

## Regulation by Calcium of *In Vivo* Synthesis of 1,25-Dihydroxycholecalciferol and 21,25-Dihydroxycholecalciferol

(rat/vitamin D/dietary Ca/intestine/serum/kidney