the concentration of uterine progesterone binding sites during the cycle have not been established.

In a recent study, Leavitt *et al.* (*38*) found an estrogen-dependent 7*S* progesterone binding component in the hamster uterus. The occurrence of cyclic variations in the cellular concentrations of progesterone binding sites was indicated by increases in the concentration of progesterone receptor during diestrus (day 3) to a maximum at proestrus on day 4 coincident with an increase in the concentration of estradiol in the serum (*38*). The number of progesterone binding sites decreased slowly after ovariectomy at proestrus and the level was rapidly restored by exogenous administration of estrogen, confirming that the increase in the number of uterine progesterone binding sites during the cycle depends on the presence of estrogen.

Transfer of the Hormone-Receptor Complex to the Nucleus

After exposure of uterine tissue to ^{3}H-labeled 17β-estradiol, two intracellular sites of hormone binding were noted: one in the cytoplasm and another in the nucleus (*39*). Subsequently, nuclear binding appeared to predominate. The pioneering studies of Jensen and his associates (*40*) and of Gorski *et al.* (*1*) have led to the concept that an estrogen-induced conformational change occurs in the uterine cytoplasmic receptor protein and that

this is followed by the translocation of the hormone-receptor complex to the nucleus. This translocation process was an attractive hypothesis since it placed the hormone-receptor complex in a compartmental location adjacent to the site of hormone-induced changes in gene expression.

That a temperature-dependent (37°C) intracellular transfer of protein-bound estradiol from the cytosol to the nucleus actually occurs was initially demonstrated in the rat uterus (*40*). Incubation of 17*β*-[^{3}H]estradiol with uterine tissue in vitro led to an accumulation of a salt-extractable (0.3*M* KCl) form of the estrogen-receptor complex from a preparation of nuclei. This nuclear hormone-receptor complex sedimented at 5*S* during sucrose gradient centrifugation and was undetectable in nuclei of target tissue not previously exposed to estrogen. While nuclear 5*S* receptor appeared during exposure to hormone, an apparent concomitant depletion in the total quantity of cytoplasmic 8*S* receptor occurred. Cell-free exposure of preparations of nuclei to [^{3}H]estradiol and cytoplasmic receptor led to accumulation of extractable 5*S* hormone-receptor complex from the nuclei, but no extractable 5*S* complex was noted when [^{3}H]estradiol alone was incubated with uterine nuclei. It was these observations that led to the "two-step" hypothesis of Jensen *et al.* (*40*) according to which the 5*S*-estradiol receptor complex extracted from nucleus is thought to represent an altered form of the cytoplasmic receptor. Recent evidence suggests that upon interacting with estrogen, the binding protein undergoes a physical change permitting it to relocate to a nuclear position (*41*). A new equilibrium is then achieved in which up to 90 percent of the estrogen bound to receptor is in the nuclear position. Because of this equilibrium, any estimation of the correlation between tissue response and estrogen binding must allow for both nuclear and cytoplasmic estrogen-receptor complexes being equally correlated with responses after equilibrium is reached.

Most of the conclusions we have discussed are based on experiments with uterine tissue in vitro or on experiments conducted under cell-free conditions. An important recent methodologic advance has permitted a better understanding of estrogen-receptor translocation to the nuclear compartment in living tissue and has thus enabled investigators to correlate the translocation process with the biologic responses of uterine tissue to various estrogenic compounds (*42*). The quantitative assay for determination of the number of nuclear estrogen-receptor sites relies on the exchange of [^{3}H]estradiol with unlabeled estradiol bound to target cell nuclei in living tissue under various physiological states. Clark *et al.* (*43*) have been able to demonstrate that the translocation of estrogen receptor to the nucleus occurs under the influence of endogenous estrogen during the estrous cycle. The number of estrogen-receptor sites that are formed is a dose-dependent phenomenon that shows a striking positive correlation with early uterine physiological responses to estrogen administration (*44*).

Because progesterone was thought to act in the nucleus to influence gene transcription, it became pertinent to establish whether this hormone was also bound to a macromolecular receptor in the nucleus of the target cell. After stimulation with the hormone, purification of oviduct nuclei and extraction with salt revealed that such a nuclear receptor was present and that it was almost identical to the receptor found in the cytoplasm of the same cells (*23*). In experiments similar to those performed with estrogen in rat uterus, it was demonstrated that no appreciable quantities of nuclear receptor were found in purified oviduct nuclei from unstimulated animals. However, upon exposure to progesterone, an increase in extractable nuclear receptor protein occurred simultaneously with a diminution in the amount of cytoplasmic receptor (*23*). It appeared again that this steroid hormone was also capable of initiating a translocation process which resulted in an accumulation of receptor-bound intracellular [^{3}H]progesterone in the target cell nucleus.

A subsequent series of experiments conducted under cell-free conditions proved to be of considerable interest. As with estrogen and uterus, direct incubation of [^{3}H]progesterone with purified oviduct nuclei led to little steroid being bound, and progesterone binding was shown to be dependent on the simultaneous presence of its receptor in the reaction medium. However, it was further shown that the progesterone-receptor complex could only bind well to nuclei of oviduct target tissue and that nuclei of nontarget tissue such as lung, spleen, heart, and intestine demonstrated little capacity to "accept" and retain the hormone-receptor complex (*23*).

This observation led to the "nuclear acceptor hypothesis," according to which target cell nuclei should contain acceptor sites with a specific affinity for the receptor molecules. Moreover, similar experiments performed with rat prostate after administration of androgens confirmed that this hypothesis was generally valid for sex steroids and their target cell nuclei (*45*).

It seemed that for the progesterone receptor to bind to the oviduct nuclei, it first had to be complexed with a progestational steroid and then allowed to undergo a time- and temperature-dependent transformation, presumably to a structural form capable of interacting with the nuclear acceptor sites. The intranuclear location of these receptor sites was confirmed when, upon fractionation of nuclei exposed to ^{3}H-labeled hormone receptor, the radioactive complex could be found attached to the chromatin (*23, 46, 47*).

Hormone-Receptor Binding to DNA

Because it has been reported that deoxyribonuclease releases bound estradiol from uterine nuclei, DNA is implicated in the nuclear binding of the hormone-receptor complex (*48*). The binding of uterine estrogen receptors to DNA has also been demonstrated to occur in vitro (*49, 50*). The binding is of sufficient strength to withstand centrifugation of the DNA through sucrose gradients to which up to 0.1*M* KCl has been added. These observations have been confirmed by means of DNA-cellulose chromatography (*51, 52*). Both cytosol and nuclear receptor forms of the complex were shown to bind to DNA, and partial purification of the receptor by ammonium sulfate precipitation appeared to enhance binding to DNA (*51*). This reaction has some of the properties of nuclear binding of estradiol in vivo and in vitro in that the binding to DNA is disrupted by 0.3*M* KCl and there exists only a limited number of high-affinity binding sites on DNA for the receptor. In fact DNA-cellulose chromatography has been used to effect receptor purification (*51, 52*). In a recent report it was calculated that if a 1 : 1 interaction occurs between estradiol and receptor, the

available DNA binding sites are saturated at a level of two receptors for every 10^7 nucleotides (DNA), or 500 receptor molecules per quantity of DNA found in a single nucleus (*50*). Although DNA has many of the characteristics one would expect of the nuclear acceptor, a lack of binding specificity is evident. In fact, estrogen receptor from rat uteri can interact with DNA from calf thymus, salmon sperm, *Escherichia coli*, and *Bacillus subtilis*. It appears then that the acceptor site is more complex and that other components of chromatin may act to modify receptor binding to DNA.

Purified chick DNA has the capacity to bind and retain the [^{3}H]progesterone-receptor complex (*53*). At high concentrations of DNA, essentially all of a limited quantity of hormone receptor could be bound to DNA. This binding affinity did not appear to be uniquely sequence-specific as DNA from a heterologous eukaryotic species (calf) showed a similar capacity to bind the chick progesterone-receptor complex (*12*).

After elution of either crude or purified progesterone receptor from a diethylaminoethyl cellulose (DEAE) column, the molecule can be resolved into two apparent subfractions or subunits termed A and B (*54*). Both subunits bind specifically with biologically active progestins for which they have a high affinity and low capacity. It is of considerable interest that only subunit A became bound to purified DNA (*53*). The B unit had no such capacity.

Although it is possible that this DNA interaction was simply a nonspecific absorption phenomenon, it was subsequently noted that binding to DNA was a high-affinity reaction ($K_d = 3 \times 10^{-10} M$) and that there were a limited number of binding sites (one receptor bound for every 10^9 nucleotide pairs of DNA). Thus, the receptor (A subunit) has a high affinity for the binding sites of oviduct DNA and the DNA has a limited number of sites.

In studies of the specificity of binding in relation to the nucleotide sequences of DNA, there appeared to be a limited degree of species specificity. Although receptor DNA interactions are of great potential interest because of the known effects of steroid hormones on gene expression, no good evidence exists to support the notion that a steroid hormone-receptor complex can bind to specific polydeoxynucleotide sequences.

Hormone-Receptor Interaction with Chromatin Nonhistone Proteins

When [^{3}H]estradiol is incubated directly with preparations of target tissue chromatin in vitro, very little of it becomes bound to the chromatin. However, incubation of the preformed [^{3}H]estradiol-receptor complex from uterus with uterine chromatin results in significant retention of the complex on chromatin (*55*). Removal of histone (basic) proteins prior to incubation exposes even more receptor binding sites (*50*). Similar results for androgen-receptor interactions with male target (for example, prostate) and nontarget tissues have been reported by researchers at three laboratories (*55, 56*).

Studies of the recombination of steroids, or steroid-receptor complexes, and chromatin in cell-free systems reveal that very little [^{3}H]progesterone becomes bound to target cell (oviduct) chromatin in the absence of receptor protein (*47, 55, 57*). Substantial amounts of [^{3}H]progesterone were recovered bound to oviduct chromatin when the [^{3}H]progesterone was first combined with the oviduct cytosol receptor. Unmetabolized [^{3}H]progesterone-receptor complex could be reextracted intact by treatment of chromatin with 0.3*M* KCl after incubation in vitro. Binding was specific for progesterone receptor alone because the binding of steroid could not be enhanced by extracellular binding proteins such as transcortin nor even by other cytosol preparations from nontarget tissues. Furthermore, these results demonstrated that progesterone receptor complexes bind to oviduct chromatin to a much greater degree than to nontarget chromatin such as that from spleen, heart, lung, or hen erythrocytes (*46*).

In attempts to determine the fraction of chromatin responsible for the binding of the hormone-receptor complex to chromosomes, histone proteins were selectively dissociated from chromatin and the chromatin was subsequently reconstituted by sequential dialysis. In this manner, "hybrid" chromatins were also prepared in which histones from other tissues or species were substituted during reconstitution. Binding of receptor to this reconstituted chromatin was similar to binding to the intact native chromatin of oviduct. Moreover, the capacity to bind the steroid-receptor complex was completely retained by hybrid chromatins containing histones from a nontarget tissue of a different species, for example, calf thymus. Finally, because oviduct chromatin from which all histones had been removed still displayed a more extensive binding than spleen chromatin containing no histones, it was concluded that histones themselves were not primarily responsible for the specificity of receptor binding (*46, 47*).

Preliminary experiments in which the dissociated chromatin was treated with ribonuclease prior to reconstitution suggested that chromosomal RNA was also not of major importance for the extensive binding. However, if during reconstitution the nonhistone (acidic) proteins of chromatin were removed, the chromatin lost most of its capacity to bind the progesterone-receptor complex (*46*).

In more detailed investigations of the importance of nonhistone proteins in regulating receptor binding, nonhistones and histones were dissociated from the chromatin of oviduct and erythrocytes. These proteins were then separated from the DNA, but it was possible to reconstitute most of the nonhistones back to the DNA by gradient dialysis. In certain instances the nonhistones and histones of the chromatin of one tissue were reconstituted with the DNA of another tissue to form hybrid chromatins. An immunochemical method employing specific antiserums against chromatin nonhistone proteins of various tissues was used to monitor the nonhistone fraction during reconstitution experiments to substantiate formation of hybrid chromatins (*58*).

Oviduct cytosol, containing the [^{3}H]-progesterone-receptor complex, was then incubated separately with the intact, reconstituted, or hybrid chromatins. Reconstituted oviduct chromatin binds progesterone-receptor complex in a manner quantitatively similar to that of intact native oviduct chromatin. However, when the nonhistone protein of erythrocyte is inserted onto the oviduct DNA during reconstitution, this hybrid loses its enhanced ability to bind receptor and resembles native erythrocyte chromatin. Conversely, insertion of nonhistone protein from oviduct into erythrocyte chromatin bestows binding capacity to this hybrid chromatin resembling that of native oviduct (*47, 58*). These experiments demonstrate that the enhanced acceptor ca-

pacity of target tissue chromatin for the hormone receptor of that tissue can be transferred to a nontarget DNA through transfer of the nonhistone (acidic) protein fraction. Additional experiments have localized this "acceptor capacity" to a certain subfraction of the total nonhistone proteins of the target cell chromatin (*58*).

As with the binding to DNA already discussed, only one of the putative progesterone receptor units, subunit B, has this capacity to interact with the nonhistone proteins of oviduct tissue (*53*). Thus, only if one considers the combined characteristics of both the A subunit (affinity for DNA) and the B subunit (affinity for nonhistone protein) of the progesterone receptor, can the full potential of crude intact receptor be accounted for. It appears then that both DNA and a nonhistone protein fraction of target cell chromatin play a positive role in forming the acceptor sites for uterine receptor in uterine chromatin and progesterone receptor in oviduct chromatin.

In summary, it can be speculated that after entry of the steroid-receptor complex into the nuclear compartment, the initial molecular interaction of the steroid-receptor complex with chromatin may occur in two parts, a high-affinity reaction occurring between the receptor subunit A and chromatin DNA and another high-affinity reaction occurring between a specifier subunit B of the intact native receptor and the nonhistone acceptor proteins (*12*). If this hormone-receptor complex is actually the inducer unit for steroid hormone modulation of nuclear RNA transcription, then this initial binding to the genome may prove to be of major importance to steroid hormone action.

Steroid Hormone Effects on the Cell Cycle

A necessary prerequisite of the study of hormone effects on mitosis in a target tissue is a knowledge of the amount and chronology of mitotic activity and cell cycle parameters in the absence of hormonal influence. In the immature oviduct, mitoses are infrequent (*59*). The mean mitotic index (MI) of the surface epithelium was 0.43 in the 7-day-old chicks (*60*). This reflects the slow natural growth of the oviduct that continues until sexual maturation. A single injection of estrogen markedly stimulated mitosis in the oviduct. There was a rapid rise in MI between 9 and 12 hours. The MI reached a peak of 2.3 at 18 hours after treatment and the amount of mitotic activity began to fall at 24 hours and continued to drop until 42 hours. The frequency of cells in mitosis 48 hours after injection with estrogen was more than twice that observed in the unstimulated oviduct.

When chicks are given a second injection of estrogen 24 hours after the first, a second rise in MI is observed. The patterns of changes in MI following single and double treatments could be explained in terms of a hormone-induced stimulation of division in a single population of cells. The cells appeared to progress through the cycle in a parasynchronous fashion. Estrogen treatment resulted in a similar stimulation of the rate of DNA synthesis in oviduct, leading to a dramatic increase in total DNA consistent with the hormonally induced cell proliferation (*60*).

Within a variety of tissues it has been shown that after an appropriate stimulus, nonproliferating cells can be stimulated to divide. In general, proliferative stimuli act to stimulate cells in the G1 (prereplicative) phase of the cell cycle to enter S phase (synthesis of DNA), G2 phase and then mitosis, or G2 cells to undergo mitosis. To ascertain the exact stage at which cells are responsive to hormone in the immature oviduct, chicks were given simultaneous injections of hormone and either fluorodeoxyuridine (FdU) or hydroxyurea (HU). If the estrogen acted to stimulate G1 cells to enter S phase, the inhibitors of DNA synthesis should have blocked the hormone-induced stimulation of mitosis. If these hormones stimulated a G2 population to undergo mitosis, the inhibitors should not block the early hormone-induced rise in MI. Both FdU and HU inhibited the stimulation of mitosis that normally occurred after treatment with estrogen; thus the stimuli causing proliferation acted prior to the completion of DNA synthesis, that is, in G1 or S. In the unstimulated oviduct, G2 = 1.75 hours and S = 7.3 hours, but a rise in MI was not observed until 12 hours after administration of the hormone. It appears, therefore, that estrogen stimulates G1 cells to enter S phase. These results are similar to those showing that estrogen stimulates cell proliferation in the uterine and vaginal epithelia of the mouse (*61*).

Progesterone also stimulated a small fraction of cells to undergo mitosis. A small, but significant rise in MI was observed 12 and 18 hours after treatment. The frequency of cells in mitosis dropped below the control level at 24 hours and remained low until 48 hours, with or without a second injection of progesterone (*60*). These data indicate that although progesterone can act as a stimulus for proliferation of a small population of cells in the surface epithelium of the immature oviduct, this steroid appears to exert its predominant inhibiting action by blocking the normal progress of proliferating cells through the cell cycle. The basis and relationship between these two actions of progesterone are not clear.

Effects of Steroids on Chromatin Composition and Conformation

In chick oviduct, differentiation of epithelial cells and synthesis of specific proteins such as ovalbumin and lysozyme occur in response to estrogen administration (*2*). Previous data have suggested that there is transcriptional control of both the differentiation process and of cell-specific protein synthesis. Based upon the hypothesis that changes in gene transcription during differentiation reflect, in part, changes in the tissue-specific pattern of gene restriction, changes occurring in the chemical composition and physical properties of oviduct chromatin were investigated during estrogen-mediated tissue differentiation.

Quantitative analysis of the chromatin from various stages of oviduct development demonstrated that while the amounts of histone varied randomly, the amounts of nonhistones increased during the first few days of differentiation and decreased gradually until completion of development (15 days of estrogen treatment) (*62*). The amounts of chromatin associated with RNA followed a similar pattern. Moreover, the capacity of the intact chromatins to serve as templates for RNA synthesis in vitro with bacterial polymerase also increased during the first few days of differentiation and then decreased during the final stages of development.

To clarify the relationship and the role of the nonhistones in the tissue-specific restriction of DNA, the antigenic properties of nonhistone protein-DNA complexes isolated from different organs were determined and then compared with those of the corresponding native (intact) chromatins. Antibodies were produced in rabbits against a preparation of oviduct nonhistone protein complexed to DNA (nucleoacidic protein) which was prepared from oviduct chromatin of chicks that had been injected with an estrogen for 15 days (*63*). The results of these studies were as follows: (i) The nucleoacidic proteins isolated from chromatin complexed to DNA were good immunogens which initiated the formation of complement-fixing antibody. (ii) The antibodies reacted strongly with the preparations from the homologous organ (chick oviduct), whereas the affinity for preparations of nucleoacidic proteins from heterologous sources (liver, heart, spleen) was very low, this indicating that the structure or composition of the antigenic sites on the nonhistone proteins in chromatin is organ specific. (iii) During the development of chick oviduct, the antigenic sites for acidic proteins underwent marked alterations which probably involved changes in the species of nonhistone proteins in addition to possible structural alterations of already existing proteins.

Structural analyses of oviduct chromosomal protein-DNA complexes were made by means of circular dichroism (CD) performed under standardized conditions. During estrogen-stimulated development of the oviduct, the chromatin DNA displayed a gradual increase in the magnitude of ellipticity measured at 275 nanometers. Other studies suggest that this increase in ellipticity may represent an "opening" of the DNA, that is, removal of proteins from areas of the DNA (*64*). Thus the CD analysis of oviduct chromatin supports the concept that an alteration in the composition or steric conformation, or both, of target cell chromatin occurs during steroid hormone-induced differentiation. These studies, together with the data showing that major quantitative and qualitative changes occur in the nucleoacidic proteins of chromatin, provide additional evidence that differentiation represents progressive alterations in chromatin biochemistry which may result in changes in cell structure and function (*62*).

Steroid Hormone Effects on RNA Synthesis

Since it is now clear that the steroid hormone-receptor complex enters the nucleus and binds to target cell chromatin, it seems logical to consider that the synthesis of RNA plays a major role in the primary mechanism of action. It has not yet been possible to demonstrate directly that the chromatin binding results in an increased rate of nuclear transcription. However, much evidence exists to support the hypothesis that steroid hormones do act upon the nucleus of target cells.

The first demonstration that estrogen stimulates the incorporation of precursor into total cell RNA came from the laboratory of Mueller and his associates (*65*). Initially, the responses to estrogen were measured in terms of hours. These studies and those from the laboratories of Gorski and Hamilton led to an argument about whether the stimulation of RNA synthesis was a cause or consequence of estrogen-mediated translation (*66, 67*). Examining the reaction more and more promptly after hormone administration, investigators finally demonstrated that within 2 minutes of a single injection of estrogen, a 40 percent increase occurred in the synthesis of rapidly labeled nuclear RNA (*68*). The estrogen stimulation of this activity was biphasic: there was an initial peak at 20 to 30 minutes, then the activity decreased abruptly and remained low for 2 hours after which time it increased once again and remained high for at least 24 hours (*69, 70*). Moreover, the first phase of this response was prevented by actinomycin D but was not blocked by cycloheximide (*71*). These data strongly suggest that the initial response of the nuclear apparatus is not dependent upon the continued synthesis of protein.

The nature of the rapidly labeled nuclear RNA formed during the first few minutes of estrogen action is still uncertain. Initial studies of the base composition of the RNA suggested that it was ribosomal-like (*69*). However, more recent studies on the sedimentation characteristics of this RNA and kinetics of stimulation of nucleoplasmic RNA polymerase II yield evidence of a more DNA-like nature (*72–74*). Moreover, stimulation of synthesis of this rapidly labeled nuclear RNA seems to be mandatory for the appearance in the cytoplasm of a group of specific estrogen-induced proteins (*75*). Within a few hours after injection, estrogen has resulted in stimulation of both ribosomal precursor RNA, 28*S*, 18*S*, and 5*S* ribosomal RNA and 4*S* transfer RNA (*4, 13, 72, 76*). Thus the synthesis of all classes of uterine RNA are eventually enhanced by estrogen.

Another approach to the investigation of changes in RNA synthesis is to utilize chromatin template activity. By this procedure one can estimate the percentage of the total genome which is available for transcription. Chromatin is used as the sole source of DNA but an excess of exogenous RNA polymerase is provided (usually from *E. coli*). Teng and Hamilton (*77*) described a 25 percent stimulation of template capacity of rat uterine chromatin within 30 minutes after the injection of estrogen to adult ovariectomized animals. Similar observations were made by Church and McCarthy (*78*) using chromatin isolated from endometrium of castrated rabbits. In these experiments, however, effects were observed as early as 10 minutes after estrogen administration. Enhancement of chromatin template activity in the rat uterus has also been observed by Barker and Warren (*79*). More recently, Glasser *et al.* (*74*) examined template activity in the rat uterus. Care was taken to monitor the chemical composition of chromatin during the isolation procedure, and in these experiments, the effects of estrogen were first demonstrable at 1 hour and the effects of estrogen on chromatin activity was paralleled by the stimulation of RNA polymerase I.

Chromatin template activity has also been shown to be stimulated by estrogen in chick oviduct (*7, 57*). By the technique of nearest-neighbor base analysis it has been shown that the qualitative changes in template activity occur in concert with quantitative differences in the nature of RNA products (*7*). Comparison of the base frequencies of RNA synthesized by chromatin from control and estrogen-treated chicks revealed considerable differences. In general, the RNA tended to become more AU-rich (adenine and uracil) during hormone treatment. Here again the data suggest that estrogen must promote the synthesis of new species of RNA during differentiation of the oviduct.

All classes of RNA are also eventually increased in the oviduct in re-

sponse to estrogen. Changes occur in the pattern of ribosomal precursor RNA as judged by polyacrylamide gel electrophoresis (*80*). Ribosomal 28*S* and 18*S* RNA species are increased (*81*) and there are marked effects on the synthesis of 4*S* and 5*S* RNA's (*7*). These increased rates of synthesis of ribosome-associated RNA species are accompanied for the first 7 days after estrogen treatment by a continuous accumulation of cytoplasmic ribosomes (*81, 82*).

Limited use has been made of the technique of DNA-RNA hybridization for studying effects of the female sex steroids on the uterine transcriptional apparatus. Church and McCarthy (*78*) employed this method to examine the effect of estrogen on the appearance of new populations of nuclear RNA sequences and obtained evidence that estrogen very rapidly induces synthesis of different populations of RNA molecules. The technique is of limited use for hormone sensitive tissues, however, because under the conditions normally used, only changes in repetitive sequences of DNA can be analyzed. Thus the portion of the genome containing unique sequences responsible for synthesis of most mRNA molecules is not assayed under the conditions used by Church and McCarthy (*78*). Recent methodological advances have allowed the genome to be effectively subdivided into unique and repeating sequences of DNA (*83*). Although no one has attempted to apply these new techniques to the investigation of the effects of estrogen on the mammalian uterus, in this laboratory we have utilized hydroxylapatite chromatography to subdivide the chick oviduct genome into unique and repetitive DNA sequences (*84*). We found no detectable differences in the renaturation profiles of oviduct DNA at various times of estrogen stimulation. The data suggest that estrogen is not acting through major gene duplication or deletion and offer evidence that the new proteins required for oviduct growth may arise from differential gene transcription.

It has also been found that DNA with unique sequences is transcribed in the oviduct with 25 to 30 percent of the resulting RNA being processed into the cytoplasm. Moreover, estrogen apparently causes an increase in the extent of transcription of unique gene sequences. Thus, [illegible] though the amount of RNA transcribed from unique sequence DNA [illegible] processed into the cytoplasm does not appear to vary as a result of estrogen treatment, qualitative differences seem to exist in the cytoplasmic mRNA populations at different stages of estrogen-induced differentiation.

Steroid Hormone Effects on RNA Polymerase Activity

Gorski was the first to report an effect of estrogen on RNA polymerase activity assayed in a crude nuclear pellet obtained from uteri of immature rats (*85*). Within 1 hour after a single injection of estradiol there was an increase in the activity of magnesium-dependent RNA polymerase. These observations were subsequently confirmed with a highly purified preparation of nuclei which had been stripped of the outer membrane by treatment with detergent (*70, 86*). A second polymerase activity was also shown to be stimulated by estrogen but required 12 hours of hormone treatment (*70, 86*). This enzyme activity required a high concentration of ions and showed a preference of Mn^{2+}. It was subsequently demonstrated that the Mg^{2+}-dependent enzyme (polymerase I) was restricted to the nucleolus and synthesized ribosomal RNA. On the other hand, the high salt enzyme activity (polymerase II) was located in the nucleoplasm and synthesized a product with a DNA-like base composition (*87*). Thus it appeared that estrogen stimulated synthesis of ribosomal RNA before any apparent effect on DNA-like RNA formation.

In further investigations of the effect of estrogen on uterine polymerase I activity, Barry and Gorski (*88*) obtained results suggesting that estrogen evokes an increase in the rate of chain elongation within 1 hour but does not affect the number of growing chains. Such experiments imply that estrogen stimulates the activity but not the number of polymerase molecules, and that its effect at this stage of the reaction is only minimally due to transcription of additional template.

Estrogen has also been shown to increase the activity of RNA polymerase in the chick oviduct during hormone-mediated differentiation (*2, 89*). In this tissue, there seems to occur a concomitant increase in polymerase I and II that is first demonstrable at about 6 hours. By 48 hours there is an eight- to tenfold increase in the polymerase I activity. It seems certain that the effects of estrogen is to enhance the activity of existing polymerase molecules as well as to increase the number of initiation sites on the DNA. This interpretation stems from the fact that during differentiation many additional cell-specific proteins appear that are under transcriptional control. Therefore, although estrogen stimulates RNA polymerase activity in both uterus and oviduct, it may do so by two different mechanisms.

For several years the rapid but transient stimulation of uterine RNA synthesis by estrogen could not be explained because neither chromatin template nor RNA polymerase seemed to be increased at the same time. However, by utilizing stringent kinetic conditions of assay, Glasser *et al.* (*74*) have shown that a rapid but transient increase in polymerase II activity can be demonstrated 15 minutes after estrogen administration. Moreover, the kinetics of this increase occur in concert with the previously documented stimulation of rapidly labeled nuclear RNA synthesis. The initial enhancement of polymerase II activity occurs prior to any detectable increase in the activities of chromatin template or polymerase I. In fact, stimulation of polymerase I first occurs at 1 hour and reaches maximum activity at 4 hours. These data therefore confirm the results of several investigators regarding the time course of activation of the nucleolar RNA polymerase I by estrogen (*85, 86*).

The studies of Glasser *et al.* (*74*) also offer evidence that may help to solve another problem of estrogen action in the uterus—whether some or all of the effects on transcription require continued protein synthesis. The activation of polymerase II is biphasic. When actinomycin D is injected 30 minutes before the estrogen, both of the increases in activity are abolished. However, the injection of cycloheximide before the injection of actinomycin D does not prevent the initial rapid increase in polymerase II. In fact, there is an increase over the effect seen with estrogen alone. Cycloheximide affects the synthesis of rapidly labeled RNA in the same way (*71*). On the other hand, the secondary increase in polymerase II which begins 2 to 3 hours after estrogen injection is completely abolished by cycloheximide. These data could explain why in the earlier studies from Gorski's laboratory (*66*), and in a more recent report from Nicolette and Babler (*90*), it was maintained that stopping

uterine protein synthesis prevented an estrogen-induced enhancement of RNA synthesis. Investigations were usually made 2 to 6 hours after hormone administration, considerably after the rapid but transient first phase of the RNA stimulation curve. Recent results from DeAngelo and Gorski (*75*) also revealed that a very early and specific event in the induction of a specific protein (induced protein) by estrogen is blo[illegible] by actinomycin D but not by pu[illegible]cin or cycloheximide. Thus it se[illegible] [illegible]t an early increase in the activi[illegible] RNA polymerase II could be [illegible]ary for the subsequent biochemi[illegible]ts in the action of estrogen o[illegible] the uterus (*74*).

Raynaud-Jammet and Baulieu (*91*) were the first workers to demonstrate an effect of estrogen on uterine RNA polymerase activity in vitro. The estrogen had to be incubated with uterine cytosol in order for the response to occur, presumably because the reaction is time- and temperature-dependent. Similar observations have been made by Arnaud *et al.* (*92*) and by Mohla *et al.* (*93*). Arnaud *et al.* (*92*) suggested that only the *5S* form of the estrogen-receptor complex would stimulate uterine RNA synthesis in vitro and that this form acted specifically on nucleolar RNA polymerase I. Moreover, these workers (*94*) suggested that phosphorylation of the *5S* receptor resulted in enhanced ability of the complex to stimulate RNA synthesis. Mohla *et al.* (*93*) also reported a 40 to 60 percent stimulation of uterine polymerase activity in vitro and showed that prior incubation of the estrogen with the cytosol is necessary for the "transformation" of the cytosol receptor to the *5S* form. Mohla *et al.* used these results to support their hypothesis that receptor transformation is an important step in estrogen action. Furthermore, they suggested that one of the biochemical functions of estradiol may be to induce conversion of the receptor protein to an active form that can enter the nucleus, bind to acceptor molecules, and induce RNA synthesis.

Recent studies in our laboratory have revealed that estrogen may indeed result in a change in chromatin template which allows an increased number of binding sites for RNA polymerase. These studies have been performed under conditions which will distinguish spurious binding on nicked regions of the DNA from true promoter regions. The role of the hormone receptor in this process, however, has yet to be elucidated.

For studies of the effect of progesterone on RNA polymerase activity, investigators have used primarily the chick oviduct as a model system (*2, 89*). Administration of progesterone to immature chicks not previously treated with estrogen produces little effect for the first 5 hours. Between 5 and 10 hours there is a considerable increase in the activities of both polymerase I and polymerase II. The maximum effect occurs at about 24 hours. Thus the increase in polymerase activity anticipates the induction of avidin synthesis.

The effect of progesterone on polymerase activity in oviducts from chicks previously treated with estrogen for 14 days is considerably different. A transient but significant decrease in activity occurs 2 hours after a single injection of progesterone (*2*); this is followed by an increase which peaks at 24 hours, and again this increased polymerase activity precedes the induction of avidin synthesis. This same type of response curve is seen for both rapidly labeled nuclear RNA and the polymerase which is presumably responsible for its synthesis (*2*). The reason for the initial decrease in activity cannot be the withdrawal of estrogen. It is probable that progesterone acts in two ways: (i) to induce mRNA specifically to synthesize avidin and (ii) to antagonize estrogen. In any event the decrease of estrogen-mediated events by progesterone is widespread among the biochemical events examined to date.

Effects of Steroids on Production and Translation of Messenger RNA

One of the most elusive problems in studies of the action of steroid hormones on the nucleus is that of obtaining direct evidence that the generation of mRNA is a rate-limiting step. Measurements of transcriptional changes have indicated that estrogen does, in fact, play a role at this level of cell regulation. Most of the major changes induced in estrogen-sensitive target tissues by administration of the hormone are blocked by prior treatment of the tissues with actinomycin D (*2, 13, 75*). Moreover, as already discussed, estrogen stimulates the synthesis of rapidly labeled nuclear RNA, and RNA polymerase activity is altered as is the template capacity of nuclear chromatin. Analysis of the RNA products by hybridization, and nearest-neighbor base analysis, also reveal marked changes in response to estrogen (*2, 77, 84, 95*). None of these studies, however, constitute direct proof of alterations in the transcription of specific structural genes.

The only definitive way to prove the existence of the ovalbumin mRNA was to demonstrate that it would support the unambiguous translation of ovalbumin in a cell-free protein synthesis system. It would then be necessary to show the absolute dependence on estrogen administration. We chose to use a modified rabbit reticulocyte lysate as the protein synthesis system. Since ovalbumin has a molecular weight of 45,000, an *8S* to *17S* RNA fraction was isolated from hen oviduct polysomes and was subsequently shown to contain ovalbumin mRNA activity (*10, 11*). Proof that the reaction product was authentic ovalbumin has been gained by several procedures: (i) interaction with a specific antiserum to purified ovalbumin; (ii) solubilization of the immunoprecipitate and analysis on sodium dodecyl sulfate gels; (iii) ion-exchange chromatography on carboxymethyl cellulose followed by reprecipitation with antiovalbumin; and (iv) the construction of peptide maps (*10, 96*).

The ovalbumin mRNA activity was specific for RNA isolated from oviduct of estrogen-stimulated chicks and was primarily found in the *8S* to *17S* fraction of polysomal RNA. The amount of synthesis was increased by addition of protein synthesis initiation factors. Moreover, inhibitors of chain initiation, such as edeine or aurin tricarboxylic acid, or of general protein synthesis, such as puromycin or cycloheximide, completely block ovalbumin synthesis directed by the oviduct mRNA fraction. Ribonuclease destroys the messenger activity whereas deoxyribonuclease does not. Steroid hormone-receptor complexes had no effect on the translation of ovalbumin mRNA.

This system for translating the ovalbumin mRNA with fidelity allowed us to look at the hormonal regulation of this specific messenger. Oviduct from laying hens, in which ovalbumin is being synthesized at its maximal rate, contains the greatest amount of ovalbumin mRNA activity. On the other hand, there is no detectable mRNA in ovalbumin from the unstimulated immature oviduct of the 7-day-old chick. Stimulation of these animals with estrogen for 4, 10, or 16 days leads to

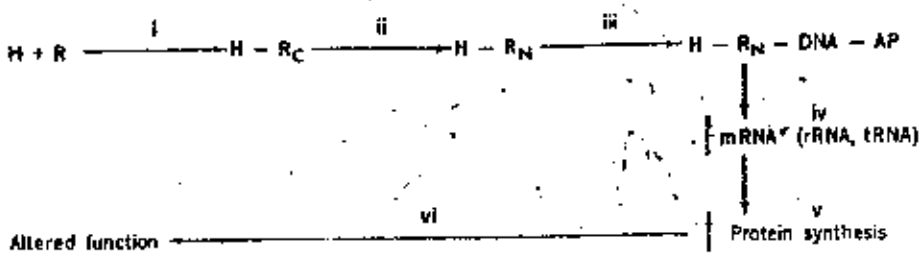

Fig. 1. Biochemical steps in steroid hormone action: (i) hormone (*H*) entering the target cell binds to a specific cytoplasmic receptor (*R*) and (ii) forms a hormone-receptor complex (H-R_c) which is transported to the nucleus (H-R_N) where (iii) it binds to specific acceptor sites on the genome [chromatin DNA and nonhistones (or acidic proteins, *AP*)]. This is followed by (iv) activation of the transcriptional apparatus that results in the appearance of new RNA species; (v) transport of the hormone-induced RNA to the cytoplasm; and (vi) the steroid-mediated functional response that is characteristic of the target tissue. Among the components of such a system which may bring about functional changes in the target cell are enzymes, structural or regulatory proteins, and nuclear events that are subject to amplification.

increasing activity of the extractable messenger. However, when chicks treated with estrogen for 16 days are subsequently withdrawn from hormone for 16 days, the ovalbumin mRNA activity again becomes very low. Finally the administration of estrogen to these animals for 1, 2, or 4 days after the 16-day withdrawal period leads once more to a progressive increase in ovalbumin messenger. These data reveal that indeed the amounts of extractable ovalbumin mRNA from oviduct are directly dependent upon estrogen stimulation. Moreover, the changes in ovalbumin mRNA in response to estrogen paralleled or slightly anticipated the changes in oviductal accumulation of ovalbumin (*97*).

To better assess the changes in mRNA after estrogen treatment, kinetic analyses of mRNA activity were made and the rate of ovalbumin synthesis was calculated (*9*). At various times after injection of estrogen to chicks subjected to a 16-day withdrawal period, the rate of ovalbumin synthesis was assayed in vitro with minces of oviduct. The rate of synthesis of this specific protein was time-dependent and peaked at 18 hours after the injection of steroid. The approximate half-life of the messenger was calculated from the descending limb of the induction curve to be 8 to 10 hours. Similar studies were then performed except that ovalbumin mRNA was extracted and the activity was quantified in the translation system. There was a remarkable parallelism between the rate of ovalbumin synthesis and the amount of available mRNA. Again, mRNA could be detected prior to ovalbumin synthesis, and the half-life of the mRNA was 8 to 10 hours.

Thus it appears that estrogen acts in the nucleus to promote the synthesis of mRNA's which code for the cell-specific proteins. Since ovalbumin represents nearly 60 percent of the protein synthesized in oviduct gland cells under the influence of estrogen, it was questioned whether the ovalbumin mRNA was transcribed from single copy DNA or whether this might represent an instance of gene amplification. The ovalbumin mRNA was purified by a variety of procedures until its activity, tested in the protein synthesis system, had been increased some 100-fold compared to a preparation of total oviduct RNA. The partially purified mRNA was incubated with RNA-directed DNA polymerase from avian myeloblastosis virus in order to produce a copy of radioactively labeled DNA which would be the complement of ovalbumin mRNA. The ^{3}H-labeled DNA produced was sized on alkaline sucrose gradients and shown to contain fragments up to 2000 nucleotides in length. When the ^{3}H-labeled DNA was reacted with excess ovalbumin mRNA, 90 percent of the labeled DNA formed a stable hybrid with the mRNA indicating that the ^{3}H-labeled DNA was indeed a complementary copy. A fraction of the ^{3}H-labeled DNA was then used in a DNA-excess hybridization experiment (*98*). Whole DNA was sheared to 400 nucleotide lengths and incubated with the ^{3}H-labeled DNA at an excess of 10^7 : 1. Complementary ^{3}H-labeled DNA hybridized to chick DNA with a $C_0t_{1/2}$ (measures of reassociation; concentration (mole) × seconds per liter) of 480. Under similar conditions of second-order kinetics, single copy or unique-sequence DNA hybridizes with a $C_0t_{1/2}$ of 420 (*84*). Thus the complementary ^{3}H-labeled DNA has hybrization properties which suggest that the ovalbumin gene is only represented one time in the oviduct genome (*99*). These data suggest, then, that estrogen may act at the level of transcription to stimulate production of numerous copies of a single gene. This type of hormonal regulation would lead to a high intracellular concentration of ovalbumin mRNA and subsequently of ovalbumin itself.

In fact, recent studies have been completed in our laboratory in which this ^{3}H-labeled complementary DNA copy of the ovalbumin mRNA has been used to calculate the exact number of mRNA copies in each oviduct cell prior to and during hormonal stimulation. After estrogen treatment, up to 15,000 molecules of ovalbumin mRNA may accumulate in a single oviduct cell. Withdrawal of the hormone results in a decreased concentration of ovalbumin mRNA to ~ 10 molecules per cell. This molecular probe (^{3}H-labeled complementary DNA) will remain as a powerful tool for monitoring transcription of specific genes.

Available data strongly suggested that estrogen acts in the nucleus to promote the synthesis of mRNA's which are necessary for the subsequent actions of this steroid on growth and differentiation. An important question that remained unanswered, however, was the general applicability of this mechanism to the actions of other hormones. In the chick oviduct, progesterone has been shown to control specifically the synthesis of the egg-white protein avidin (*2, 100*). Unlike estrogen, no marked changes occur in total cell RNA synthesis, and polysome profiles are seemingly unaltered (*2, 101*). Avidin represents no more than 0.1 percent of the total egg-white protein. Consequently, it followed that the mRNA for this protein might also be present in small amounts. Extraction of total RNA from estrogen-stimulated hen oviducts proved to be less than satisfactory as a means of quantitation. When such RNA preparations were tested in the heterologous protein synthesis system, it was not always possible to demonstrate avidin synthesis by a specific immunoprecipitation procedure. In order to assure reproducible results, it was necessary to effect a partial purification of the messenger fraction. We were able to take advantage of the fact that many mRNA's including the one for avidin contain, at the 3′ terminal end, an extensive sequence of poly-

adenylate residues. The presence of a poly(A) sequence was shown by Brawerman *et al.* (*102*) to allow the mRNA to be selectively adsorbed to nitrocellulose filters. Application of this procedure to oviduct RNA results in a one-step 50-fold purification of avidin (and ovalbumin) mRNA (*103*). This simple procedure allowed us to measure routinely and consistently the avidin mRNA activity which appears in oviduct in response to progesterone (*104*).

Avidin mRNA is only present in the 8*S* to 17*S* fraction of oviduct polysomal RNA. Moreover, it has been shown by sucrose gradient analysis to have an average sedimentation of 9*S*. This would be expected if the message were to code for a protein of approximately 15,000 daltons. This is, in fact, the molecular weight of a single subunit of avidin. Avidin mRNA activity is also abolished by ribonuclease and no avidin is synthesized when inhibitors of peptide chain initiation or elongation are present in the cell-free system. Again, direct addition of progesterone, estrogen, or adenosine 3′,5′-monophosphate has no effect on the translation process.

Avidin mRNA activity is highest in oviducts of mature laying hens where progesterone stimulation is maximal. No activity can be demonstrated in the unstimulated immature chicks or in oviducts from animals which have received multiple injections of estrogen. However, after a single injection of progesterone, avidin mRNA activity can be detected within 6 hours (*9*). Maximum concentrations are achieved by 18 hours, which is considerably before the maximum amount of avidin appears in the tissue. These data suggest that both estrogen and progesterone act on the nucleus of target cells to promote the synthesis of mRNA's. This response seems to be a rate-limiting step in the subsequent production of specific proteins.

Summary

The data discussed herein demonstrate the great variation in target-tissue response that can occur after administration of steroid hormones. The female sex steroids can exert regulatory effects on the synthesis, activity, and possibly even the degradation of tissue enzymes and structural proteins. Each response, nevertheless, appears to be dependent on the synthesis of nuclear RNA. In many instances, the steroid actually promotes a qualitative change in the base composition and sequence of the RNA synthesized by the target cell, implying a specific effect on gene transcription. Most important is our direct quantitative evidence that sex steroids cause a net increase in the intracellular amounts of specific mRNA molecules in target tissues.

It thus appears that we are discovering a pattern of steroid hormone action which includes (Fig. 1): (i) uptake of the hormone by the target cell and binding to a specific cytoplasmic receptor protein; (ii) transport of the steroid-receptor complex to the nucleus; (iii) binding of this "active" complex to specific "acceptor" sites on the genome (chromatin DNA and acidic protein); (iv) activation of the transcriptional apparatus resulting in the appearance of new RNA species which includes specific mRNA's; (v) transport of the hormone-induced RNA to the cytoplasm resulting in synthesis of new proteins on cytoplasmic ribosomes; and (vi) the occurrence of the specific steroid-mediated "functional response" characteristic of that particular target tissue.

To elucidate fully the mechanism of steroid hormone action we must study the biochemistry of the process by which information held by the steroid hormone-receptor complex is transferred to the nuclear transcription apparatus. If our assumptions are correct, we should ultimately be able to discover how this hormone-receptor complex exerts a specific regulatory effect on nuclear RNA metabolism. Such regulation might be achieved (i) by direct effects on chromatin template leading to increased gene transcription and thus RNA synthesis; (ii) by activation of the polymerase complex itself; (iii) by inhibition of RNA breakdown; or (iv) by intranuclear processing of large precursor molecules so that smaller biologically active sequences are produced, and (v) by transport of RNA from the nucleus to the cytoplasmic sites of cellular protein synthesis.

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25-Hydroxycholecalciferol: Direct Effect on Calcium Transport

Abstract. *The perfused small intestine from a vitamin D deficient rat exhibits about one-half the calcium transport of the intestine from a rat given vitamin D. These levels of calcium transport can be maintained for at least 4 hours. Addition of 2.5 micrograms of 25-hydroxycholecalciferol added to the vitamin D deficient intestine via the arterial blood perfusate induces a rise in calcium transport to +D levels within 2 hours. In contrast, 250 micrograms of vitamin D_3 given in the same manner has no effect on the calcium transport level over a 4-hour period. These data provide strong evidence that 25-hydroxycholecalciferol represents the metabolically active form of vitamin D_3.*

Since the identifications of vitamins D_2 and D_3 by Windaus *et al.* (*1, 2*) and Askew *et al.* (*3*), it has been assumed that the unaltered vitamin D itself carries out the vitamin's physiologic functions. In 1966 Lund and DeLuca established the existence of a highly biologically active vitamin D metabolite in rats (*4*). This metabolite has recently been isolated and identified as 25-hydroxycholecalciferol (25-HCC) (*5, 6*) and has been chemically synthesized (*7*). When injected into the jugular vein of a vitamin D deficient rat, 25-HCC is approximately 1.4 times more active than vitamin D in curing rickets (*6*). More importantly, 25-HCC exhibits its in vivo activity in stimulating bone mobilization and intestinal calcium transport more rapidly than an equivalent dose of vitamin D_3 (*8*). This time lag suggests that vitamin D_3 must be converted to 25-HCC before its biological activity is manifested. Studies of early vitamin D metabolism implicated the liver in this conversion (*9*) and recently, by the use of hepatectomized rats, it has been demonstrated that the liver is the major if not the only site of 25-HCC production (*10*). In addition, a liver enzyme system has been obtained which catalyzes the conversion of vitamin D_3 to 25-HCC (*11*).

Direct evidence is now available in

support of the hypothesis that 25-HCC is responsible for vitamin D action. Trummel *et al.* have demonstrated that 25-HCC at a concentration of 0.9 unit/ is effective in stimulating in vitro ase of Ca^{45} from bone tissue cultures, while 400 units of vitamin D_3 per milliliter has little or no effect in this system (*12*). The results in the present report demonstrate that 25-HCC is highly effective in stimulating calcium transport in an isolated perfused intestine, while large amounts of vitamin D_3 have no effect.

Male, 20-day-old albino rats were maintained on a vitamin D deficient diet with normal mineral content (0.47 percent Ca and 0.3 percent P) as described earlier (*13*). After 4 weeks the animals were vitamin D deficient, as shown by reduced growth and low serum calcium. All experimental animals were starved in a tubular restraining case (to minimize coprophagy) for 24 hours prior to use. At 12 hours prior to use, vitamin D treated rats received a single dose of 2000 units of vitamin D_3 in 0.1 ml of cottonseed oil via a blunt needle inserted well into the esophagus. The experimental rats were anesthetized with sodium pentobarbital the small intestine was isolated for fusion as described (*14*). A solution of 5 percent blood serum from vitamin D deficient rats, 4.7 percent dextran of high molecular weight (*15*), and 0.1 percent glucose in Krebs-Ringer bicarbonate buffer was pumped at 120 pulses/min under a pressure of about 100 mm-Hg into the superior mesenteric artery. After passing through the capillary bed of the small intestine this blood perfusate drained from the portal vein at 2 to 3 ml/min. A reservoir of the same solution containing Ca^{45} was pumped (Harvard pump) at 0.08 ml/min through the intestinal lumen from the duodenum to the ileum.

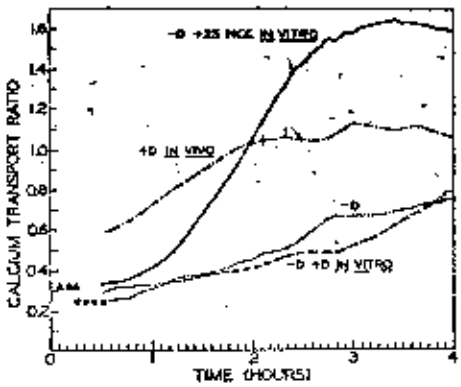

Fig. 1. Time course of calcium transport in perfused rat intestines in response to vitamin D_3 and 25-HCC. Intestines were treated as described in the text. The solid triangles and squares represent infusion of 25-HCC and vitamin D_3, respectively.

It took 10 to 20 minutes for this solution to fill the intestine and begin to drain from the ileal cannula. After this time, parallel 5-minute fractions of the blood and lumen perfusates were collected. The ratio of the amount of Ca^{45} in the blood perfusate to the concentration of Ca^{45} in the lumen effluent was taken as an index of calcium transport. (The use of the word "transport" is not intended to imply that the observed flux of Ca^{45} is necessarily the result of active transport. This experimental design will not differentiate between any of the processes involved in intestinal absorption.)

Intestines from deficient rats were treated with 25-HCC or vitamin D_3 as follows. One-fourth microgram of 25-HCC or 250 μg of vitamin D_3 dissolved in 10 μl of 95 percent ethanol was suspended in 15 ml or 20 ml, respectively, of the blood perfusate. This solution was infused into the arterial perfusate at approximately 1 ml/min.

The time course of calcium transport as measured by the amount of Ca^{45} in the blood perfusate divided by the concentration of Ca^{45} in the lumen (calcium transport ratio) is shown in Fig. 1. Small intestines from vitamin D deficient rats have a low rate of calcium transport as characterized by a calcium transport ratio of about 0.3 at ½ hour, gradually rising to a calcium transport ratio of about 0.7 at 4 hours. The classical effect of vitamin D on calcium transport is shown by intestines from vitamin D deficient rats which have been dosed with 2000 units of vitamin D_3 12 hours prior to the intestinal isolation. The calcium transport ratios of these intestines were about 0.6 at one-half hour and rose to about 1.1 after 4 hours. Thus a high but physiological dose of vitamin D doubled the calcium transport as observed in this system.

Vitamin D deficient intestines which have been exposed to 100 units of 25-HCC during the first 15 minutes of perfusion exhibit a pronounced change in calcium transport over the 4 hours of the perfusion. Initially these intestines have a low calcium transport ratio, as is expected for vitamin D deficient intestines. However, 45 to 50 minutes after the introduction of 2.5 μg of 25-HCC into the arterial blood perfusate, the level of calcium transport begins to rise, and within 2 hours after 25-HCC administration the calcium transport of these intestines is at the level exhibited by intestines from vitamin D treated animals. This elevated calcium transport is maintained for the remainder of the 4-hour perfusion. In direct contrast, intestines from vitamin D deficient rats exposed to 10,000 units of vitamin D_3 during the first 30 minutes of the perfusion showed the same time course of calcium transport as untreated intestines from vitamin D deficient animals. The confidence limits of the results presented in Fig. 1 are given in Table 1. An "overshoot" in calcium transport at 3 hours in 25-HCC treated intestine is frequently noted, although not highly significant statistically (Fig. 1). Several possible reasons for this can be visualized. Since it is unknown if such an overshoot is noted in vivo in response to 25-HCC, additional comment would seem premature.

These results clearly demonstrate that within the time of the perfusion the isolated small intestine responds readily to 25-HCC while massive amounts of vitamin D_3 produce no response. If vitamin D_3 were capable of stimulating calcium transport in an isolated intestine, there is good evidence

Table 1. The influence of vitamin D_3 and 25-HCC on calcium transport in perfused intestine. Intestines were treated as described in the text. The numbers in parentheses represent the number of rats used to determine the mean and the standard error of the mean.

Time (hours)	Calcium transport ratios			
	− D (vitamin D deficient)			+D (not deficient) (N = 5)
	No treatment (N = 5)	+ 10,000 units of vitamin D_3 (N = 5)	+ 100 units of 25-HCC (N = 4)	
½	0.33 ± 0.01	0.31 ± 0.02	0.31 ± 0.02	0.73 ± 0.04*
1½	.38 ± .01	.37 ± .02	.67 ± .05*	.89 ± .02*
2	.45 ± .03	.41 ± .02	1.05 ± .09*	1.03 ± .05*
3	.67 ± .02	.52 ± .03	1.58 ± .12*	1.12 ± .07*

* Different from the −D (no treatment) transport ratio at better than the 99 percent confidence level. † Different from the −D (no treatment) transport ratio at better than the 97.5 percent confidence level.

that this stimulation would have been observed within 4 hours of perfusion. It has been shown that rats which received 20,000 units of vitamin D_3 intrajugularly have increased intestinal calcium transport within 4 hours (*16*), and rats dosed with only 2000 units of D_3 intraperitoneally have increased intestinal transport by 6 hours after the dose (*17*). Furthermore, experiments with ^{3}H-labeled vitamin D_3 show that the intestine receives about 2 percent of an intrajugular dose of vitamin D_3 (*18*). In this light the 10,000 units given to the isolated intestines is equivalent to a considerably larger dose given to the whole animal (approximately 500,000 international units).

This is the first report of an in vitro effect of a vitamin D on intestinal calcium transport. This effect complements the in vitro effect of the metabolite in mobilizing calcium from bone tissue culture (*12*). These in vitro data, together with the results of earlier in vivo experiments showing the effectiveness of 25-HCC in bone mobilization and intestinal calcium transport (*8*), provide an important insight into the basic mechanism of vitamin D action. It seems clear that at the tissue level 25-HCC is the active form of vitamin D_3. To exert its calcium-mobilizing and intestinal-transport effects it is apparent that vitamin D must be converted to this active metabolite. Neither the intestine nor the bone tissue seems capable of catalyzing this reaction, and most likely the liver is the only organ capable of significant conversion of the vitamin D_3 to 25-HCC (*9–11*). However, it must be noted that it is possible that in the target tissues 25-HCC may be converted further to another metabolite before it is active. This possibility requires further study.

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Biological Activity of 1,25-Dihydroxycholecalciferol*

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ABSTRACT: A biologically active vitamin D_3 metabolite (1,25-dihydroxycholecalciferol) was generated in deficient chicks and isolated using Sephadex LH-20 chromatography. The use of silicic acid chromatography for the isolation of 1,25-dihydroxycholecalciferol from chicks dosed with high levels of vitamin D (6.5 μmoles) resulted in a heterogeneous metabolic fraction, as revealed by LH-20 chromatography. The concentration of 1,25-dihydroxycholecalciferol in the intestine was found to be constant at 13 pmoles/chick, regardless of vitamin dosage. Use of a sensitive *in situ* technique revealed that 1,25-dihydroxycholecalciferol (325 pmoles) stimulated intestinal calcium transport more rapidly and to a greater extent than an equivalent dose of 25-hydroxycholecalciferol. In contrast, 1,25-dihydroxycholecalciferol appeared less than half as effective as 25-hydroxycholecalciferol when assayed for antirachitic activity in the rat. This result was not unexpected since 1,25-dihydroxycholecalciferol has an apparently short half-life *in vivo*. Serum calcium concentration of rats on a low-calcium, vitamin D deficient diet was also increased by 1,25-dihydroxycholecalciferol (65 pmoles), but to a lesser degree than a similar dose of 25-hydroxycholecalciferol. Results indicate that the 1,25-dihydroxycholecalciferol is the apparent metabolically active form of the vitamin in catalyzing intestinal calcium transport.

It had been assumed for some 30 years following the elegant work concerning the structural identification of vitamin D_2 (Askew *et al.*, 1931; Windaus *et al.*, 1936) and D_3 (Schenck, 1937) that vitamin D *per se* was the functional form of the vitamin. This question remained unexamined until the synthesis of moderate to high specific activity radioactive vitamin D_3 (Norman and DeLuca, 1963; Neville and DeLuca, 1966). It was first possible to demonstrate with the moderate specific activity material that indeed biologically active metabolites of vitamin D are formed *in vivo* (Norman *et al.*, 1964). However, the concept of a metabolically active form of vitamin D did not make its appearance until Fraser and DeLuca (1969) demonstrated quite conclusively the existence of a metabolite fraction that possessed biological activity equivalent to that of the parent vitamin. Of great importance was the fact that this fraction contained a metabolite or metabolites which acted more rapidly than the parent vitamin (Morii *et al.*, 1967). Blunt *et al.* (1968a) subsequently isolated in pure form the major metabolite of vitamin D from the plasma of pigs and identified its structure as 25-HCC.[1] Not only did this metabolite exceed the biological activity of vitamin D_3, but it acted more rapidly in inducing intestinal calcium transport and bone mineral mobilization (Blunt *et al.*, 1968b). Also important was the finding that 25-HCC acts directly in isolated target tissues of vitamin D whereas vitamin D_3 itself does not (Trummel *et al.*, 1969; Olson and DeLuca, 1969), demonstrating that vitamin D must be hydroxylated in the 25 position before it is effective. The fact that 25-HCC is the major metabolite of vitamin D in the blood (Ponchon and DeLuca, 1969a) provided evidence that this could well represent a hormonal form of the vitamin. The 25-HCC is synthesized by a very tightly controlled enzymatic process in which the liver serves as a type of endocrine organ for the secretion of active vitamin D (Ponchon and DeLuca, 1969b; Ponchon *et al.*, 1969; Horsting and DeLuca, 1969). The synthesis of radioactive 25-HCC (Suda *et al.*, 1971) now allowed the study of whether 25-HCC itself is the active form or whether it must be converted further before it is effective. Accordingly, Cousins *et al.* (1970a,b) demonstrated that in fact there is a very rapid appearance of a polar metabolite in intestine and in other tissues which could well represent the metabolically active form. This metabolite was labeled as peak V because of its behavior on silicic acid columns. The generation of this metabolite from vitamin D_3 was shown by Haussler *et al.* (1968) followed by Lawson *et al.* (1969) and Ponchon and DeLuca (1969a). Myrtle *et al.* (1970) have reported a marked effect of this metabolite on intestinal calcium absorption. More recently Holick *et al.* (1971a,b) and Lawson *et al.* (1971) have identified this polar metabolite as 1,25-DHCC.

Examination of the biological properties of 1,25-DHCC seemed of great importance since it may represent the metabolically active form in the intestine. At present there is uncertainty concerning the biological activity of 1,25-DHCC, due in part to the questions which arose regarding the methods used in estimating the metabolites' effect on intestinal calcium transport. Therefore, a reliable *in situ* method for examining intestinal calcium transport response in the chicken to 1,25-DHCC and other forms of vitamin D was developed. Using this it was possible to show that 1,25-DHCC initiates intestinal calcium absorption much more rapidly than does either 25-HCC or vitamin D_3. However, 1,25-DHCC is less than half as active as 25-HCC in the cure of rickets in rats and is somewhat less effective than 25-HCC in stimulating bone mineral mobilization. These results suggest that 1,25-DHCC may well represent the metabolically active form of the vitamin, at least in the intestine. It is the purpose of this report to present these results.

Materials and Methods

Chicks. White Leghorn cockerel chicks were obtained from

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[1] Abbreviations used are: 25-HCC, 25-hydroxycholecalciferol; 1,25-DHCC, 1,25-dihydroxycholecalciferol; 21,25-DHCC, 21,25-dihydroxycholecalciferol.

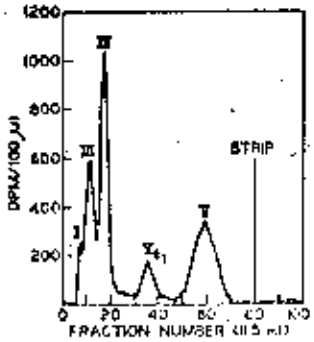

FIGURE 1: Sephadex LH-20 column (65% chloroform in Skellysolve B) chromatographic profile of intestinal extract from chicks dosed (iv) with 6.25 µg (250 IU) of [1,2-³H]vitamin D₃ 24 hr earlier.

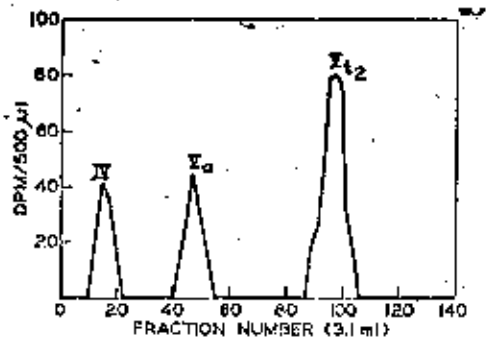

FIGURE 3: Chromatography of silicic acid peak V region on a Sephadex LH-20 (65% chloroform in Skellysolve B) column. The peak V region was obtained from chicks given 2.5 mg (100,000 IU) of [³H]vitamin D₃ each orally 24 hr earlier.

Northern Hatcheries of Beaver Dam, Wis. They were maintained in cages at 38°, shielded from ultraviolet light, and fed *ad libitum* one of two rachitogenic diets. Diet A was a corn soy protein diet which produced rachitic symptoms at approximately 14 days of age (Thornton and Omdahl, 1969). Diet B was a vitamin D deficient purified diet composed of the following in per cent: soy protein, 25.6; sucrose, 56; DL-methionine, 0.6; glycine, 0.4; cellulose, 3; cottonseed oil, 4; $CaHPO_4$, 0.52; KH_2PO_4, 1.55; $CaCO_3$, 2.25; NaCl, 0.8, with the trace salts and vitamins added as previously reported (Imrie *et al.*, 1967b). Chickens used for generation of 1,25-DHCC were fed diet A for three weeks followed by diet B for one week. Bioassay chicks received diet B throughout the course of the experiment (3.5 weeks).

Rats. Male, weanling rats were purchased from the Holtzman Co., Madison, Wis. They were fed *ad libitum* either the rachitogenic diet of Steenbock and Black (1934) with added crystalline water-soluble vitamins as described by Suda *et al.* (1970c) or the low-calcium, vitamin D deficient diet of Blunt *et al.* (1968b). They were housed individually in hanging wire cages. The rachitic rats were used for the antirachitic line-test assay (U. S. Pharmacopoeia, 1955). The rats fed the low-calcium diet were used for the bone mineral mobilization test (Blunt *et al.*, 1968b) of vitamin D activity.

Radioactive Vitamin D₃. [1,2-³H]Vitamin D₃ (0.6 Ci/mmole) was prepared as previously described (Neville and DeLuca, 1966) while [4-¹⁴C]vitamin D₃ (19.2 mCi/mmole) was a gift from the Philips-Duphar Company of the Netherlands. Both preparations were purified using Sephadex LH-20 chromatography (Holick and DeLuca, 1971) and dissolved either in cottonseed oil for oral administration or in 95% ethanol for iv injection (0.05 ml).

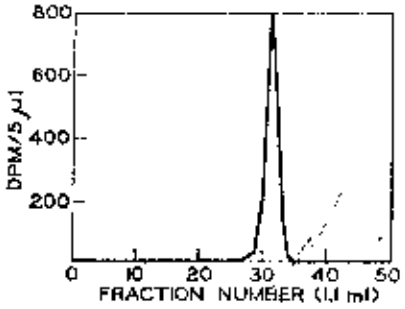

FIGURE 2: Rechromatography of peak V (*i.e.*, 1,25-DHCC) from Figure 1 on a Sephadex LH-20 column in methanol.

Preparation of Extracts Containing Vitamin D Metabolites. A. CHICKS GIVEN 6.25 µg OF (250 IU) VITAMIN D₃. Four-week-old, vitamin D deficient chickens were dosed iv with 6.25 µg of [1,2-³H]vitamin D₃ (240,000 dpm/µg) and killed 24 hr later. The whole small intestine was excised, flushed with ice-cold distilled water, and rapidly frozen on solid CO_2. The combined tissue from 600 chickens was extracted with chloroform and methanol as previously described (Lund and DeLuca, 1966).

B. CHICKS GIVEN 2.5 mg (100,000 IU) OF VITAMIN D₃. The procedure was identical with the 6.25-µg/chick experiment except as noted. Vitamin D deficient chickens (100) were injected iv with 2.5 mg of [1,2-³H]vitamin D₃ (1460 dpm/µg) and killed 24 hr later.

Chromatography of the Intestinal Extracts. The intestinal lipid extract from chicks dosed with 6.25 µg (250 IU) of [³H]vitamin D₃ was dissolved in a minimal volume of 65% chloroform in Skellysolve B (a petroleum fraction, bp 67–69°) and applied to a 60 g Sephadex LH-20 column (2.0 × 60 cm) which contained 2 cm of Celite on top of the gel bed. The column was equilibrated and then eluted with 880 ml of the solvent mixture, resulting in the detection of five radioactive peaks (Figure 1). The column was stripped with 200 ml of 75% chloroform in Skellysolve B. Fractions of 11.5 ml were collected and a 0.1-ml aliquot of each was taken to determine radioactivity. Peaks V and V_a (Figure 1) were then rechromatographed individually on a 20-g (1.1 × 60 cm) Sephadex LH-20 column (65% chloroform in Skellysolve B) followed by a MeOH Sephadex LH-20 column as previously described (Suda *et al.*, 1970a,b) (Figure 2). Cochromatography of peaks V and V_a (Figure 4) was carried out using the 20-g LH-20 column equilibrated with 65% chloroform in Skellysolve B.

The intestinal extract from chicks dosed with 2.5 mg (100,000 IU) of [³H]vitamin D₃ was initially applied to a large multibore silicic acid column as previously described (Suda *et al.*, 1970a,b). The peak V region from this column, which appeared homogeneous on silicic acid was applied to a 20-g Sephadex LH-20 column described above (Holick and DeLuca, 1971). The column was eluted with 300 ml of the solvent (65% chloroform in Skellysolve B). Fractions of 3.1 ml were collected and an aliquot of each was taken for ³H determination. Three radioactive peaks were detected, showing the silicic acid peak V region to be heterogeneous (Figure 3).

Double-Label [³H/¹⁴C] Experiment. Three vitamin D deficient chicks were injected iv (6.25 µg/chick) with a mixture of [1,2-³H]- (2.5 × 10⁶ dpm/µg) and [4-¹⁴C]- (1 × 10⁵ dpm/µg) vitamin D₃ which had a ³H/¹⁴C ratio of approximately 5.3:1. Chicks were killed at 24 hr and the peak V isolated using

65% chloroform in Skellysolve B LH-20 column (20 g, 1.1 × 60 cm) as described above. Radioactivity was estimated and the $^3H/^{14}C$ ratio determined by use of a Packard Model 3375 liquid scintillation spectrometer.

Bioassay of Vitamin D Metabolites. The enhancement of intestinal calcium absorption in vitamin D repleted chickens was the basis of the bioassay. Calcium (*i.e.*, ^{45}Ca) absorption was estimated by using a duodenal loop *in situ* method. Vitamin D deficient chicks weighing between 170 and 190 g were dosed (oral or iv) with 25-HCC, vitamin D_3, or peak V metabolite at various time periods prior to experimentation. Chicks were fasted 15 hr before experimentation and then anesthetized with chloral hydrate (35 mg/100 g body weight, ip). A 10 cm portion of the duodenal loop was ligated and its lumen rinsed with 5 ml of a phosphate-free bicarbonate solution (120 mM NaCl, 4.9 mM KCl, and 9.2 mM $NaHCO_3$; pH adjusted to 7 with CO_2) after which the proximal ligature was tied off. The distal ligature was tightened around a blunted 19 gauge needle through which 0.2 ml of phosphate-free [^{45}Ca]calcium bicarbonate solution (0.5 μCi of ^{45}Ca/ml; 10 mM $CaCl_2$) was injected into the lumen of the duodenal loop. The needle with the distal ligature was secured to the loop replaced in the peritoneal cavity for 30 min at which time the animal was killed. The loop was rapidly excised and subsequently dry-ashed in porcelain crucibles at 600° for 15 hr. The ashed product was dissolved in 2 N HCl, neutralized with 2 N Tris and 0.05-ml aliquots spotted on filter paper disks (2.3-cm diameter). Radioactivity was estimated by placing the paper disk in a 20-ml scintillation counting vial, adding 10 ml of toluene counting solution (Neville and Dietrich, 1966), and counting in a Tri-Carb liquid scintillation spectrometer (Model 3375). Results were expressed as per cent ^{45}Ca absorption and were calculated using the following formula: % $^{45}Ca_{ab}$ = $[1 - {}^{45}Ca_r/{}^{45}Ca_i](100)$, where $^{45}Ca_r$ is the amount of ^{45}Ca remaining in the duodenal loop following the *in situ* incubation and $^{45}Ca_i$ is the amount of ^{45}Ca initially added to the loop preparation.

Antirachitic Activity Assay. Rachitic rats were injected iv with either 260 pmoles (4 IU) of standard vitamin D_3, 260 pmoles of 25-HCC, or 260 pmoles of 1,25-DHCC iv in 0.02 ml of 95% ethanol. Controls received only 0.02 ml of 95% ethanol. Seven days later the rats were killed, their radii and ulnae were removed and stained with 1.5% silver nitrate solution, and the apparent calcification was scored. The antirachitic activity was estimated according to the U. S. Pharmacopoeia (1955).

Bone Mineral Mobilization Activity Assay. The low-calcium, vitamin D deficient rats were injected (iv) with 0.02 ml of ethanol containing 65 pmoles of either 25-HCC or peak V. Controls received 0.02 ml of ethanol carrier. Blood samples were taken at 6, 24, 48, 72, and 96 hr for calcium determination. In another experiment identically carried out, a blood sample was taken 12 hr after either 1,25-DHCC or 25-HCC. No response was observed at 12 hr to either peak V or 25-HCC.

Serum Calcium Determination. Calcium was estimated using atomic absorption spectrophotometry (Perkin-Elmer Model 214). Serum samples (0.05 ml) were dissolved in 2 ml of 0.1% $LaCl_3$ and assayed directly for calcium. Blank and standard solutions were made to approximate diluted serum by the addition of NaCl (3.42 mM) and KCl (0.19 mM).

Results

In order to estimate the amount of 1,25-DHCC it was necessary to use known specific activity radioactive vitamin D_3 in vitamin D deficient chicks for generation of 1,25-DHCC *in vivo*. This assumes that the specific radioactivity of the metabolite is the same as the parent vitamin. The vitamin D_3 in the present experiments was labeled in the 1 and 2 positions which necessitated a preliminary experiment concerning possible ^3H loss in the 1 position (Lawson *et al.*, 1969) during generation of 1,25-DHCC. Injection (iv) of vitamin D deficient chicks with a mixture of [1,2-^3H]- and [4-^{14}C]cholecalciferol ($^3H/^{14}C$ of 5.3:1) resulted in a decreased $^3H/^{14}C$ ratio from 5.3 to 2.6 for the peak V metabolite (Table I), in agreement with Lawson *et al.* (1969). This was taken into account by decreasing the specific activity by a factor of two when calculating the amount of generated peak V metabolite.

TABLE I: Loss of Tritium During the Conversion of [1,2-^3H]-Vitamin D_3 to 1,25-DHCC in Intestine.

Compound[a]	$^3H/^{14}C$
Vitamin D_3	5.4
25-HCC	5.2
1,25-DHCC	2.6
Peak V_a	5.2

[a] A mixture of vitamin D_3 labeled either at positions 1 and 2 with ^3H or position 4 with ^{14}C was given iv to rachitic chicks and 24 hr later the mucosa from the small intestine was removed, extracted, and chromatographed on LH-20.

Generation of peak V metabolite in sufficient quantity to perform biological measurements required large groups of deficient chicks (*i.e.*, 600 chicks/group) due to a metabolite yield of 13 pmoles per chick (or approximately 1 ng/g of tissue).

Sephadex LH-20 column chromatography proved to be a valuable asset affording excellent separation of vitamin D_3 metabolites.[5] Peaks V and V_a could be isolated free of less polar metabolites (Figure 1) following chromatography on the LH-20 column (60 g, 65% chloroform in Skellysolve B). Both metabolites were individually rechromatographed on a smaller LH-20 column (20 g) (65% chloroform in Skellysolve B) and then on a methanol LH-20 column (20 g) (Suda *et al.*, 1970a,b). Each compound migrated as a single peak on the methanol column, exemplified by the profile in Figure 2.

Preliminary studies in our laboratory employing silicic acid chromatography and various vitamin D_3 levels, revealed that high dosages (*e.g.*, 100,000 IU or 6.5 pmoles) could effect an increase in production of peak V metabolite. However, the peak V from silicic acid was heterogeneous and could be resolved into three radioactive peaks by Sephadex LH-20 column chromatography (Figure 3). One of the peaks (V_a) migrated in the peak V region; however, cochromatography indicated it was different from peak V (Figure 4). The peak V metabolite was not detected in chicks dosed with the large amount of vitamin D_3, due to the low specific activity of the preparation; strengthening the concept that 1,25-DHCC is present in the intestine only to a finite amount (about 1 ng/g) regardless of vitamin D dosage.

The *in situ* method for detection of changes in calcium

[5] In our laboratory the identified metabolites of vitamin D_3 and their corresponding peak numbers are as follows: vitamin D_3 ester, peak I; vitamin D_3 or cholecalciferol, peak II; 25-HCC, peak IV; 21,25-DHCC, peak Va; 1,25-DHCC, peak V.

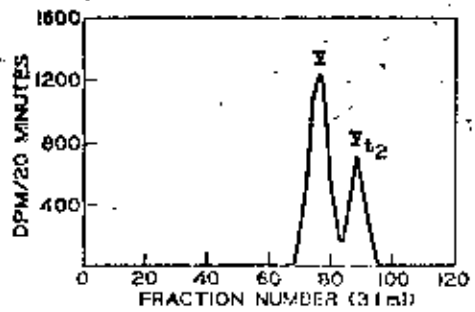

FIGURE 4: Chromatography of peaks V from Figure 1 and V_{t_2} from Figure 3 using Sephadex LH 20 (65% chloroform in Skellysolve B).

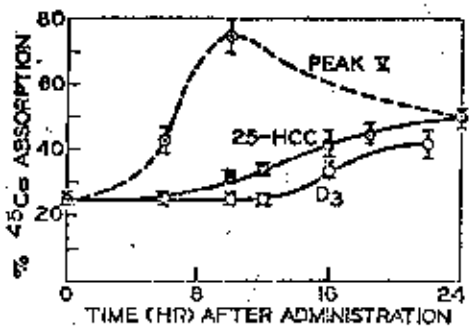

FIGURE 6: Response of chick intestinal calcium absorption to 25-HCC, vitamin D_3, and peak V (1,25-DHCC). Each point represents the average of 4–7 chicks ± standard error. (O——●) 325 pmoles given iv; (O- - - -O) 325 pmoles given iv; (O——○) 325 pmoles administered orally.

absorption proved to be quite sensitive (Figure 5). As little as 195 pmoles of 25-HCC gave a significant increase in calcium absorption. The retrograde movement of calcium (*i.e.*, blood to intestine) ranged between 0.7 and 1.4% of the calcium placed in the intestine, a factor which added negligible error to the calculations. A time-course profile was obtained for vitamin D_3, 25-HCC, and 1,25-DHCC (Figure 6). It was observed that the effect of 25-HCC preceded that of vitamin D_3 by approximately 5 hr, both compounds demonstrating similar calcium absorption profiles. Such results complement earlier studies using rats (DeLuca, 1969). The 1,25-DHCC, however, demonstrated strikingly novel characteristics by superseding 25-HCC in brevity of lag time and extent of response of calcium absorption. The 1,25-DHCC gave a maximal response at approximately 10 hr, which was twice the maximal response of 25-HCC as measured at 24 hr and then declined until equal in activity to 25-HCC at 24 hr (Table II). Two other metabolites (*i.e.*, V_{t_1} and V_{t_2}) isolated by Sephadex LH-20 chromatography failed to stimulate intestinal calcium absorption (Table II).

Since 1,25-DHCC gave a faster and larger response than either 25-HCC or vitamin D_3, it became of interest to see if a similar situation existed for the polar metabolite concerning antirachitic and bone mobilization activity.

Using the rat line test, the 1,25-DHCC assayed only 0.4 as effective as 25-HCC in curing rickets (Table III). Peak V_{t_2}, isolated from high vitamin D dosed chicks, demonstrated no detectable activity in the cure of rickets.

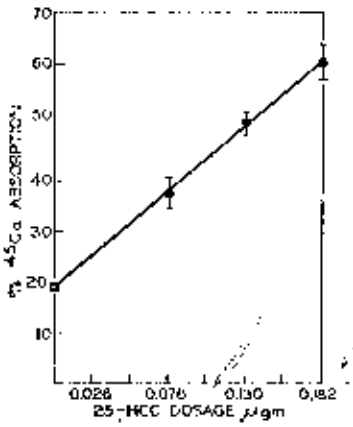

FIGURE 5: Dose-response curve for 25-HCC and calcium absorption in chicks. Chicks were injected iv 24 hr prior to use. Each point represents the average of 6 chicks. The vertical bars represent standard error.

Bone mobilization activity was conducted using small doses of 25-HCC and 1,25-DHCC (*i.e.*, 65 pmoles or ~1 IU). Serial blood samples revealed a significant rise in serum calcium at 24 hr and 72 hr for both the 25-HCC and 1,25-DHCC; however, the 25-HCC response was significantly larger ($p < 0.02$) at both time periods (Figure 7). The serum calcium showed a maximal increase of approximately 3 mg/100 ml of serum for both 25-HCC and 1,25-DHCC when measured at 48 hr, whereas at 96 hr both groups had returned to control levels. These results demonstrate that 1,25-DHCC does possess bone mobilizing activity in contrast to another report (Kodicek *et al.*, 1970).

Discussion

A highly refined multibore silicic acid chromatographic system and a new liquid-liquid Celite partition chromatography system (Suda *et al.*, 1970a) have made it clear that the peak V metabolite fraction from hog and chick plasma is extremely heterogeneous. Two of these metabolites have been recently isolated in pure form and identified as 24,25- and 25,26-dihydroxy derivatives of the parent vitamin (Suda *et al.*,

TABLE II: Stimulation of Intestinal Calcium Absorption by 25-HCC, 1,25-DHCC, and Peak V Metabolites.

Compound	Dosage (pmoles)	Dosage Method	*ta* (hr)[a]	RTAM (*ta*)/25-HCC (t_{24})[b]
25-HCC	195–455	Iv and oral	24	1.00
Peak V_{t_1}	227	Oral	24	0
Peak V_{t_2}	258	Iv	24	0
1,25-DHCC	455	Iv	24	0.72
1,25-DHCC	455	Oral	24	1.09
1,25-DHCC	325	Oral	24	0.97
1,25-DHCC	325	Oral	10	2.00

[a] *ta* represents: time of assay following dosage. [b] (Net % ^{45}Ca absorption for metabolite at time *ta*)/(net % ^{45}Ca absorption for 25-HCC at time 24 hr) where net % ^{45}Ca absorption = % ^{45}Ca absorption for metabolite − % ^{45}Ca absorption for control. [c] RTAM = relative transport activity metabolite.

TABLE III: Antirachitic Activity of 1,25-DHCC in Rats.

Compound	Activity (IU/nm)
Vitamin D_3	15.4 ± 0.1[a]
25-HCC	21.5 ± 0.1
1,25-DHCC	8.1 ± 0.2
Peak V_a[b]	0

[a] Standard error. Each value represents the average of 7 animals. [b] See Figure 4.

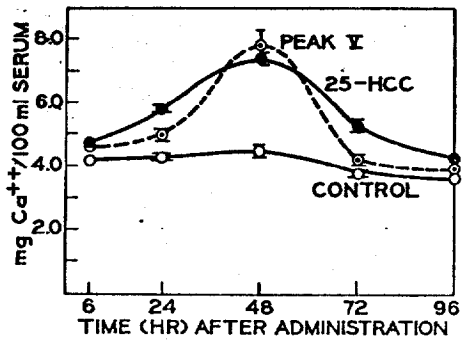

FIGURE 7: Plasma calcium response of vitamin D deficient rats on a low-calcium diet to a 65-pmole dose of 25-HCC or peak V (1,25-DHCC). The vertical bars represent the standard error of the mean from 4 to 6 animals.

1970a,b). Recently a new chromatographic procedure using Sephadex LH-20 in a mixed solvent of chloroform in Skellysolve B also demonstrated the heterogeneity of rat plasma peak V (Holick and DeLuca, 1971). More importantly, it showed that the peak V in the intestine which was generated from 0.25 μg of vitamin D_3 in chicks was homogeneous (*i.e.*, 1,25-DHCC), whereas at a dose of 2.5 mg of vitamin D_3 the peak V region, which appeared to be homogeneous on silicic acid, was resolved into three distinct peaks (Figure 3). One of the peaks (Va) was shown to be 21,25-DHCC by cochromatography in two different chromatographic systems (M. F. Holick, T. Suda, and H. F. DeLuca, 1970, unpublished data).

Lawson *et al.* (1969) initially observed that the peak V[3] metabolite which loses the 1-^{3}H cannot be increased in the intestine by increasing the dose of vitamin D_3. This observation has also been confirmed by Norman and Midgett (1970) and by our laboratory (this report). The discrepancies involving the biological activity of peak V appear to be due in part to the heterogeneity of the metabolite fraction. In particular, at high doses of vitamin D_3 three other metabolites predominate (peaks V_{t_1}, V_{t_2}, V_a). Since silicic acid cannot separate these three metabolites from the desired peak V, the apparent recovery of peak V from silicic acid is much higher than predicted. Ponchon *et al.* (1970) reported that the peak V metabolite isolated from the intestine is only about 50% as active as vitamin D_3 in intestinal Ca^{2+} absorption. Because they dosed the chicks with 250 μg, the recovered "apparent" peak V from the silicic acid column was about 10 times higher than would be expected based on 1 ng/g of tissue. Since V_{t_1} and V_{t_2} have no biological activity for intestinal Ca^{2+} transport, these metabolites diluted the effect of the peak V. Also 21,25-DHCC elicits an intestinal Ca^{2+} transport response that is one-half of that of vitamin D_3. Only when physiological doses of vitamin D_3 are given to the animals is a homogeneous peak V (*i.e.*, 1,25-DHCC) obtained on silicic acid chromatography of intestinal extracts.

Previous studies concerning 1,25-DHCC and stimulation of calcium absorption have used a method which estimates disappearance of ^{45}Ca from the intestine by measuring its appearance in blood (Ponchon *et al.*, 1970; Myrtle *et al.*, 1970; Lawson *et al.*, 1969). Although a linear response is claimed by the authors, several disadvantages are apparent. Such disadvantages include: (1) high standard deviation within treated groups, necessitating a low sensitivity; (2) the reality that several tissues, aside from intestine, influence plasma ^{45}Ca concentration (*e.g.*, a new metabolite could give an increased plasma ^{45}Ca by having its effect on bone or kidney rather than intestine itself). If stimulation of calcium absorption is the desired effect then measurement of calcium movement from the gut *per se* should be desirable. Such a method, using the duodenum *in situ*, was employed with success during this study. The activity of 1,25-DHCC appeared equivalent to 25-HCC in stimulation of calcium absorption when assayed 24 hr following dosage. Previous studies employing the 24-hr time period but using plasma ^{45}Ca for estimation of absorption, have revealed values ranging from 1 to 3.5 that obtained using 25-HCC (Myrtle *et al.*, 1970; Kodicek *et al.*, 1970). In a more recent study (Myrtle and Norman, 1971), a time-course profile for 1,25-DHCC was obtained wherein its activity was equivalent to 25-HCC at 24 hr, consistent with this report. Inconsistent measurements of the biological activity of 1,25-DHCC may well stem from the method used for measuring calcium absorption or the procedure for isolation of the metabolite.

A most revealing observation of 1,25-DHCC, however, concerns the rapid onset and decline of the metabolites' action in the intestine (Figure 6). The maximal response of 1,25-DHCC is elicited at approximately 10 hr, compared to 24 hr or longer for 25-HCC. This time for maximal response is in agreement with other laboratories (Haussler *et al.*, 1971; Myrtle and Norman, 1971).

A shorter response time than that for 25-HCC combined with studies using cycloheximide and actinomycin D (Tanaka and DeLuca, 1971; Gray and DeLuca, 1971) suggest that 1,25-DHCC is the metabolically active form of vitamin D_3, at least in the intestine. Although 1,25-DHCC acts in the intestine, recent results suggest that it is synthesized in the kidney (Fraser and Kodicek, 1970; Gray *et al.*, 1971) and then sequestered by the target tissue.

An attempt to quantitatively compare the total activity of 1,25-DHCC to that of 25-HCC or of vitamin D_3 in the stimulation of calcium absorption seems rather tenuous at this time. However, integration of areas under the time-course profiles could give a reasonable estimate to the biological potency of vitamin D and its metabolites, but has not yet been carried out to the authors' knowledge. Vitamin D repletion of rachitic rats promotes calcification in the epiphysis which can be quantitated. This is the basis of the sensitive rat-line test used for vitamin D and related compounds. This assay is based on the entire effect of the administered substance and thereby measures the total effect of the vitamin or metabolite in in-

[3] The term peak V is used in this communication as regards to a polar metabolite of vitamin D_3 found in intestine after a physiological dose of either vitamin D_3 or 25-HCC. Other laboratories use a different notation for the same metabolite (Haussler *et al.* (1968) used 4b and Lawson *et al.* (1969) used P). This polar metabolite has recently been identified as 1,25-DHCC (Holick *et al.*, 1971a,b).

ducing calcification. Although 1,25-DHCC acts much more rapidly in stimulating intestinal calcium transport and gives a larger maximal response than 25-HCC, it is obviously of short duration. 25-HCC and vitamin D_3, on the other hand, are of long duration. The cumulative effect of the 1,25-DHCC is probably less than either vitamin D_3 or 25-HCC. This possibility is reflected in the line test result which revealed 1,25-DHCC to be only 0.4 as active as 25-HCC.

Kodicek *et al.* (1970) have reported no change in plasma calcium concentration 24 hr following 25-HCC or 1,25-DHCC administration (325 pmoles). In contrast, previously mentioned results (Figure 7) show that 1,25-DHCC is active in bone mineral mobilization, although somewhat less effective than 25-HCC. Experiments presently in progress may well provide the necessary information to allow a deduction as to which is the metabolically active form of vitamin D in bone mineral mobilization.

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Modification of USP Vitamin D Assay to Remove Interference of Vitamin E

By MODEST OSADCA and ELMER DE RITTER

When tocopherol is incorporated in multivitamin products at the 10–30 mg. levels, corresponding to the daily adult requirement, serious interference is encountered in determining vitamin D by the USP method. A modification of the USP assay method is described in which the tocopherol is separated by column chromatography on secondary magnesium phosphate prior to the normal USP chromatographic purification steps. Proper conditioning and performance testing of the secondary magnesium phosphate are essential to assure good separation of tocopherol and quantitative recovery of vitamin D.

THE RECOGNITION of a daily adult requirement for tocopherol in the range of 10–30 mg. (1) has led to increased usage of vitamin E in multivitamin supplements. For example, the formulas for decavitamin capsules and tablets in the USP XVII (2) include a suitable form of α-tocopherol at a level equivalent to 15 mg. of *dl*-α-tocopheryl acetate per capsule or tablet. Vitamin D levels, on the other hand, have tended to be lowered in such products due to increased emphasis on the desirability of avoiding intakes of vitamin D in excess of the recommended daily allowance (1). As a result of these developments, the ratio of vitamin E to vitamin D in many products has increased markedly to the point where the vitamin E causes considerable over-estimation of vitamin D by the USP assay method (2).

A modified vitamin D assay procedure has been developed in which tocopherol is removed from the solvent extract by column chromatography on secondary magnesium phosphate prior to the normal chromatographic purification steps in the USP method. The use of secondary magnesium phosphate for separations in the assay of vitamin E has been described by Bro-Rasmussen and Hjarde (3). Careful conditioning of the adsorbent and performance testing before use are of critical importance in the vitamin E assay and also in the vitamin D assay described below.

Mulder *et al.* (4) separated tocopherols from vitamin D by column chromatography on alkaline alumina prior to passage through the diatomaceous earth[1] and Fuller's earth[2] columns, but the oils tested contained slightly less than 1 mg. of α-tocopheryl acetate per 1,000 units of vitamin D. Multivitamin mixtures containing 10–30 mg. of vitamin E plus 400 units of vitamin D represent ratios of vitamin E to vitamin D about 25 to 75 times as great as those reported by Mulder *et al.* (4). In our experience with alkaline alumina, when quantitative recovery of vitamin D was obtained, the removal of these higher levels of vitamin E was not complete and significant interference was encountered in the vitamin D assay.

EXPERIMENTAL

Reagents and Apparatus—These are described in USP XVII (2) with the following additions.

Diethyl Ether—must be free of peroxides; Mallinckrodt's black label has been suitable.

Petroleum Ether—Merck reagent grade benzin, boiling range 30–65°; purify by passing through a silica gel column until no absorption is found at 301 mμ.

Secondary Magnesium Phosphate—The following products have been used successfully when conditioned as directed below.

(*a*) Magnesium phosphate, dibasic, pure, E. Merck A. G., Darmstadt, West Germany—distributed by Brinkmann Instruments, Inc., Westbury, L. I., N. Y. (*b*) Magnesium phosphate, C.P., dibasic—distributed by Amend Drug and Chemical Co., Inc., New York, N. Y.

Conditioning Procedure for Secondary Magnesium Phosphate—Dry about 250 Gm. for 1 hr. at 180° and cool over concentrated H_2SO_4. Boil the powder for 10 min. with 2 L. of a solution of 16 Gm. of secondary sodium phosphate (anhydrous, reagent grade) in H_2O. Cool slightly, filter through Whatman No. 1 paper on a Buchner funnel, and wash with about 1.5 L. of H_2O. Dry the secondary magnesium phosphate for 48 hr. at 180° and cool to room temperature over concentrated H_2SO_4. Grind in a mortar, screen through a 120-mesh sieve, and store in a tightly closed container. Prior to use, transfer a weighed amount of this material to a jar, add 7% H_2O, close jar, and shake thoroughly to break up all lumps and distribute the water. Store in a tightly closed jar. Let stand at least 2–3 hr. before use and do not keep for more than 2 days after adding the H_2O.

Preparation of Column—Plug the lower, constricted end of a 17–20 mm. i.d. chromatography tube about 25 cm. long with glass wool. With the stopcock closed, fill about two-thirds with petroleum ether. Add conditioned secondary magnesium phosphate portionwise, allowing it to flow slowly into the tube and settle without pressure or vacuum until a column height of 10–12 cm. is

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[1] Celite, Johns-Manville Corp., New York, N. Y.
[2] Florex, Floridin Co., Pittsburgh, Pa.

obtained. Drain off the petroleum ether until the solvent is about 3 mm. above the surface of the adsorbent.

Column Performance Check—Using a mixture of 75 mg. of α-tocopheryl acetate and 25,000 USP units of vitamin A as acetate or palmitate, obtain a sample preparation as described under *Procedure*. Transfer the 10 ml. of petroleum ether extract onto the prepared column and open the stopcock. Wash down with two 5-ml. aliquots of petroleum ether, being careful not to let the surface of the column run dry. Elute with a 6:94 (v/v) mixture of diethyl ether-petroleum ether until the vitamin A zone (seen as a green-yellow fluorescence with the aid of a long wavelength UV light) reaches the glass wool plug. This should require 35-60 ml. of eluant and all of the tocopherol which precedes vitamin A is then in the eluate receiver. Change the receiver and elute the vitamin A with a 3:7 (v/v) mixture of diethyl ether-petroleum ether. Vitamin A elution as observed with the UV light should be complete with about 50 ml. of eluant.

Check recovery of vitamin D from a second, similar column of secondary magnesium phosphate by adding 10 ml. of a petroleum ether solution containing 2,000 USP units of vitamin D and no vitamin A or vitamin E. Proceed with chromatography as described above, using the volume of 6:94 diethyl ether petroleum ether mixture determined above to be necessary to bring the vitamin A down to the glass wool plug. Change the receiver and elute the vitamin D with 70 ml. of the 3:7 mixture of diethyl ether-petroleum ether. Evaporate this second eluate and measure the vitamin D colorimetrically as per USP XVII. Quantitative recovery of vitamin D should be obtained with this procedure.

When a new lot of secondary magnesium phosphate is received, a portion should be conditioned and checked for performance as described above. Drying times and moisture content of the adsorbent influence its activity. If the above conditions are not optimal for a particular lot of secondary magnesium phosphate, they may have to be modified until the above performance criteria are met. If vitamin A spreads all through the column and moves down too rapidly, the adsorbent is not sufficiently active. Longer drying or less water addition may then be needed. If the vitamin A does not move down or moves too slowly, addition of more water is indicated.

Procedure—Obtain a sample preparation as described on p. 802 of USP XVII except to add 50 mg. of hydroquinone instead of 2 ml. of cottonseed oil prior to saponification and to take up the residue after evaporation of the solvent hexane extract in 10 ml. of petroleum ether rather than solvent hexane. The vitamin D level in the original sample taken may be as low as 2,000 units. Transfer an aliquot of the petroleum ether extract containing 2,000-4,000 USP units of vitamin D onto the prepared secondary magnesium phosphate column. Open stopcock and allow solution to drain into column without pressure or suction. Do not let top of column run dry. Wash down with two 5-ml. portions of petroleum ether. Elute tocopherol with 6:94 mixture of diethyl ether-petroleum ether until the vitamin A zone reaches the glass wool plug. Change the receiver and elute vitamin D along with vitamin A with 70 ml. of a 3:7 mixture of diethyl ether-petroleum ether. Evaporate the eluate just to dryness under a stream of nitrogen with the flask immersed in a water bath at 40°. Dissolve the residue in 5 ml. of solvent hexane. Take a 2 or 4-ml. aliquot containing about 1,600 USP units of vitamin D for "first column chromatography" as described in USP XVII and continue to follow the USP procedure.

TABLE I—COMPARISON OF USP AND MODIFIED USP ASSAYS FOR VITAMIN D IN PRESENCE OF VITAMIN E

Vitamin D, USP units	Vitamin E, mg. α-Tocopheryl Acetate	Vitamin A, USP units	Vitamin D (or Equivalent) Found, USP units: USP XVII	Modified USP XVII	Difference
		Model Systems			
0[a]	15	0	150	10	140
			270	20	250
0[a]	30 (*dl*-α-Tocopherol)	4,000	300	30	270
400	15	5,000	580	401	179
			700	404	296
		Multivitamin Tablets			
(Added per tablet[b]) A 600	15	25,000	790	580	210
			820	630	190
			810	600	210
			810	610	190
B 600	15	5,000	710	580	130
		Multivitamin Drops			
(per 0.6 ml.[b]) 600	10	2,500			
		Initial	612	420	192
		2 mo. at 45°	654	450	204
		3 mo. at 45°	636	444	192

[a] Samples without vitamin D were treated as if they contained 400 USP units per specified quantity of vitamin E. [b] Vitamin D content represents the actual amount added in manufacturing; vitamin A and E values represent label claims.

Applications of Procedure. Both the USP XVII procedure for vitamin D assay and the present modified procedure have been applied to (a) samples of vitamin E only, (b) a laboratory mixture of vitamins A, D, and E, (c) multivitamin tablets and drops, the latter including samples aged at 45°. In all cases the equivalent of 2,000 USP units of vitamin D based on label claim was taken as the original sample and the entire 2,000 units taken for the first column chromatography in both procedures.

RESULTS

The results are summarized in Table 1. When only vitamin E is carried through the USP vitamin D assay procedure, a significant amount of brown color is obtained and measured as vitamin D. The additional chromatography on secondary magnesium phosphate in the modified procedure reduces this nonspecific blank to a very low level. In the model mixture of vitamins A, D, and E, the USP method overestimated the 400 units of vitamin D by 45–74% in the presence of 15 mg. of α-tocopheryl acetate. Recoveries in the two trials of the modified procedure with this combination of vitamins were 100 and 101%. Similarly, with multivitamin products the USP method overestimates the vitamin D content to a considerable degree (130–210 USP units on a total of 533–600 units). The results by the modified procedure are close to the actual levels of addition of vitamin D.

The magnitude of the interference due to tocopherol in the USP assay for vitamin D is not sharply reproducible from one sample or product to the next. On the basis of the results in Table 1, it does not appear that a generally applicable correction to the USP assay value could be calculated based on the relative proportions of tocopherol and vitamin D and the dilutions used. Preliminary removal of the tocopherol *via* column chromatography on secondary magnesium phosphate provides an effective and reliable method of removing this source of interference.

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Keyphrases

Vitamin D analysis
Vitamin E—interference elimination
Column chromatography—separation
Magnesium phosphate, secondary—column adsorbent
UV light—vitamin A fluorescence
Colorimetry—analysis

4450

ROYAL NETHERLANDS EMBASSY

WASHINGTON D.C. 20008

OFFICE OF THE AGRICULTURAL COUNSELOR
4200 LINNEAN AVENUE, N.W.

February 21, 1974

CABLES: NEDAGRICO
TEL. 202 - 244 - 5300

YOUR REFERENCE:

OUR REFERENCE: L/194

ENCLOSURES:

SUBJECT:
Vitamin D

Mr. Humphrey F. Sassoon, Ph.D.
Tracor Jitco, Inc.
1300 East Gude Drive
Rockville, MD 20851

Dear Dr. Sassoon:

From the Netherlands Food Inspection Service I received a reply to your question regarding the use of vitamin D as a food additive. It reads as follows:

> "In the Netherlands the addition of vitamins to foods is in general forbidden. In special cases, where the need for and usefulness of supplementation have been clearly proven, the Minister of Health will designate suitable vehicles for obligatory supplementation (Food Law, General Decree, Art. 10 bis).
> In view of eating habits and climatic conditions in the Netherlands the need for extra vitamin D has been accepted as proven.
> As a vehicle for supplementation "margarin" (an edible water in oil emulsion with 80% fat) has been designated and 3 I.U. of vitamin D3 have to be present per gram of margarin (Food Law, Margarin Decree, Art. 2, Par. 2).
> In addition "Infant formula" shall contain a minimum of 60 I.U. and a maximum of 120 I.U. of vitamin D per 100 kcal.
> Apart from those two exceptions ("margarin" and "infant formula") no additions of vitamin D in foods for sale in the Netherlands are permitted."

I hope this information will be useful in preparing your monograph. I would appreciate it very much, if you would send me a copy of your study in due time.

Sincerely,

A. TAUZER
for the Counselor (Agricultural)

Separation of vitamin D esters by thin-layer chromatography*

There is evidence that vitamin D, like other sterols[1,2], is esterified during intestinal absorption[3]. The nature of the esters thus formed is currently being investigated in this laboratory with ^{14}C vitamin D_3 in rats. This communication describes methods for separating esters of vitamins D_2 and D_3 with thin-layer chromatography.

Methods

Esters were prepared by a modification of the method of KUKSIS AND BEVERIDGE[4] either from commercially available fatty acid chlorides (butyryl, hexanoyl, octanoyl, decanoyl, lauroyl and oleoyl, J. T. Baker Chemical Co., and myristoyl, palmitoyl and stearoyl, Matheson Scientific, Inc.) or from acid chlorides synthesized from fatty acids (linoleic, linolenic and arachidonic, Sigma Chemical Co.) and oxalyl chloride (Eastman Organic Chemicals).

To synthesize the acid chlorides, 0.58 mmole fatty acid was dissolved in 3.5 ml petroleum ether and refluxed for one hour with 1.45 mmole oxalyl chloride. Excess oxalyl chloride was removed after cooling by extracting with 0.5 ml water. To synthesize the esters, 0.5 mmole vitamin D_2 or D_3 (Mann Research Laboratories, Inc.) was refluxed with 60 ml benzene to which 0.58 mmole acid chloride and 0.05 ml pyridine had been added. The solvent was removed under vacuum and the residue was chromatographed in the dark under N_2 on 20 × 20 cm plates covered with Silica Gel H (Brinkmann Instruments) in the system hexane–benzene 1:1. The esters were visible under U.V. light as a wide band near the solvent front and were eluted with methylene chloride and stored in the dark under N_2 at 4°. A more detailed description of the synthesis and the physical characteristics of the esters will be published elsewhere.

Thin layers 250 μ thick were prepared on 20 × 20 cm plates from a slurry of Silica Gel H (Brinkman Instruments) and 55 ml 0.02% Rhodamine G in distilled water. For separation of the unsaturated fatty acid esters, 20 g Silica Gel G (Brinkman Instruments) made up with 50 ml 1.4% silver nitrate ($AgNO_3$) and 0.02% Rhodamine G in distilled water was used.

Results

The results are shown in Tables I and II.

The mobility of the esters was greater when the proportion of benzene in the solvent system was increased (Table I). The R_F values were found to be dependent upon the chain length of, as well as the number of unsaturated double bonds in, the fatty acid (Table I).

TABLE I

R_F VALUES* FOR ESTERS OF VITAMINS D_2 AND D_3 CHROMATOGRAPHED ON SILICA GEL IN HEXANE–BENZENE 1:1 (A) AND 1:2 (B)

Ester	A		B	
	D_2	D_3	D_2	D_3
Acetate	0.15	0.21	0.26	0.29
Butyrate	0.29	0.29	0.38	0.41
Hexanoic	0.36	0.35	0.50	0.51
Octanoic	0.44	0.40	0.58	0.55
Decanoic	0.46	0.42	0.65	0.60
Laurate	0.47	0.47	0.67	0.65
Myristate	0.49	0.49	0.71	0.69
Palmitate	0.52	0.54	0.73	0.73
Stearate	0.55	0.55	0.75	0.73
Oleate	0.52	0.53	0.73	0.70
Linolate	0.48	0.48	0.71	0.69
Linolenate	0.43	0.42	0.61	0.64
Arachidonate	0.43	0.43	0.69	0.66

* Mean of three or four determinations.

* Supported (in part) by U.S.P.H.S. Grant No. AM 09016-01 and (in part) by U.S.P.H.S. General Research Support Grant No. 1-S01-FR-05070-01.

The esters were more clearly separated on the basis of the degree of unsaturation of the fatty acid when chromatographed in silica gel impregnated with $AgNO_3$ (Table II).

There was essentially no difference in mobility of the same esters of vitamins D_2 and D_3 (Tables I and II).

TABLE II

R_F VALUES FOR ESTERS OF VITAMINS D_2 AND D_3 CHROMATOGRAPHED ON SILICA GEL IMPREGNATED WITH $AgNO_3$ IN HEXANE–BENZENE 1:2

Ester	D_2	D_3
Stearate	0.79	0.78
Oleate	0.57	0.58
Linolate	0.41	0.41
Linolenate	0.30	0.30
Arachidonate	0.18	0.20

Discussion

The results show that the migration of vitamin D esters on silica gel is dependent on the chain length as well as the degree of unsaturation of the fatty acid moiety, the longer chain length increasing, and the greater degree of unsaturation decreasing, mobility (Table I). These findings are thus similar to those obtained with thin-layer chromatography of esters of cholesterol[5,6] and of β-sitosterol[7]. The effect of unsaturation gives rise to "critical pairs" of esters with the same rates of migration: palmitate–oleate, myristate–linolate and laurate–linolenate (Table I).

The unsaturated esters can be separated from the saturated ones with longer chain lengths on the basis of the number of unsaturated double bonds by thin-layer chromatography on silica gel impregnated with $AgNO_3$ (Table II). This method has previously been used to fractionate cholesterol esters in the same manner with much the same results[8,9].

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Company Code: [redacted]
FPC No. : 0246

4511

PET INCORPORATED

FORM 6

INGREDIENT SPECIFICATION

NUMBER: 0625540
DATE: AUGUST 4, 1970
CANCELS: JAN. 15, 1970

INGREDIENT: VITAMIN D_3 - USED FOR EVAPORATED MILK ONLY

ALL DELIVERIES OF VITAMIN D_3 SHALL COMPLY IN EVERY RESPECT TO THE REQUIREMENTS OF THE FEDERAL FOOD, DRUG AND COSMETIC ACT, AS AMENDED AND REGULATIONS PROMULGATED THEREUNDER, AS WELL AS ANY OTHER STATE, FEDERAL OR LOCAL FOOD REGULATORY AGENCIES. IN ADDITION, THE PRODUCT SHALL COMPLY TO THE FOLLOWING SPECIFICATIONS AND ALL LOTS ARE SUBJECT TO THE APPROVAL OF THE QUALITY CONTROL DEPARTMENT OF PET INCORPORATED.

		PROCEDURE
COLOR & FORM:	WHITE TO CREAM COLOR - FREE FLOWING POWDER	VISUAL
ODOR:	NONE	
+IDENTIFICATION OF VITAMIN D_3	MUST MEET CODEX EXCEPT NEED NOT BE CRYSTALLINE	CODEX - FIRST ED. PAGES 715-716
POTENCY:	1) 200 MILLION USP UNITS/LB. 2) 440,917 USP UNITS/GRAM	
SOLUBILITY:	DISPERSIBLE IN WATER	
PARTICLE SIZE:	100% THRU NO. 20 80% THRU NO. 80 MIN.	TYLER ROTAP U.S.S. SIEVES
COMPONENTS:	VITAMIN D_3 DEXTRIN BHA BHT LECITHIN	
LABEL COLOR:	ORANGE BACK-GROUND WITH BLACK PRINT	
ASSAY REPORT:	LABORATORY BIOASSAY REPORT TO BE FURNISHED FOR EACH LOT WHICH IS PREPARED AND SENT TO: DR. A. Z. HODSON CHEMISTRY DEPARTMENT PRODUCT DEVELOPMENT SERVICES GREENVILLE, ILLINOIS 62246	
STORAGE:	KEEP IN COOL, DRY AREA PROTECTED FROM LIGHT.	
SHIPMENT:	EACH SHIPMENT SHALL NOT CONTAIN MORE THAN TWO LOT OR BATCH NUMBERS. LOT OR BATCH NUMBER TO BE INDICATED ON LABEL.	
PERFORMANCE:	MUST BE COMPARABLE IN PERFORMANCE BY USAGE TEST TO PRODUCT PREVIOUSLY APPROVED.	

+LATEST CHANGE MARKED WITH A PLUS.

VITAMIN D_2 (type 500) (F.C.C.)

SAMPLING: Representative samples will be obtained from each lot delivered, and composited into a clean, dry and air-tight container.

ESSENTIAL LABORATORY TESTS:	Minimum:	Maximum:	Methods:
Visual inspection	No foreign matter, water dispersible		M-1.01
Odor	No off		M-1.11
Color	White		Visual
Granulation	Free flowing		Visual
COMPLEMENTARY LABORATORY TESTS:			
Insects	Irreducible minimum*		M-2.15
Rodent contamination	Irreducible minimum*		M-2.15
Filth	Irreducible minimum*		M-2.28

* Contamination must be at a minimum and meet the requirements of the Food, Drug and Cosmetic Act as interpreted by the Food and Drug Administration.

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PEDIATRICS 13: 178, 1954.

EFFECTS OF VITAMIN D ON THE SERUM CALCIUM AND PHOSPHORUS LEVELS IN INFANTS DURING THE FIRST WEEK OF LIFE

By J. B. Pincus, M.D., I. F. Gittleman, M.D., A. E. Sobel, Ph.D., and E. Schmerzler, B.S.
Brooklyn

A previous communication[1] reported that cow's milk enriched with vitamin D administered to infants during the first week of life tends to lower the serum calcium levels.

As it was not possible to find reports of similar studies for comparison, the study of the effect of vitamin D on serum calcium and phosphorous was therefore extended to include observations on groups of infants fed powdered milk formulas or breast milk.

The infants thus studied were divided into five groups. Two groups received breast milk from their mothers; one of these groups received besides a daily supplement of 600 USP units of vitamin D in an aqueous multivitamin preparation. These infants were used as "controls." The other three groups received a powdered milk formula of the following composition: The calcium to phosphorus ratio was 1.5 to 1 and the calcium and total phosphate content were 77 mg. and 51 mg./100 cc. formula, respectively. Group 1 was fed a powdered milk formula free of vitamin D. Group 2 received a similar powdered milk formula but containing 400 USP units of vitamin D per quart. Group 3 was fed a powdered milk formula free of vitamin D, as were the infants in Group 1; but they received a daily supplement of 600 USP units of vitamin D in an aqueous multi-vitamin preparation.

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Aided by grants from the United States Public Health Service and the M & B Laboratories of Columbus, Ohio.

A preliminary report was read before the Society for Pediatric Research at Old Point Comfort, Va., in 1952.

(Received for publication July 8, 1953)

Blood samples were initially obtained in each case on the first day of life before feedings were begun and also on the day of discharge—four hours after the last feeding. The technic of obtaining blood and the methods employed for the measurement of calcium and phosphorus were described previously.[1]

Analysis of Results

The data and a statistical analysis of the results are presented in tables 1-7.

Tables 1 and 2 present the findings in two breast-fed groups. There is no statistical difference in the calcium and phosphorus levels on the fifth day of life in the two groups. The authors did, however, note that the mean serum calcium was lower and the mean phosphorus was higher on the fifth day of life in the group receiving breast milk supplemented daily with 600 USP units of vitamin D. On the other hand, the serum phosphorus and calcium levels in the breast-fed infants who did not receive vitamin D supplement remained practically unchanged.

Tables 3, 4 and 5 present the findings in infants fed powered milk formulae with and without vitamin D. A statistical analysis of these data for the change in the calcium and phosphorus levels are given in tables 6 and 7. The authors found no statistically significant difference between the phosphorus levels on the fifth day of life in the three groups of infants fed powdered milk formulae. In all of them the mean phosphorus level was significantly higher on the fifth day of life but the groups did not vary from each other in the degree of rise.

The calcium levels, however, showed a

TABLE 1

SERUM CA AND P LEVELS IN INFANTS RECEIVING BREAST MILK NOT SUPPLEMENTED WITH VITAMIN D

Case	Ca mg./100 cc. 1 d.	Ca mg./100 cc. 5 d.	P mg./100 cc. 1 d.	P mg./100 cc. 5 d.
1	10.5	10.4	5.5	5.6
2	7.9	9.9	5.9	4.9
3	10.5	10.0	6.5	4.7
4	8.2	8.4	5.6	4.9
5	8.3	8.1	5.1	5.1
6	8.0	8.4	9.6	6.0
7	8.8	8.8	5.6	5.7
8	9.0	9.6	5.9	5.4
9	9.0	8.9	5.3	5.9
10	9.2	9.3	4.8	4.9
11	9.5	9.[illegible]	5.5	5.5
12	9.6	9.4	5.5	5.1
13	8.9	8.5	5.6	5.0
14	8.2	8.4	5.2	6.6
15	9.0	9.2	5.3	5.6
Mean	8.96	9.05	5.74	5.06

TABLE 2

SERUM CA AND P LEVELS IN INFANTS RECEIVING BREAST MILK SUPPLEMENTED WITH 600 U.S.P. UNITS OF VITAMIN D DAILY

Case	Ca mg./100 cc. 1 d.	Ca mg./100 cc. 5 d.	P mg./100 cc. 1 d.	P mg./100 cc. 5 d.
1	10.0	8.9	5.75	6.7
2	9.0	9.6	6.0	5.6
3	8.9	8.8	5.9	5.4
4	10.0	9.4	6.1	6.15
5	9.3	8.9	5.35	5.95
6	8.6	9.0	5.55	6.1
7	9.2	9.2	6.0	5.0
8	9.0	8.4	6.3	7.3
9	9.9	9.7	5.85	4.85
10	9.0	8.2	6.2	7.6
11	8.8	8.6	5.8	4.8
12	8.8	8.5	7.2	7.0
13	8.7	8.6	5.3	5.9
Mean	9.2	8.9	5.95	6.1

TABLE 3

SERUM CA AND P LEVELS IN INFANTS RECEIVING POWDERED MILK NOT CONTAINING VITAMIN D

Case	Ca mg./100 cc. 1 d.	Ca mg./100 cc. 5 d.	P mg./100 cc. 1 d.	P mg./100 cc. 5 d.
1	9.0	9.1	5.8	7.4
2	10.2	10.0	5.7	5.7
3	11.0	10.6	4.7	7.1
4	10.8	10.6	5.1	6.8
5	10.4	10.6	5.7	8.2
6	8.4	9.9	5.6	6.9
7	9.9	9.4	6.9	7.3
8	9.2	9.0	4.1	7.4
9	8.9	8.2	5.6	8.0
10	9.6	9.0	5.5	8.8
11	8.2	6.9	5.8	7.9
12	9.3	9.4	6.0	7.2
13	10.3	10.3	6.2	6.6
14	8.8	9.1	6.8	7.4
15	10.2	8.0	5.2	8.2
16	8.8	9.7	6.3	8.2
17	9.6	9.8	5.9	7.2
18	9.8	9.2	6.2	7.0
19	9.6	8.6	5.9	7.8
20	8.8	7.4	6.5	9.0
21	9.2	8.1	4.5	9.2
22	9.8	9.3	6.0	9.8
23	8.9	8.5	6.7	7.8
24	9.5	9.5	6.4	8.5
25	9.5	9.5	7.2	8.3
26	9.0	9.5	5.5	8.0
27	9.7	10.2	5.7	8.0
28	8.8	8.9	6.8	8.6
29	9.5	8.7	4.4	8.5
30	9.5	10.3	6.1	8.2
31	9.6	9.4	4.5	8.4
32	9.2	8.6	5.7	7.2
33	9.8	7.4	4.8	6.7
34	9.4	8.7	6.9	10.1
35	9.1	9.8	7.9	8.1
36	9.6	9.0	5.4	7.7
37	9.0	9.4	6.5	6.5
38	9.2	10.3	6.4	6.0
39	8.9	8.3	5.8	7.0
40	9.0	9.5	6.8	7.1
41	9.6	9.8	6.9	7.6
42	9.8	9.6	6.5	7.2
43	9.8	8.6	6.0	8.7
44	9.6	7.4	5.2	8.0
45	9.0	9.4	6.2	8.5
46	10.4	7.7	6.0	8.6
Mean	9.45	9.14	5.90	7.83

TABLE 4

SERUM CA AND P LEVELS IN INFANTS RECEIVING POWDERED MILK CONTAINING VITAMIN D (400 U.S.P. UNITS/1000 CC. FORMULA)

Case	Ca mg./100 cc. 1 d.	Ca mg./100 cc. 5 d.	P mg./100 cc. 1 d.	P mg./100 cc. 5 d.	Case	Ca mg./100 cc. 1 d.	Ca mg./100 cc. 5 d.	P mg./100 cc. 1 d.	P mg./100 cc. 5 d.
1	11.4	9.4	2.9	4.2	28	9.9	9.4	4.7	7.1
2	10.1	8.4	6.3	7.2	29	8.9	8.1	5.0	7.4
3	11.6	7.6	4.7	9.6	30	9.0	8.1	5.1	8.2
4	8.2	7.5	6.8	7.4	31	8.2	8.0	6.1	9.0
5	10.6	8.1	4.5	9.3	32	8.1	8.2	4.6	7.2
6	10.4	10.7	4.1	7.3	33	8.0	8.0	4.7	7.5
7	9.7	10.6	3.8	7.7	34	8.0	7.8	6.0	7.5
8	10.1	7.7	5.9	8.3	35	9.2	9.6	4.8	8.4
9	9.1	4.8	4.6	7.7	36	10.1	9.4	4.6	8.4
10	9.0	8.8	5.2	7.8	37	9.2	9.2	4.5	8.2
11	8.0	9.2	5.5	7.8	38	10.2	9.7	7.0	7.2
12	9.0	8.6	5.7	8.2	39	8.4	8.0	5.0	6.8
13	9.4	7.7	5.5	7.0	40	9.5	8.5	3.8	5.7
14	8.4	8.4	6.1	6.1	41	8.5	9.0	5.1	8.5
15	8.6	7.4	5.0	7.5	42	8.8	9.0	5.0	6.6
16	10.4	9.8	4.1	8.1	43	8.8	9.1	4.6	7.5
17	9.4	9.8	5.9	6.0	44	9.5	9.2	2.5	8.5
18	8.4	10.1	5.9	8.5	45	8.6	8.4	6.0	8.2
19	9.2	9.6	5.6	8.2	46	8.2	8.0	5.7	6.6
20	9.0	8.9	6.1	8.6	47	8.3	8.2	5.3	8.8
21	9.4	9.6	5.7	8.2	48	9.6	8.6	3.6	6.7
22	8.4	8.7	4.7	8.5	49	9.9	9.4	4.2	7.4
23	10.4	7.4	6.6	9.8	50	8.8	8.4	5.7	6.4
24	9.1	8.9	5.4	5.3	51	8.1	8.0	5.1	6.8
25	8.1	8.7	4.8	5.8	52	8.6	7.0	4.7	7.2
26	8.4	8.1	4.7	7.5					
27	9.3	9.3	6.1	7.5	Mean	9.12	8.61	5.14	7.48

significant fall when Group 1 was compared with Group 3 or when Group 2 was compared with Group 3. There was no statistically significant difference in the calcium fall between Groups 1 and 2.

The incidence of serum calcium levels below 8 mg./100 cc. is given in table 8. It is evident that in Group 1 there is an incidence of 10.9%, while in Group 2 the incidence is 17.3% and in Group 3 the incidence is 30%.

DISCUSSION

It has generally been accepted that vitamin D aids in the absorption of calcium salts from the intestinal tract.[2, 4] It has also been established that vitamin D, when given to experimental animals fed diets containing varying calcium to phosphorus ratios, exerts a regulatory effect upon the concentration of these elements in the blood serum.[7, 9]

In the newborn infants studied the authors failed to observe such beneficial influence; quite the contrary, it was found that vitamin D tends to depress the serum calcium level during the first week of life.

In order to explain this "anomalous" effect of vitamin D in early neonatal life several of the factors that operate in maintaining a normal calcium to phosphorus relationship in the serum have been considered.

1. The reciprocal relationship that exists between serum calcium and phosphorus levels.

Observations have been made[10, 12] that when the serum phosphorus level is raised to abnormal heights the calcium level drops. The present investigators[1] and others[13-17] have found that when newborn infants are given a high phosphorus-containing diet

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TABLE 5

SERUM CA AND P LEVELS IN INFANTS RECEIVING "VITAMIN D FILLED" POWDERED MILK. THIS GROUP RECEIVED DAILY SUPPLEMENT OF 600 U.S.P. UNITS OF VITAMIN D

Case	Ca mg./100 cc. 1 d.	Ca mg./100 cc. 5 d.	P mg./100 cc. 1 d.	P mg./100 cc. 5 d.	Case	Ca mg./100 cc. 1 d.	Ca mg./100 cc. 5 d.	P mg./100 cc. 1 d.	P mg./100 cc. 5 d.
1	8.4	9.0	6.1	6.6	17	9.5	8.9	6.3	7.7
2	8.9	6.6	6.0	8.0	18	9.1	8.0	6.85	8.3
3	9.0	8.4	5.8	7.05	19	8.1	7.5	5.6	8.85
4	9.4	8.8	5.65	8.7	20	9.3	5.2	5.15	10.3
5	10.0	7.9	5.0	9.2	21	9.2	7.2	5.8	7.65
6	9.1	9.0	6.7	7.95	22	9.1	8.1	6.2	7.3
7	8.8	6.7	5.85	8.15	23	8.8	9.2	7.1	7.65
8	9.0	9.8	4.1	5.75	24	8.6	7.4	6.2	8.25
9	8.8	9.2	6.75	7.5	25	8.2	8.0	6.25	9.1
10	10.0	8.4	6.0	6.95	26	9.2	9.2	5.5	7.6
11	9.8	8.0	7.9	7.9	27	9.2	9.1	6.6	7.6
12	8.8	6.0	5.75	8.6	28	9.1	8.1	6.1	9.1
13	9.0	9.0	6.85	8.1	29	8.9	8.0	5.95	6.8
14	10.0	9.3	6.75	7.1	30	8.9	6.1	5.5	8.0
15	9.7	9.0	5.3	7.65					
16	8.6	8.2	5.5	8.75	Mean	9.11	8.17	6.11	7.91

there is an immediate elevation of the serum phosphorus level. This elevation of the serum phosphorus is not associated with a consistent depression of the calcium level. In the present studies it was found that the serum phosphorus level rises in each of the three experimental groups while the calcium level is most significantly depressed in the group receiving the largest amount of vitamin D. Individual cases in every group did show the effect of the reciprocal relation between calcium and phosphorus, i.e., when the phosphorus was unusually high the serum calcium was low. It seems therefore fair to state that a reciprocal relationship of calcium to phosphorus appears to operate in some cases but the over-all effect is that of a statistically significant independent drop in the calcium levels in the group receiving 600 USP units of vitamin D. The authors therefore cannot accept as an explanation that the tendency of the serum calcium to fall in the infants studied is based on a reciprocal rise of the serum phosphorus.

2. Rapid precipitation of lime salts in the newborn infant.

It has been observed[9, 18] that tetany may be precipitated in rachitic infants and experimental animals by either the administration of small amounts of vitamin D or by exposure to ultraviolet radiation. This phenomenon has been explained on the rapid withdrawal of lime salts from the blood to be precipitated in the bone. In all the instances when tetany was produced the serum phosphorus rose and the calcium fell, or both the serum calcium and phosphorus fell.[18, 9] The authors failed to find a relationship between the mean drop in calcium and a corresponding rise in the phosphorus. Nor was it possible to see a corresponding fall in both elements. Therefore, a rapid withdrawal of lime salts from the blood hastened by vitamin D cannot adequately explain these findings.

3. Transient state of hypoparathyroidism.

In order to explain the increased incidence of tetany of the newborn infant which was observed in recent years many investigators postulated[12, 14, 21-23] that a state of transient hypoparathyroidism in the newborn period exists. The degree of hypoparathyroidism would probably vary from infant to infant. In infants fed breast milk, thus receiving a relatively low phosphorus load, the parathyroids would be "able" to maintain homeostasis of the serum calcium

TABLE 6

STATISTICAL ANALYSIS OF CHANGE IN SERUM CALCIUM DURING FIRST 5 DAYS OF LIFE ON REGIMEN OF POWDERED MILK WITH AND WITHOUT VITAMIN D SUPPLEMENT

Group*	Ca_5-Ca_1 > 0.2†	Ca_5-Ca_1 < -0.2†	Total
I	23	23	46
II	26	26	52
Total	49	49	98

$\chi^2=0$ $P‡>0.99$

Group*	Ca_5-Ca_1 > -0.5†	Ca_5-Ca_1 < -0.5†	Total
I	28	18	46
III	10	20	30
Total	38	38	76

$\chi^2=5.51$ $P‡<0.02$

Group*	Ca_5-Ca_1 > -0.4†	Ca_5-Ca_1 < -0.4†	Total
II	31	21	52
III	10	20	30
Total	41	41	82

$\chi^2=5.26$ $P‡>0.02$

* Group I was fed processed milk free of vitamin D.

Group II was fed processed milk containing 400 USP units of vitamin D per quart of diluted milk. The total intake of vitamin D in 5 days did not exceed 300 USP units/infant.

Group III was fed processed milk plus a supplement of 600 USP units of vitamin D/day contained in a multivitamin preparation in aqueous medium.

† Ca_5 = serum calcium level on 5th day of life.

Ca_1 = serum calcium level on 1st day of life.

Differences in calcium levels expressed in mg./100 cc.

‡ P is for one degree of freedom.

TABLE 7

STATISTICAL ANALYSIS OF CHANGE IN SERUM PHOSPHORUS DURING FIRST 5 DAYS OF LIFE ON REGIMEN OF POWDERED MILK WITH AND WITHOUT VITAMIN D

Group*	P_5-P_1 2.3†	P_5-P_1 <2.3†	Total
I	19	27	46
II	30	22	52
Total	49	49	98

$\chi^2=2.62$ $P‡>0.10$

Group*	P_5-P_1 >1.0†	P_5-P_1 <1.0†	Total
I	25	21	46
III	13	17	30
Total	38	38	76

$\chi^2=0.88$ $P‡>0.30$

Group*	P_5-P_1 >2.05†	P_5-P_1 <2.05†	Total
II	30	22	52
III	11	19	30
Total	41	41	82

$\chi^2=3.36$ $P‡>0.05$

* *See* * footnote under table 6.

† P_5 = serum phosphorus level on 5th day of life.

P_1 = serum phosphorus level on 1st day of life.

Differences in phosphorus levels expressed in mg./100 cc.

‡ P is for one degree of freedom.

The distribution of serum Ca changes from day 1 to day 5 in groups I, II, and III was examined by setting up three contingency tables (Fisher, R. A., Statistical Methods for Research Workers, ed. 6, London, Oliver & Boyd, Ltd., 1936, p. 99) comparing groups I and II, groups I and III, and groups II and III. The critical values of serum Ca changes were selected by determining the median change in serum Ca in each pair of groups. The differences in serum Ca changes between groups I and II are not statistically significant: $\chi^2=0$, and $P‡>0.99$. The differences in serum Ca changes between groups I and III are statistically significant: $\chi^2=5.51$, and $P‡<0.02$. The differences in serum Ca changes between groups II and III are also statistically significant: $\chi^2=5.26$, and $P‡<0.03>0.02$.

TABLE 8

INCIDENCE OF CALCIUM LEVELS BELOW 8 mg./100 cc. IN INFANTS FED BREAST AND POWDERED MILK WITH AND WITHOUT VITAMIN D DURING FIRST FIRST WEEK OF LIFE

Milk	No. of Infants Studied	Calcium Levels Below 8 mg./100 cc.	Per cent
Powdered Milk No Vitamin D	46	5	10.9
Powdered Milk +Vitamin D 400 U.S.P. Units/quart	52	9	17.3
Powdered Milk +Vitamin D 600 U.S.P. Units/day	30	9	30
Breast Milk	15	0	0
Breast Milk +Vitamin D 600 U.S.P. Units/day	13	0	0

and phosphorus. Infants receiving fresh cow's milk or processed milk in which the phosphorus load may be from 3 to 4 times that of breast milk may not be able to control physiologically the serum calcium and phosphorus levels.

The authors observed no instance of a serum calcium level below 8 mg./100 cc. in the breast-fed infants while in the group fed powdered milk free of vitamin D, they did observe an incidence of 10.9% of serum calcium levels below 8 mg./100 cc. by the end of the fifth day of life. When small amounts of vitamin D were added, the incidence was 17.3% and when the dose was further increased to 600 units per day, the incidence rose to 30%.

Several investigators[1, 2] observed that an apparent antagonism between parathormone and vitamin D exists under certain circumstances. If we agree that these observers are correct in their assumption, it becomes possible to give a plausible explanation for the finding that vitamin D aggravates the fall of the serum calcium.

If a state of transient hypoparathyroidism exists in infants during the neonatal period the administration of vitamin D to these infants would further diminish the available parathormone, thus reducing the calcium and raising the phosphorus level. This effect is admittedly not equal in all infants but statistically we find that when vitamin D is administered the tendency for the aggravation of the lowering of the calcium is increased. There was no apparent effect of vitamin D on the mean phosphorus levels when Groups 3 and 1 and 3 and 2 are compared. However, when one considers that the mean phosphorus rise in Group 1 was 24% higher on the fifth day when compared with the initial values, and with few exceptions, there is generally a marked rise in the serum phosphorus level in all cases studied, the effect of the dosage of vitamin D which was administered may have been masked.

It is of interest to note in this connection that this effect of vitamin D is discernible, though to a lesser degree, in the breast-fed infants receiving 600 USP units of vitamin D daily. When the two series of breast fed infants are compared, it is noted that in the one receiving vitamin D the mean phosphorus level rises and the mean calcium falls. Though it is not a statistically significant change the trend is unmistakable.

SUMMARY

Five groups of infants, all less than one week old, were studied. Two groups were fed breast milk and were considered as controls. Three other groups were given powdered milk formulas. The diet varied only in the vitamin D content. One of the control groups received a daily supplement of 600 USP units of an aqueous multi-vitamin preparation. One of the experimental groups received a powdered milk free of vitamin D; another group was fed a powdered milk containing 400 USP units of vitamin D per quart of diluted formula, and a third group received a vitamin D-free powdered milk formula, and a daily supplement of 600 USP units of vitamin D in an

aqueous multi-vitamin preparation.

The authors found no instance of a serum calcium level below 8 mg./100 cc. in the group of infants receiving breast milk. The incidence of serum calcium levels below 8 mg./100 cc. in the experimental groups of infants varied from 10.9% in the group fed powdered milk vitamin D-free to 30% in the group receiving the largest amount of vitamin D. A statistical analysis of the results revealed that there was a significantly greater fall of the calcium level on the 5th day in Group 3 when compared with either Group 1 or 2. While the mean phosphorus level was significantly higher on the fifth day of life in each of the three experimental groups there was no significant difference in the phosphorus levels on the fifth day of life when the three groups are compared with each other.

Conclusion

Vitamin D, when administered during the first week of life to infants fed a diet containing 3 to 4 times the amount of phosphorus found in human milk, tends to lower serum calcium levels.

A trend towards a lower mean serum calcium and higher serum phosphorus levels was noted in the breast-fed infants receiving 600 USP units of vitamin D daily as compared to the series of infants receiving breast milk only.

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SPANISH ABSTRACT

Efectos de la Vitamina D sobre los Niveles Sanguíneos de Calcio y Fósforo del Recién Nacido

Habiendo observado que la leche de vaca enriquecida con vitamina D y administrada a los niños durante la primera semana de la vida acentuaba la tendencia hacia la hipocalcemia, los autores trataron de analizar si realmente dicha vitamina afectaba los niveles de calcio y fósforo sanguíneos, para lo cual observaron varios grupos de niños alimentados al pecho o con fórmulas de leche de vaca, distribuyéndolos así:

1. Grupo alimentado al pecho exclusivamente.
2. Grupo alimentado al pecho al que se le daban diariamente 600 unidades de vitamina D en preparación acuosa multivitaminada.
3. Un grupo con fórmula de leche en polvo sin vitamina D.
4. Grupo con la misma fórmula láctea agregada de 400 unidades de vitamina D por litro.
5. Grupo con la misma fórmula, recibiendo además 600 unidades de vitamina D diariamente.

Los dos primeros grupos sirvieron de control.

En términos generales se ha aceptado que la vitamina D favorece la absorción de las sales de calcio del tracto digestivo así como que al proporcionarla a animales de experimentación sujetos a dietas de variable relación calcio-fósforo, ejerce una acción reguladora sobre la concentración de estos elementos en el suero sanguíneo.

En los recién nacidos estudiados por estos autores no se observó la influencia benéfica señalada; por el contrario, encontraron que la vitamina D tendía a bajar el nivel calcico durante la primera semana de la vida. Esto los obligó a analizar algunos de los factores que intervienen en la conservación de las cifras normales de la relación calcio fósforo del suero: la influencia recíproca que existe entre los niveles sanguíneos calcio-fósforo, que no aceptan los autores; la precipitación brusca de las sales de calcio en el recién nacido, que tampoco les satisface como explicación y, por último, el estado transitorio de hipoparatiroidismo, factor que puede considerarse como el más probable: la vitamin D acentúa la caída del calcio sanguíneo por su aparente antagonismo bajo determinadas circunstancias con la paratormona, de por sí deficiente ya en forma transitoria.

No se observó hipocalcemia en ninguno de los grupos alimentados al pecho; en los grupos de experimentación la incidencia de hipocalcemia varió del 10.9% (grupo con fórmula artificial sin vitamina D) al 30% (grupo que recibió la mayor cantidad de vitamina D). El análisis estadístico de los resultados muestra una diferencia significativa en el descenso de niveles de calcio entre los grupos uno y tres y grupos dos y tres. El nivel medio del fósforo se encontró significativamente más elevado al quinto día de la vida en cada uno de los tres grupos de experimentación, pero no hubo diferencia significativa al quinto día de la vida en los tres grupos entre sí.

Los autores concluyen que el proporcionar vitamina D al recién nacido durante la primera semana de la vida, siendo alimentados con leches que contienen altas cifras de fósforo, acentúa la tendencia a la hipocalcemia. Se observó tendencia a cifras medias bajas de calcio y cifras medias altas de fósforo en los niños alimentados al pecho que recibían 600 Unidades de vitamina D diariamente, en comparación con las de los niños que recibían sólo leche materna.

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Metabolism and Biological Activity of Vitamin D

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In the last five years, it has been established that vitamin D_3 (cholecalciferol) needs to be metabolized into an active form in order to yield its biologic effects (3). This metabolite has been identified as the 25-hydroxycholecalciferol (25-OH-D_3) (1). Animal experiments have shown that 25-OH-D_3 is highly potent in stimulating the intestinal absorption of calcium (2, 5) and the bone resorption (2, 8) in vitro as well as in vivo. Moreover clinical investigations indicate that 25-OH-D_3 is able to alleviate rickets and hypoparathyroid hypocalcemia resistant to vitamin D (4, 6). It appears therefore that the biologic activity of vitamin D is controlled by its intermediary metabolism, and it becomes of major importance to study the biosynthesis and degradation of 25-OH-D_3. A first advance was to show that the liver is physiologically the main if not the only site of hydroxylation of D_3 into 25-OH-D_3 in the rat (7).

In chicken more than 6 metabolites of D_3 could be detected with chromatographic characteristics similar to those found in rats. However the production as well as degradation of 25-OH-D_3 was faster. This might explain the shorter lag of time between the administration of D_3 and its biologic effects. More polar metabolites represented a greater fraction of the radioactivity found in various tissues and plasma. One of these products, "peak V" appeared in increasing quantities after 8 hours in the small intestine and became the major metabolite in this organ within 24 hr. It is possible that "peak V" was made in the small intestinal mucosa since its formation was not prevented by bile duct cannulation and its relative concentration was higher in this tissue.

Using [26, 27-^{3}H]-25-OH-D_3 prepared by chemical synthesis in this laboratory (T. Suda and H. F. DeLuca, in preparation), it has been possible to investigate more accurately the metabolic fate of 25-OH-D_3. Two hours after i.v. injection a significant proportion of 25-OH-D_3 was converted into "peak V" and a more polar metabolite "peak VI" which was found only in the duodenal part of the small intestine. Hence 25-OH-D_3 appeared as an immediate precursor of the non-identified "peaks V" and "VI".

"Peak V" isolated from chicken intestine was found approximately half as potent as 25-OH-D_3 in stimulating the intestinal absorption of calcium. In contrast "peak V" from other origin showed no biopotency, nor did "peak VI". Despite its apparent biologic activity, the rather late appearance of "peak V" does not support the idea of a critical role in initiating the early effects of vitamin D. Rather it is proposed that "peaks V" and "VI" represent an important control of the effects of vitamin D, by quick inactivation of its biologically active form 25-OH-D_3 in the small intestine.

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"Activation" of Vitamin D by the Liver

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Abstract. Isolation of the liver from the circulation of rats eliminates almost completely their ability to convert [1,2-^{3}H] vitamin D_3 into its biologically active metabolite, 25-hydroxycholecalciferol, as well as certain other metabolites. It is concluded that the liver is the major if not the only physiologic site of hydroxylation of vitamin D_3 (cholecalciferol) into 25-hydroxycholecalciferol.

The osteodystrophy and the higher requirements for vitamin D observed in hepatic insufficiency may be due to an inability of the liver to transform vitamin D into its metabolically active form.

INTRODUCTION

A major step in understanding the mechanism of action of vitamin D has been the recent identification of its biologically active metabolite, 25-hydroxycholecalciferol (1, 2). This substance is more active than vitamin D_3 itself in curing rickets when administered in vivo and acts more rapidly in stimulating the intestinal absorption of calcium as well as mobilization of bone (3).

Whereas it has never been possible to demonstrate a direct effect of small doses of vitamin D on bone or intestine, 25-hydroxycholecalciferol has proved to be directly active in vitro in trace amounts on bone resorption (4) and intestinal transport of calcium (5). These new evidences support the concept that 25-hydroxycholecalciferol may be the metabolically active form of vitamin D. It is therefore of major concern to determine the site of its biosynthesis.

Investigations in man (6) and in rats (7) have shown first the disappearance of ^{3}H from the plasma after an intravenous dose of tritiated vitamin D_3 is interrupted by a very characteristic rebound of radioactivity after 2-4 hr. It has been possible to show that the liver is responsible for this transient increase in plasma radioactivity by quickly releasing into the blood a large portion of the dose which had been initially cleared from the plasma and accumulated in the liver. At the very same time, 25-hydroxycholecalciferol starts to accumulate in the plasma and in the tissues (7). These observations led us to postulate the liver as the site of hydroxylation of vitamin D_3 into its active metabolite, 25-hydroxycholecalciferol.

The very high incidence and severity of bone dystrophies in patients with severe liver disease has been associated with a controversy concerning the possible role of the liver in the metabolism of calcium and phosphorus. Impaired intestinal absorption of calcium (8, 9) and vitamin D (10-14) due to the lack of bile salts has been generally implicated in the etiology of the osteodystrophy. However, several reports indicate that in addition to an occasional poor intestinal absorption of vitamin D, the metabolism and the effects of this vitamin were directly altered by the liver disease. This was based mainly on the higher requirements for vitamin D usually observed in chronic hepatic insufficiency (15-23). In 1956, Atkinson, Nordin, and Sherlock, after careful investigation of 25 cases of prolonged obstructive jaundice stated "it seems possible that destruction of liver cells may interfere with storage or metabolism of vitamin D" (20).

More direct evidence that failure of liver function results in an abnormal metabolism of vitamin D in man has been provided by Avioli and coworkers (6). They demonstrated an unusually slow plasma disappearance of vitamin D_3-^{3}H associated with a decrease in the vitamin D_3 glucuronide fraction in the urine of cirrhotic patients.

The present work demonstrates that in rats the liver is the major, if not the only, physiological site of hydroxylation of vitamin D_3 into its active form, 25-hydroxycholecalciferol. It is suggested that severe liver insufficiency in man results in a lack of 25-hydroxycholecalciferol, or of an equivalent active metabolite of vitamin D. This would provide an experimental explanation of the higher requirements for vitamin D to alleviate the hepatic osteodystrophy.

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METHODS

The experiments were designed to investigate the ability of hepatectomized rats to convert vitamin D_3-^3H into 25 hydroxycholecalciferol-^3H. Since total ablation of the liver was not technically possible, surgical vascular disconnection of the liver was performed and considered equivalent to an actual hepatectomy.

Holtzman male rats (average weight 140 g) were fed the vitamin D-deficient diet 11 of Gascon, DeLuca, and Steenbock (Ca, 0.47%; P, 0.30%) (24) for a period of 4-8 wk. At this time they were judged vitamin D-deficient because of lack of growth and low serum calcium values (25). They were subjected to surgery under ether anesthesia. A portocaval shunt was created by vascular suture, followed by the ligation and section of the hepatic artery and bile duct two 4 days later. Because the liver was intimately attached to the vena cava, it was not possible to separate these two tissues and to ligate the hepatic vein. The rats recovered extremely well from the first operation, and lost less than 10% of body weight between the two surgeries. Just at the end of the second operation, 10 IU (0.25 μg) [1,2-^3H] vitamin D_3 (26) (SA 24,600 dpm/IU or 0.44 μc/μg) dissolved in 0.05 ml of plasma from vitamin D-deficient rats was injected intravenously via the jugular vein (27). The radiochemical purity of [1,2-^3H] vitamin D_3 was assessed by chromatography on silicic acid[1] columns, UV spectrophotometry (λ maximum 265 mμ; ϵ, 16,300), and biological assay using the line test method in rachitic rats (28). "Hepatectomized" rats had a very limited survival, and were sacrificed after 4 hr. As already described (7, 26) plasma, liver, and the two target tissues of vitamin D action, bones and small intestines, were analyzed for total tritium content after combustion analysis (^3H) and after lipid extraction in chloroform-methanol-water system (30). The "chloroform-soluble" metabolites of vitamin D_3 were separated by chromatography on silicic acid[1] columns (55 × 1.5 cm). Samples dissolved in 5-10 ml of redistilled petroleum ether (bp 67°C) were applied on columns and subsequently chromatographed by gradients of solvents of increasing polarity in the given order: (*a*) exponential gradient obtained by superimposing a holding chamber containing 250 ml of 75% diethyl ether in petroleum ether on a constant volume, Erlenmeyer mixing chamber containing 250 ml of 100% petroleum ether; (*b*) exponential gradient by adding 450 ml of 100% diethyl ether to the empty holding chamber; (*c*) exponential gradient by adding to the holding chamber 300 ml of 5% methanol in diethyl ether; (*d*) exponential gradient by adding to the holding chamber 200 ml of 50% methanol in diethyl ether; and (*e*) batch elution with 200 ml of absolute methanol applied on the column without the intermediate mixing chamber.

10-ml fractions were collected and their radioactivity measured. The chromatographic peaks were estimated as per cent of the radioactivity chromatographed, and the total amount of these metabolites in each plasma or tissues was expressed as per cent of the vitamin D_3-^3H dose. "Aqueous-soluble" and "nonextractable" radioactivities were separately recorded.

Tritiated water resulting from combustion was taken up in combustion counting solution, made of 4 g of 2,5-diphenyloxazole (PPO), 50 mg of dimethyl *p*-bis[2-(5-phenyloxazolyl)] benzene (dimethyl-POPOP), 200 ml of absolute ethanol, and toluene AR ad 1 liter (26). The other samples were dried in counting vials and dissolved in toluene counting solution consisting of 2.0 g of PPO and 100 mg of dimethyl POPOP in 1 liter of toluene. Some samples were dissolved in 0.5-2.0 ml of "NCS" solubilizer[2] (31) before adding the toluene counting solution. All the radioactivity measurements were performed in triplicate with a Packard Tri-Carb 3003 liquid scintillation counter equipped with an automatic external standardization system 3951 (32).

The metabolism of vitamin D_3-^3H into 25-hydroxycholecalciferol-^3H and other metabolites during a 4 hr period was compared in three sets of control experiments. Using vitamin D-deficient rats of equivalent weight (*a*) a group of 4 "intact rats" were injected intravenously with 10 IU [1,2-^3H]-vitamin D_3 without previous surgery. Plasma and tissues were analyzed as in the "hepatectomized rats." (*b*) A group of three "stress rats" were bled to ½ of their blood volume by cardiac puncture under ether anesthesia, then operated by laparotomy and manipulation of the viscera immediately before the intravenous injection of labeled vitamin D_3. Only plasma was analyzed. (*c*) Two rats received a portocaval shunt without ligation of the hepatic artery 4 days before the administration of the tritiated vitamin D_3 ("shunt rats")

RESULTS

Plasma and tissue metabolites in "hepatectomized" and "intact" rats. Table I shows that there was no difference in the plasma radioactivities and its partitioning between "hepatectomized" (HPX) and intact rats. The presence of 9.6% of the dose in the livers of "hepatectomized" rats whose portal vein and hepatic artery were severed was surprising. Since all peritoneal and visceral adherences were freed during the operation, the most likely explanation is a reflux of blood from the vena cava to the liver through the nonligated hepatic vein. Small intestines and bones of intact rats seemed to contain more tritium and aqueous-soluble radioactivity than the hepatectomized rats but the difference might not be significant.

TABLE I

Plasma and Tissue Radioactivities 4 hr after Intravenous Injection of 10 IU Vitamin D_3-^3H

Samples		Total RA*	Chloroform-soluble	Aqueous-soluble	Non-extractable
		% dose	% of sample radioactivity		
Plasma	HPX	22.8 ± 1.5	99.2	0.8	0
	Intact	20.6 ± 0.9	99.6	0.4	0.05
Liver	HPX	9.6	99.0	0.9	0.1
	Intact	31.7	99.1	0.5	0.4
Small intestine	HPX	1.1	93.3	7.1	0
	Intact	2.5	85.4	12.0	2.6
Bones	HPX	8.7	91.0	5.9	4.5
	Intact	12.8	85.4	14.9	0.6

HPX = "hepatectomized" rats.

* Mean ± S.E.M. or range for the "total radioactivity" in plasma. Other data represent value of pooled samples of 3 rats.

[1] Silicic acid, 100 mesh, Mallinckrodt, St. Louis, Mo.

[2] Nuclear-Chicago Corporation, Chicago, Ill.

TABLE II

Plasma and Tissue Chromatographic Fractions 4 hr after Intravenous Injection of 10 IU Vitamin D_2-^3H

Samples		I	II_a	II	III	IV_a	IV	V_{a+b}	VI_{a+b}	VII_{a+b}
Plasma	HPX	0.2 ±0.06		1.6 ±0.2	93.2 ±0.4	0.7	1.2 ±0.1	1.0 ±0.2	0.7 ±0.2	0.5 ±0.1
	Intact	0.3	0.2	1.9	73.1	1.1	16.0	4.3	1.4	0.9
Liver	HPX	0.6	0.2	11.3	81.4		1.2	1.4	0.8	1.1
	Intact	1.1	1.1	3.1	88.8	--	1.7	1.2	1.2	0.3
Small intestine	HPX	0.4	0.5	6.2	83.6	-	1.8	3.3	0.6	2.5
	Intact	1.5	0.1	3.0	65.1	1.6	14.1	4.8	1.5	3.2
Bones	HPX	2.7	-	7.3	80.1		0.8	3.4	2.1	1.5
	Intact	1.8	0.3	5.0	75.7	1.4	8.9	3.3	1.3	0.5

The data are expressed as per cent of chromatographed radioactivity. Mean ±SEM are given for the plasma of hepatectomized rats. Other data represent values from pooled samples of 4 rats.

It will be reported separately that the present chromatographic system separates up to 12 distinct radioactive peaks from the chloroform extracts of plasma and tissues after injection of tritiated vitamin D_2.

Only peaks I, III, and IV have been so far identified as respectively vitamin D_2 esters (33), unaltered vitamin D_2 (34), and 25-hydroxycholecalciferol (1, 2). In addition to the most biologically active metabolite 25-hydroxycholecalciferol and unaltered vitamin D_2, only the esters and peak II display consistent but smaller antirachitic activity (3, 34, 35).[1]

4 hr after intravenous vitamin D_2-^3H, most of the radioactivity in the plasma and tissues of intact rats was accounted for by the unchanged vitamin (peak III) and 25-hydroxycholecalciferol (peak IV) (Table II). In contrast, the proportion of peak IV is vanishingly small in the plasma and tissues of "hepatectomized" animals. The significance of such small amounts is questionable, since it is difficult to clearly define a faint chromatographic peak as shown in Fig. 1. This figure compares the chromatographic profile of lipid extracts of plasma from one "hepatectomized" rat with that from a pool of four intact rats. The individual chromatographic profiles obtained in each "hepatectomized" rat were very similar as indicated by the small SEM in Table II. It is quite possible that the traces of peak IV observed in our hepatectomized rats originate from the incompletely isolated liver. The radioactivity found in these livers (Table I) suggests that some blood has refluxed in through the nonligated hepatic vein. The minimal amounts of 25-hydroxycholecalciferol thus formed in functionally impaired hepatocytes might find their way out to the plasma as well.

It may be concluded that hepatectomized rats do not hydroxylate a significant proportion of vitamin D_2 into 25-hydroxycholecalciferol. However, the possibility that surgical stress might interfere with the hydroxylation of vitamin D_2 in other tissues required further investigation.

Plasma vitamin D_2 metabolites in "stress" and "shunt" rats. Since 25-hydroxycholecalciferol accumulated mainly in the plasma compartment the following experiments were limited to the plasma. In addition to the evaluation of the surgical stress, the ability of the rats with only a portocaval shunt to metabolize vitamin D

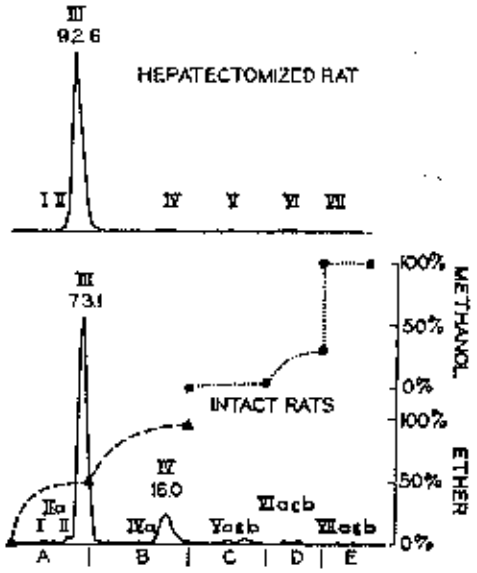

FIGURE 1. Silicic acid column chromatographic profile of radioactivity from lipid extract of plasma obtained 4 hr after i.v. injection of 10 IU [1,2-^3H]-vitamin D_2 to vitamin D-deficient "hepatectomized" and "intact" rats. The upper profile represents the plasma of one individual "hepatectomized" rat, and the lower profile corresponds to a pool of plasma from four intact rats.

[1] Ponchon, G., and H. F. DeLuca. Metabolites of vitamin D_2 and their biologic activity. *J. Nutr.* in press.

TABLE III

Plasma Chromatographic Fractions in "Stress" and "Shunt" Rats 4 hr after Intravenous Injection of 10 IU Vitamin D_3 ^3H

Groups of rats	I	IIa	II	III	IVa	IV	Va+b	VIa+b	VIIa+b
Stress	0.2	0.2	1.0	72.1	0.4	18.3	2.6	1.7	2.0
Shunt	0.5	0.1	1.7	82.7	1.0	6.4	3.2	1.2	1.5

The data are expressed as per cent of chromatographed radioactivity. The values are from pooled samples of 3 "stress" and 2 "shunt" rats.

was compared with the "intact" and "hepatectomized" animals. It is evident from Table III that the surgical stress did not interfere with the normal conversion of vitamin D_3 into 25-hydroxycholecalciferol. The plasma radioactivity found in the form of 25-hydroxycholecalciferol (18.3% in peak IV) was similar to the 16.0% seen in "intact" animals. On the other hand, the creation of a portocaval shunt seemed to handicap the ability of the rats to hydroxylate vitamin D_3 into 25-hydroxycholecalciferol. Only 6.4% of the plasma radioactivity was represented by this metabolite in the shunted group.

The "stress" rats which have lost more than 2 of their blood volume have less radioactivity in their plasma compartment [11.6 ±1.5% (3)* of dose] than intact rats [20.6 ±0.9% (4)]. Since vitamin D and 25-hydroxycholecalciferol are known to bind to certain plasma proteins (36, 39), a decrease of these carriers by blood loss likewise might well reduce the proportion of the dose held in the plasma compartment.

"Shunt" rats seem to have a lower level of plasma radioactivity (17.3 and 15.5% of dose) than the "intact" animals. As a result of their smaller conversion of vitamin D_3 into 25-hydroxycholecalciferol, the quantity of this metabolite in plasma is much reduced (1.0% of dose).

DISCUSSION

Whereas the role of the mammalian liver as a storage organ for vitamin D is still a matter of debate (40), its function in the early metabolic events of vitamin D becomes more and more evident. Kodicek, Cruickshank, and Ashby (41) were the first to call the attention to the very rapid and large uptake of injected labeled vitamin D by the liver (more than 50% of the dose in 30 min). This is apparently the case with doses ranging from 0.1 to 1000 μg (26, 42, 43). The quick release of this radioactivity from the liver strongly suggests an important metabolic function of the liver (7, 40). Previous observations led us to suggest that the liver was responsible for the conversion of vitamin D_3 into its active metabolite, 25-hydroxycholecalciferol (7).

The present experiments demonstrate that the liver is necessary indeed for the biologic hydroxylation of vitamin D_3 into its metabolite, 25-hydroxycholecalciferol, in rats. "Hepatectomy" results in an absence of this metabolite in the plasma and tissues of rats while impaired liver function by portocaval shunt reduced its proportion. That this effect is not due to the metabolic disorders induced by surgical stress is shown by an entirely normal production of 25-hydroxycholecalciferol in shunt-operated and bled rats. However, one cannot definitely refute the criticism that metabolic alterations due to acute liver insufficiency itself might interfere with the normal conversion of vitamin D possibly occurring in other tissues. It seems unlikely however that it would result in a total absence of 25-hydroxycholecalciferol. On the other hand the possibility that some tissues other than the liver could hydroxylate vitamin D_3 at very high concentrations of vitamin D_3 substrate may not be regarded as a physiologic mechanism and is out of the scope of this work.

Theoretically the liver could participate in the formation of 25-hydroxycholecalciferol in two different ways: (a) by converting vitamin D into an intermediate compound subsequently transported to other tissues where 25-hydroxycholecalciferol would be made, or (b) by direct hydroxylation of vitamin D_3 in the hepatocytes. The formation of an intermediate is not substantiated by the failure to observe such a precursor in blood or tissues. Another possibility suggested earlier (39) is that the liver might liberate vitamin D from its lipoprotein complex and facilitate its binding to specific plasma proteins. These carriers would transport efficiently vitamin D to peripheral tissues where the hydroxylation would occur. However, vitamin D binds also to these proteins when added to serum in vitro, and therefore this function of the liver would not be essential.

Rather, all evidence supports the idea that the liver directly hydroxylates vitamin D_3 into 25-hydroxycholecalciferol. Previous work (7) has shown that the appearance of 25-hydroxycholecalciferol in plasma is coupled with the release of radioactive material from the liver without a time lag. A delay would have been expected if an intermediary metabolite had been formed. Moreover, work in progress in our laboratory (44) shows that perfused isolated liver of rats converts vitamin D into 25-hydroxycholecalciferol and in vitro incubation of rat liver homogenate effects the same hydroxylation.

Surprisingly, only a very small proportion of the liver radioactivity is represented by 25-hydroxycholecalciferol, whereas the bulk of it is still unchanged vitamin D_3-^3H.

* Mean ±SEM (number of rats).

Although this proportion increased with time after the injection of vitamin D_3-^3H, the liver has characteristically a much lower concentration of 25-hydroxycholecalciferol than the plasma, bone, and intestine.[3] It may be suggested that the accumulation of 25-hydroxycholecalciferol is dependent upon specific receptor sites found in the target tissues but not in the liver (45, 46).

It has long been recognized that severe liver insufficiency results in abnormalities of the skeleton and disturbances in calcium-phosphorus metabolism (14-16, 18-23, 47-52). However, unclear definition of the bone lesions and hepatic disease was a cause of controversy. The skeleton becomes consistently osteopenic with various degrees of osteoporosis and osteomalacia or rickets. Although it appears that osteoporotic changes do not usually respond to vitamin D therapy alone (18, 20, 49, 52), several clinical observations emphasize the higher requirements for vitamin D even in the cases of osteomalacia or rickets (15, 17, 18-23). Since osteoporosis and malacia are most often associated we will use the general term of "hepatic osteodystrophy" for the sake of simplicity.

The main question is how the liver is involved in these metabolic disorders. Simple biliary deficiency as encountered in the experimental or clinical biliary fistulae should be distinguished from alteration in the hepatic parenchyma (liver cirrhosis). Unfortunately, severe chronic liver insufficiency usually associates in various proportions with cirrhosis and bile retention. It is now clear that bile salts improve absorption of calcium (8, 9) and are necessary for the absorption of vitamin D (10-14). This certainly accounts to a large extent for the osteodystrophy resistant to oral administration of vitamin D observed in biliary obstruction without major liver parenchymal damage or in bile fistulae (11, 12, 53, 54). However, as pointed out by Atkinson et al. (20) the evolution of the bone lesions in severe hepatocellular deficiency is much faster than one would expect from simple malabsorption of vitamin D. The authors stated that in such cases "it seems possible that destruction of liver cells may interfere with storage or metabolism of vitamin D." Parenteral treatment with large doses of vitamin D, in addition to other measures, was advocated to match the higher requirements for vitamin D.

In 1931, Greaves and Schmidt showed that vitamin D_2 given orally or subcutaneously to jaundiced rachitic rats does not heal the rickets as it does in the normal rachitic animals. But they suggested this was due to metabolic impairment of the osteogenic cells by the jaundice (55).

The present experiments provide evidence that the liver contributes and (or) is necessary in the rats to the effect of vitamin D_3 by converting it into its active form, 25-hydroxycholecalciferol. Since the metabolic pathway of vitamin D appears qualitatively similar (6, 40) we suggest that gross liver insufficiency in man results in a deficient formation of 25-hydroxycholecalciferol or of an equivalent vitamin D metabolite.

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Metabolites of Vitamin D_3 and Their Biologic Activity [1,2]

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ABSTRACT The metabolism of vitamin D_3 has been studied after intravenous injection of 10 IU [1, 2-^{3}H] vitamin D_3 to vitamin D-deficient rats. A new chromatographic system achieved the separation of unchanged vitamin D and 11 radioactive metabolites of which only one (25-hydroxycholecalciferol) exhibits intense antirachitic activity. An important proportion of the radioactivity in bones and small intestine is represented by this active metabolite. By way of contrast, liver and kidneys accumulate selectively unchanged D_3. Plasma is expected contains large amounts of 25-hydroxycholecalciferol.

An elucidation of the mechanism of action of vitamin D requires an understanding of its pathway of metabolism including the identification of its products and intermediates (1). Kodicek (2) was the first to report biologically inactive "breakdown products" of vitamin D all of which remain unidentified. Work done in our laboratory demonstrated clearly the presence of at least three metabolic fractions (peaks I, II and IV) derived from vitamins D_2, D_3 and D_4 in lipid extracts of blood and various tissues of vitamin D-deficient rats (1, 3–6). Of these, peak I has been positively identified as an ester of vitamin D and long-chain fatty acids (5). Peak II still remains unidentified but is of minor significance. Peak IV, however, proved to be of great interest. Not only was it as effective as vitamin D itself in curing rickets but it acted more rapidly than vitamin D in stimulating intestinal transport of calcium. Thus, it was thought to contain the metabolically active form of the vitamin (1, 4, 7). This fraction, however, appeared to be heterogeneous, composed of more than one metabolite.[3] Hence, it was necessary to develop a chromatographic system which would resolve these components. It is the purpose of this report to describe such a procedure which separates peak IV into six components. Of these six, only one retains the ability to cure rickets in rats, which probably represents the metabolically active form of vitamin D. The new chromatographic system presented here achieves the separation of 11 metabolites of the vitamin in the rat. This separation has now made possible the successful isolation and identification of 25-hydroxycholecalciferol, the metabolically active form of vitamin D (8, 9).

MATERIALS AND METHODS

Radioactive vitamin D_3. [1, 2-^{3}H]-Vitamin D_3, synthesized in this laboratory (10), was purified by chromatography on a column of silicic acid and stored in benzene solution. The purity of the radioactive preparation was assessed by ultraviolet spectrophotometry, biological assay (Ionest) (11), and chromatographic behavior as previously described (10). The specific activity was 24,053 dpm/IU or 0.44 μCi/μg. For intravenous injections, an appropriate amount of the benzene stock solution was dried under N_2 and redissolved in plasma from vitamin D-deficient rats to achieve a concentration of 200 IU/ml.

[4-^{14}C]-Vitamin D_3[4] was purified by silicic acid chromatography (10) and stored in benzene at 4°. Proper dilution with nonradioactive crystalline vitamin D_3[5] reduced the specific activity to 51.8 dpm/IU or 9.33×10^{-4} μCi/μg. The intravenous doses were prepared by removing the solvent under nitrogen and redissolving the vitamin D_3 in absolute ethanol at a concentration of 200,000 IU/ml.

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[3] Neville, P. F., J. Lund, H. Morii and H. F. DeLuca 1967 Biologically active metabolites of vitamin D Federation Proc., 26: 697 (abstract).
[4] Purchased from Philips-Duphar, Weesp, The Netherlands.
[5] Cholecalciferol, USP XVII, Philips Duphar.

Animals. Male, weanling rats of the Holtzman strain, housed in individual hanging wire cages were fed the vitamin D-free diet no. 11 (0.4% Ca, 0.3% P) of Guroff et al. (12) for 3 to 5 weeks. At this time the rats were vitamin D-deficient but not rachitic and weighed an average of 150 g. Throughout the experimental period, they were fed the same diet.

Extraction of tissues. Extractions were carried out with a mixture of chloroform–methanol–water according to the procedure of Bligh and Dyer (13) modified in this laboratory (14). The chloroform extracts were evaporated to dryness in a flash evaporator under water-pump vacuum at 35° and redissolved in 5 or 10 ml redistilled (67°) petroleum ether (Skellysolve-B) before chromatography. Three aliquots of lipid extract in Skellysolve-B were pipetted into counting vials for measurement of the chloroform-soluble radioactivity. The aqueous soluble phase and nonextractable precipitates were separately collected for radioactivity measurements. Before counting, the precipitates were washed three times with distilled water which was added to the aqueous soluble phases (Fig. 1).

Chromatography of vitamin D metabolites. Tissues and plasma extracts in redistilled Skellysolve-B solution were subjected to column chromatography on silicic acid. Prior to use, silicic acid, 100 mesh,[a] was treated according to Hirsch and Ahrens (14). Glass columns cooled to 10° by a water jacket were filled with a slurry of 35 g silicic acid in Skellysolve-B. Packed under a pressure of 3 psi, the adsorbent formed a column of 50 cm by 1.5 cm which gave a flow rate of approximately 1.5 ml/minute. Samples were applied to the column in 5 to 10 ml Skellysolve-B, followed by a 5-ml Skellysolve-B rinse. Adsorbed samples were then eluted with the following solvent system in the order given: 1) a hyperbolic gradient zero to 50% diethyl ether in Skellysolve-B was obtained by superimposing a holding chamber containing 250 ml of 75% diethyl ether in petroleum ether on a constant volume Erlenmeyer mixing chamber containing 250 ml of 100% petroleum ether (15); 2) a hyperbolic gradient 50 to 94% diethyl ether in Skellysolve-B was achieved by adding to the empty holding chamber 400 ml of 100% diethyl ether; 3) a hyperbolic gradient from 6% Skellysolve-B, 94% ether to 1.6% Skellysolve-B, 94.8% ether, 3.6% absolute methanol was obtained by adding 300 ml of 5% methanol in diethyl ether to the empty holding chamber, 4) a hyperbolic gradient from 1.6% Skellysolve-B, 94.8% ether, 3.6% methanol to 0.7% Skellysolve-B, 68.7% ether, 30.6% methanol was then utilized by adding 200 ml of 50% methanol in diethyl ether to

[a] Mallinckrodt Chemical Works, St. Louis, Mo.

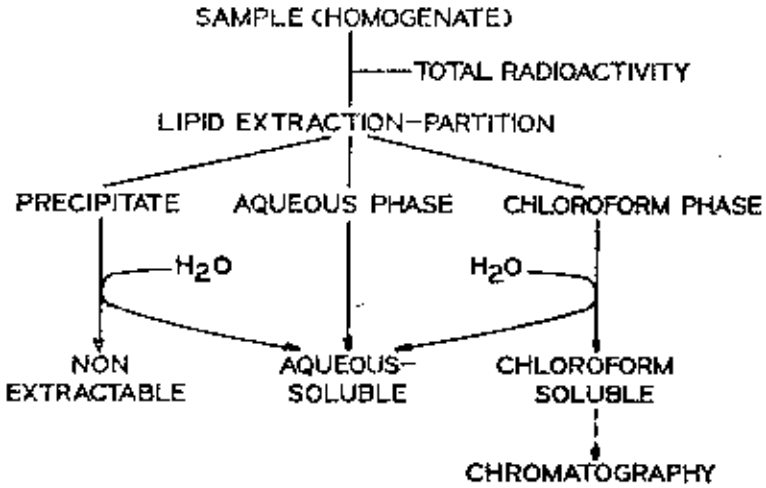

Fig 1 Flow-sheet of tissue radioactivity measurements.

the empty holding chamber; and 5) finally, an addition of 200 ml absolute methanol directly applied on the column without the intermediate mixing chamber concluded the elution.

The effluents were collected as 10-ml fractions directly in glass counting vials for radioactivity measurements. When biological assays were performed, 10-ml fractions were collected in test tubes and 0.5 ml was transferred to glass counting vials for radioactivity measurements while the remaining 9.5 ml was kept for bioassays.

Biological assays. After the radioactivity profile had been determined, the fractions from each peak were pooled; the solvent was removed with a flash evaporator and the residue redissolved in a small volume of diethyl ether. On the basis of their measured radioactivity and the specific activity of the parent ^{14}C-vitamin D_3 (51.8 dpm/IU), a calculated amount of cottonseed oil[5] was added, and the ether removed with nitrogen giving a solution containing the equivalent of 40 IU vitamin D/ml oil. Until used for bioassays, the oily preparations were kept frozen at −10°.

The newly isolated metabolites were assayed for their antirachitic activity in the rat according to official USP procedures (11) (line-test). The rats were dosed with the equivalent of 4 IU vitamin D in 0.1 ml oil by gastric tube, on the assumption that the specific activity of each metabolite is equal to that of the parent ^{14}C-vitamin D_3. In addition, positive reference rats were fed 4 IU standard vitamin D_3 in 0.1 ml cottonseed oil, and negative controls received 0.1 ml pure cottonseed oil alone.

Radioactivity measurements. The fractions were dried in an air stream under an infrared lamp and counted in a toluene counting solution consisting of 2.0 g PPO and 100 mg dimethyl POPOP/liter toluene Alt.

The "dose-standards" were measured by delivering into counting vials in triplicate the same dose of labeled vitamin D as was injected into the rats. [1, 2-^{3}H]-Vitamin D_3, 0.05 ml in plasma was evaporated to dryness and dissolved in 0.5 ml Nuclear Counting Solution reagent[6] (16) before adding the toluene counting solution.

The radioactivity in each lipid extract submitted to chromatography was also determined and is referred to as chloroform-soluble radioactivity. Aliquots of the aqueous phases from each extraction were dried in counting vials, redissolved in a few drops of distilled water and 2 ml N.C.S. reagent[7] and finally mixed with toluene-counting solution. This gave the aqueous-soluble radioactivity. The nonextractable radioactivity was obtained by dissolving the washed precipitate in N.C.S. reagent, and counting in toluene-counting solution. To determine the total radioactivity in different organs, tissue samples in triplicate were combusted according to the procedure of Kelly et al. (17), using a Thomas Ogg solvent igniter.[8] Weighed liver or kidney slices and small intestinal segments were inserted in cellophane combustion envelopes[9] and lyophilized before combustion. Whole femurs were weighed, split to remove the bone marrow, and dried by lyophilization in preparation for combustion. The tritiated water resulting from the combustion was dissolved and counted in combustion-counting solution consisting of 6 g PPO, 50 mg dimethyl POPOP, 300 ml absolute ethanol, and toluene Alt to 1 l (10). Alternatively, total radioactivity in tissues was calculated as the sum of the chloroform-soluble, aqueous soluble and nonextractable radioactivity.

Total plasma radioactivity was determined in triplicate on samples dissolved in N.C.S. reagent[7] and mixed with toluene-counting solution. All radioactivity measurements were performed with a liquid scintillation counter[10] equipped with an automatic external standardization system 3951 (18). Counting efficiencies for ^{3}H were of the order of 25 to 35% with the toluene-counting solution, and 15 to 25% with the combustion-counting solution.

Experimental procedure. Ten international units [1,2-^{3}H]-vitamin D_3 in 0.05 ml plasma solution were injected intravenously, via the jugular vein, to eight vita-

[5] Wesson Oil, Wesson Sales Company, Fullerton, Calif.
[6] Nuclear Chicago Corporation, Des Plaines, Ill.
[7] See footnote 6.
[8] A. H. Thomas Company, Philadelphia, Penna.
[9] Ivers-Lee Company, N. J.
[10] See footnote 6.
[10] Packard Tri-Carb, Packard Instrument Company, Inc., Downers Grove, Ill.

min D-deficient rats under light ether anesthesia. After 4 and 24 hours, respectively, two groups of four rats each were exsanguinated by cardiac puncture under ether anesthesia. The plasma was separated from the heparinized blood by centrifugation within 2 hours and its total radioactivity was determined. The radioactivity in the total blood plasma was calculated from an estimated plasma volume equivalent to 3% of body weight. Storage of the blood plasma as well as of any tissue was done at −10° until chromatography.

Liver and kidneys were removed by dissection, rinsed in 0.9% NaCl, blotted on absorbent paper, and weighed before being frozen until extraction.

The long bones (femurs, tibias, humeri, ulnas and radii) were dissected from adhering muscles and ligaments; they were weighed, split to remove the bone marrow, and frozen until they could be extracted.

The small intestine was removed, dissected from the mesentery, cut into segments which were gently squeezed with forceps to expel the intestinal contents, and rinsed with 0.9% NaCl before being frozen.

To collect large amounts of metabolites for bioassays, 10,000 IU of [4-^{14}C]-vitamin D_3 in 0.05 ml absolute ethanol were injected intravenously via the jugular vein to 10 vitamin D-deficient rats. After 18 hours, the animals were exsanguinated through cardiac puncture under ether anesthesia. Plasma and tissues were prepared, extracted and chromatographed as described above. The isolated radioactive fractions were subsequently tested for their biopotency, as described under biological assays.

RESULTS

As shown by its chromatographic pattern (fig. 2), the labeled [1, 2-^{3}H]-vitamin D_3 dissolved in plasma was free of any polar contaminants, although it contained a small amount (3.6%) of less polar material. This represents presumably some previtamin D_3 in thermal equilibrium with the vitamin (19). Nevertheless, the ultraviolet spectrum and the biological activity determined by the line-test assay were consistent with a specific activity of 24,453 dpm/IU.

The distribution of the radioactivity in plasma and tissues after intravenous injection of 10 IU [1, 2-^{3}H]-vitamin D_3 to vitamin D-deficient rats is given in table 1 and 2. Four hours after the dose (table 1), the liver had accumulated more radioactivity than the blood or any other tissue (34.2%). Following in decreasing order are the plasma compartment (20.6%), the skele-

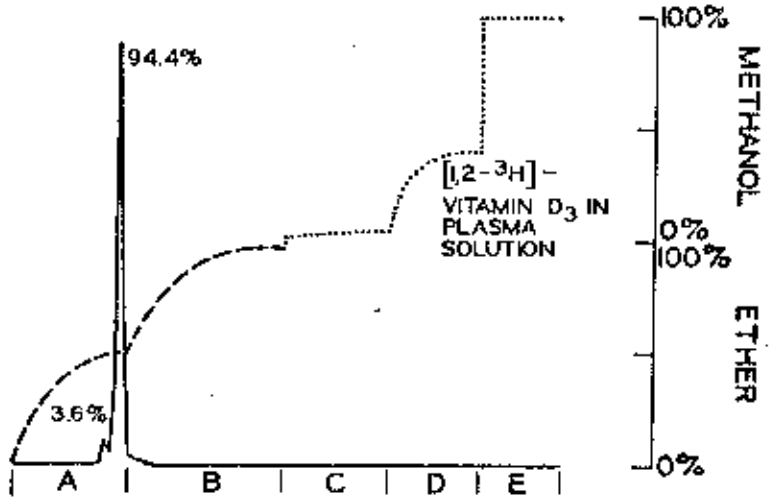

Fig. 2 Silicic acid column profile of radioactivity from [1, 2-^{3}H]-vitamin D_3 dissolved in D-deficient plasma (dose).

[illegible] (12.3%), the kidneys (7.9%), and the small intestine [illegible]. Practically all the radioactivity in plasma, liver and kidneys (over 90%) was lipid soluble, and partitioned in the chloroform phase during the extraction. In contrast, a significant amount of water-soluble radioactive material was present in the small intestine (12.0%) and the bone (11.0%). Almost no radioactivity remained unextracted [illegible] in the [illegible] of the small intestine.

Twenty-four hours after dosage [illegible] activities [illegible] 10.0 and [illegible] respectively [illegible] and [illegible] significant [illegible] smaller [illegible] radioactivity [illegible] all the radioactivity was still [illegible] in increasing portion [illegible] 8% [illegible] radioactivity [illegible] the precipitates.

The relative incorporation [illegible] the fraction [illegible] the tissue [illegible] of the concentration of [illegible] and [illegible] a striking concentration [illegible] liver (20.5%) [illegible] kidneys (21.1%) compared [illegible] concentrations in small [illegible] and bone (1.2%). While [illegible] incorporation did not change [illegible] at 24 hours in the small [illegible] and bones (1.2%) [illegible] in the kidneys to reach the very [illegible] of 31.2% [illegible].

In highly heterogeneous tissues [illegible] bone and small intestine [illegible] to expect the [illegible] of dose in the cells [illegible] (10), bone cells [illegible] of the dry weight of [illegible] and would have [illegible] of 30 and [illegible] after 4 and 29 hours [illegible] The small intestine is assumed [illegible] of 80% mucosa and 70% muscle [illegible] tissue. It has already [illegible] that muscle has a very [illegible] incorporation decreasing [illegible]

TABLE 2

Total radioactivity in plasma and tissues 24 hours after intravenous injection of 0.25 μg [1,2-^{3}H]-vitamin D_3 to vitamin D-deficient rats

	Plasma		Small intestine		Skeleton		Liver		Kidneys	
	Sample radioactivity	Dose	Sample radioactivity	Dose	Sample radioactivity	Dose	Sample radioactivity	Dose	Sample radioactivity	Dose
	%	%	%	%	%	%	%	%	%	%
Chloroform soluble	99.1[1]	10.3	83.5	1.5	90.6	14.3	87.2	13.0	93.3	17.5
Aqueous soluble	0.9	0.09	7.6	0.1	3.2	0.8	4.9	0.7	1.2	0.2
Nonextractable			8.9	0.2	4.2	0.7	7.9	1.2	5.6	1.1
Total radioactivity	100	10.6 ±0.67(4)[2]	100	1.6	100	16.0 ±0.97(4)	100	14.9 ±1.8(4)	100	18.8 ±1.1(4)

[1] Single data represent values for pooled samples from four rats. SEM are given for separate measurements.
[2] Mean ± SEM (number of rats).

elapses after dose (10). One can roughly estimate that the mucosal cells accumulate about 10.9 and 12% of dose/g of dry tissue after 4 and 24 hours, respectively.

The chloroform phases of plasma and tissue extracts, obtained 4 and 24 hours after dose, have been chromatographed as described in Methods. Figure 3 illustrates a characteristic chromatographic pattern obtained from the plasma 24 hours after dose. All the chromatographic patterns are similar except for the relative proportion of radioactive peaks, which are given at 4 hours (table 4) and 24 hours (table 5) after dose. Twelve radioactive fractions were consistently observed, although some of them were incompletely separated (Va and Vb, VIa and VIb), or represented only minimal amounts (IIa, IVa, VIb, and VIIb). In such instances, their combined amounts were reported.

Fractions I, II and III had already been separated in our laboratory, using a chromatographic technique similar to the present one in its starting solvent system (3, 5). Fractions I and III have been identified as vitamin D esters and unchanged vitamin D, respectively, while fraction II has not yet been characterized. The newly isolated fraction IIa was previously included either in fraction I or II. Its composition is not known. The so-called "peak IV" active metabolite of vitamin D previously discovered by the same authors has now been resolved into six sub-fractions IVa, IV, Va and Vb, VIa and VIb, by means of a refined gradient system. Fractions IVa and IV were eluted at a concentration of about 90% ether in Skellysolve-B. Fractions Va and Vb were eluted by a very small concentration of approximately 3% methanol in ether (95%)–Skellysolve-B (2%), while VIa and VIb came out with 25% methanol in ether (74%)–Skellysolve B (1%). As shown later, the biological activity was specific to the purified fraction IV. These fractions have not been characterized, except the biologically active "peak IV" which has been most recently identified as 25-hydroxycholecalciferol in our laboratory (8, 9).

Fractions VIIa and VIIb were eluted by 100% methanol, and were probably not brought off the column with the solvent

TABLE 3

Relative incorporation of dose in tissues 4 and 24 hours after intravenous injection of 10 IU [1,2-^{3}H]-vitamin D_3 to vitamin D-deficient rats

Time interval	Small intestine	Skeleton	Liver	Kidneys
hr		% dose/g dry tissue		
4	3.4 [1] 10.9 [3]	1.2±0.6(4) [2] 30.0 [3]	20.8	24.8
24	3.7 12.0 [3]	2.2±0.2(4) 55.0 [3]	13.7±1.7(4)	81.3±5.6(4)

[1] Single data represent values for pooled samples from four rats. SEM are given for separate measurements.
[2] Mean ± SEM (number of rats).
[3] Corrected values for small intestine mucosal cells and bone cells (see text).

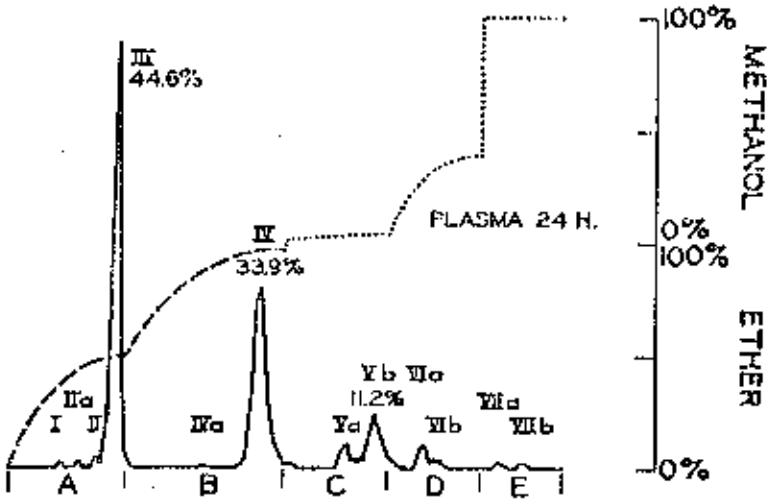

Fig. 3 Silicic acid column profile of radioactivity from pooled lipid extracts of plasma obtained 24 hours after intravenous injection of 10 IU [1,2-^{3}H]-vitamin D_3 to four vitamin D-deficient rats.

TABLE 4

Chromatographic fractions of plasma and tissues extracts 4 hours after intravenous injection of 10 IU [1,2-^{3}H]-vitamin D_3 to vitamin D-deficient rats

Chromatographic fractions	Plasma	Small intestine	Skeleton	Liver	Kidneys
I	0.3 [1]	1.5	1.8	1.1	1.0
IIa	0.2	0.4	0.3	1.1	0.8
II	1.9	3.0	5.0	3.1	3.2
III	73.1	62.2	75.7	88.8	76.6
IVa	—	1.6	1.4	--	1.0
IV	16.0	14.1	9.0	1.7	3.9
Va	1.6	2.0	1.0	0.8	2.8
Vb	2.6	2.8	2.3	0.6	2.4
VIa	0.9	4.0	0.9	0.7	3.1
VIb	0.4	0.5	0.4		
VIIa	0.7	2.5	0.4	0.3	1.9
VIIb	0.3	0.7	0.1	--	0.3

[1] All values are percent of total chromatographed radioactivity from pooled samples of four rats.

TABLE 5

Chromatographic fractions of plasma and tissues extracts 24 hours after intravenous injection of 10 IU [1,2-^{3}H]-vitamin D_3 to vitamin D-deficient rats

Chromatographic fractions	Plasma	Small intestine	Skeleton	Liver	Kidneys
I	0.5 [1]	4.4	1.5	2.8	2.7
IIa	0.6	2.1	2.0	1.5	0.8
II	0.8	4.0	4.2	3.2	4.0
III	44.6	38.8	53.5	75.9	73.6
IVa	0.4	—	2.1	—	—
IV	33.9	16.9	16.0	10.6	8.1
Va	2.5	16.3	9.0	2.5	1.8
Vb	11.2				
VIa	2.0				
VIb	0.8	2.2	2.5	—	1.8
VIIa	0.3	2.9	2.2	1.1	3.3
VIIb	0.3	2.4	1.7	0.4	2.4

[1] All values are percent of total chromatographed radioactivity from pooled samples of four rats.

gradient used previously (3, 4) which did not produce over 57% methanol concentration in the effluent. These fractions have not been identified. Further stepwise elution with 150 ml water-methanol (10:90) did not bring any more radioactivity from the column from any of the extracts.

Overall recoveries of radioactivity from chromatographed material varied between 95 and 102%.

The four major fractions (III, IV, Va and Vb) usually represent over 80% of the chromatographed radioactive material. It is evident that 24 hours as well as 4 hours after dose, the major radioactive fraction in plasma and all tissues is the "peak III," unchanged vitamin D_3. This fraction decreased significantly in all cases, between 4 and 24 hours after dose. Concomitantly, there was a relative increase in the proportion of peaks IV and Va + b (tables 4 and 5). It should be noted that plasma, small intestine and skeleton contained the highest proportion of peak IV and Va + b both 4 and 24 hours after dosage. The minor fractions, I, IIa, II, VIa +b, and VIIa + b, tended to increase in all samples, from 4 to 24 hours after dosage. The relative proportion of these substances was higher in all tissues than in plasma.

Tables 6 and 7 give the absolute amounts of fractions I, II, III, IV and Va + b in plasma and tissues 4 and 24 hours after dose, respectively. Four hours after dose, the liver had accumulated the largest fraction of the unchanged vitamin D_3, 30.1% of the injected dose (table 6). The remainder of the unchanged vitamin D_3 was distributed mostly in the plasma compartment (15.0% of dose), skeleton (8.2%) and kidneys (6.0%). The small intestine, owing to its very small accumulation of dose, contained only a small fraction of the vitamin D and metabolites. Most of the "peak IV" metabolite was present in the plasma, where it represented 3.3% of the dose. Fractions I, II and Va + b were less than 1% of the dose in any of the tissues or plasma examined. Twenty-four hours after an intravenous dose (table 7) the amount of unchanged vitamin D had decreased considerably in the plasma (17.1 down to 4.7% of dose) and the liver (30.1 to 9.0% of dose), compared with the 4-hour period. It also decreased in small intestine (1.31 to 0.6%), but did not change much in skeleton. Unexpectedly, the kidneys doubled their content of vitamin D (6.0 to 12.9% of dose), and contained more vitamin than the plasma or any other tissue investigated.

The amount of "peak IV" metabolite increased slightly in plasma and all tissues except the small intestine. Plasma still contained the largest part of "peak IV" (3.6% of dose). There was no consistent change in the quantities of peak I and II. Fractions Va + b increased slightly in plasma (0.9 to 1.4% of dose), and skeleton (0.4 to 1.3%), but did not change in the small intestine, liver and kidneys.

The antirachitic activity of the vitamin D metabolites was assessed by the rat line-test method (11), and the results are sum-

TABLE 6

Amounts of vitamin D_3 and its major metabolites in plasma and tissues 4 hours after intravenous injection of 10 IU [1,2-^{3}H]-vitamin D_3 to vitamin D-deficient rats

Chromatographic fractions	Plasma	Small intestine	Skeleton	Liver	Kidneys
I	0.06 [1]	0.03	0.2	0.4	0.1
II	0.4	0.06	0.5	1.0	0.2
III	15.0	1.3	8.2	30.1	6.0
IV	3.3	0.3	1.0	0.6	0.3
Va+b	0.9	0.1	0.4	0.4	0.4

[1] All values are percent of administered dose from pooled samples of four rats.

TABLE 7

Amounts of vitamin D_3 and its major metabolites in plasma and tissues 24 hours after intravenous injection of 10 IU [1,2-^{3}H]-vitamin D_3 to vitamin D-deficient rats

Chromatographic fractions	Plasma	Small intestine	Skeleton	Liver	Kidneys
I	0.05 [1]	0.07	0.2	0.4	0.5
II	0.08	0.06	0.6	0.4	0.7
III	4.7	0.6	7.8	9.9	12.9
IV	3.6	0.2	2.3	1.4	1.4
Va+b	1.4	0.2	1.3	0.3	0.3

[1] All values are percent of administered dose from pooled samples of four rats.

marized in table 8. As specified under Methods, these fractions were obtained from a pool of 10 rats injected with 10,000 IU [4-^{14}C]-vitamin D_3/rat, and killed 18 hours later. Positive reference rats, fed 0.1 µg crystalline vitamin D_3 in 0.1 ml cottonseed oil gave the required 40 IU/µg response whereas the negative controls fed 0.1 ml pure cottonseed oil showed no line-test.

Among the fractions assayed, only peak III (unchanged vitamin D_3) and peak IV gave consistently a response close to that of crystalline vitamin D_3. There was some variation in the response to peak IV material from various tissues, with plasma peak IV as the most active (125% of crystalline vitamin D_3 activity). Fractions IIa from liver and kidneys had some antirachitic activity (10 and 42%, respectively, of that of vitamin D_3). Peak IVa from liver also showed some activity (18% of vitamin D activity), but it was not possible to confirm it with other extracts. Peak Va + b from plasma and skeleton gave no response, but kidney peak Va + b was apparently active (37% of vitamin D activity). Fractions VIa + b were found inactive in plasma and liver. Fractions VIIa + b were inactive in plasma and bone, and showed questionable activity (less than 10% of vitamin D_3 activity) in liver extract.

DISCUSSION

Much attention has been paid in this laboratory to the study of a highly biologically active metabolite of vitamin D_3 in rats initially isolated as a peak IV fraction (3, 4). Further methodologic investigations provided indications that this fraction was nonhomogeneous [14]. We report here the successful resolution of the former "peak IV" fraction into six different subfractions: IVa, IV, Va, Vb, VIa, VIb. Only the newly purified metabolite IV exhibits intense biological activity. It was very recently identified by Blunt et al. (8, 9) as 25-hydroxycholecalciferol, and is thought to be the active principle of vitamin D_3. In addition, one low polarity fraction IIa, and two very polar chromatographic fractions VIIa and b, have been separated. The new chromatographic system achieved the separation of 11 different metabolic fractions instead of the 3 previously obtained from plasma and tissue extracts of rats injected with tritiated vitamin D. It should be noted

[14] See footnote 2.

TABLE 8

Antirachitic activity of chromatographic fractions of plasma and tissues extracts

Chromatographic fractions	Plasma	Small intestine	Skeleton	Liver	Kidneys
IIa	—	—	—	4[1]	17
III	40	—	—	—	—
IVa	—	—	—	7	—
IV	50	34	30	34	41
Va+b	0	—	0	—	15
VIa+b	0	—	—	0	—
VIIa+b	0	—	0	3	—

[1] The biological activity is expressed in IU/μg.
The amount of each fraction was estimated on the basis of the specific activity of the parent [4-^{14}C] vitamin D_3, i.e., 2,072 dpm/μg (51.8 dpm/IU).

that some resolution of the impure "peak IV" material has also been achieved by means of a dichloromethane-ether or dichloroethane-ether solvent system[15] (20). That these fractions do not represent degradation products occurring during storage, extraction or chromatographic procedure, was demonstrated by their absence in extracts of 3H vitamin D_3 added to plasma and by their increase in amount as a function of time after dose.

Of these 12 fractions, only peaks I, III and IV have been identified so far as, respectively, vitamin D esters (6), unchanged vitamin D (3), and 25-hydroxycholecalciferol (6, 9).

As far as biological activity is concerned, only the unchanged vitamin D_3 fraction (peak III) and the 25-hydroxycholecalciferol (peak IV) showed consistently an antirachitic effect comparable to that of crystalline vitamin D_3. Pure 25-hydroxycholecalciferol exhibits 140% of the vitamin D_3 antirachitic activity (9). In our experiments, however, plasma peak IV did not show more than 125% of vitamin D_3 activity, and the tissue extracts were somewhat less active than vitamin D itself. It is possible that this discrepancy is due either to radioactive contaminants very closely related chemically but biologically inactive, or to nonradioactive substances interfering with the antirachitic activity of 25-hydroxycholecalciferol. One should also consider that chemical degradation is likely to affect, to a higher extent, the very small amounts (less than 1μg/pooled tissue extract) isolated in the present experiments than the milligram quantities on hand for the previous formal determinations of biological activity. In previous work, vitamin D esters (peak I) and unidentified peak II have been found partially active (3–5).

Unchanged vitamin D_3 which accumulated first in liver and plasma disappeared rapidly from 4 to 24 hours. During the same period, it disappeared more slowly from the small intestine and bones, while it doubled its concentration in the kidneys. Concomitant with the decrease of unchanged vitamin D_3 was a slight increase in the amount of 25-hydroxycholecalciferol and of the more polar metabolites, in most tissues and plasma. The largest quantities of 25-hydroxycholecalciferol were found in plasma and skeleton. In contrast, liver and kidneys had always the smallest proportions of 25-hydroxycholecalciferol. Incidentally, peaks I and II did not change significantly between 4 and 24 hours. They always represented small fractions of the radioactivity (less than 5%) and were present mostly in liver, skeleton and kidneys.

The distribution of the total radioactivity among the tissues examined revealed a striking accumulation of the dose in the kidneys 24 hours after the intravenous 10 IU [1, 2-^{3}H]-vitamin D_3. While the radioactivity disappeared rapidly from liver and plasma and slowly from small intestine between 4 and 24 hours, it tended to increase in skeleton and doubled in the kidneys. At 24 hours the kidneys contained a higher proportion of the dose than any other tissue. Most of this radioactivity was lipid soluble and represented unchanged vitamin D_3. This radioactive uptake by the kidneys is much higher than previously reported (10). Such a difference may be related to the injection vehicle used in the

[15] Philips Dupher, personal communication.

former experiments, namely 0.1% Tween 20[14] in 0.9% NaCl. The higher accumulation of dose in our observations might be due to the persistence of higher plasma levels of radioactivity when the labeled vitamin D_3 is injected in a plasma solution (21). Another, more unlikely, possibility is that Tween 20 was interfering locally with the kidney uptake of ^{3}H-vitamin D_3. The present report of an important uptake of vitamin D_3 by the rat kidneys supports the concept of kidney as a target for vitamin D action, because it is not related to the very small urinary excretion of vitamin D and metabolites in rats (22). Kidneys, however, do differ from the well-known "target organs," bones and small intestine, by their slower accumulation of dose and the much higher proportion of unchanged vitamin D_3. This might indicate a delayed effect, storage, or possibly a different mechanism of action of the vitamin in the kidney than in bones and small intestine.

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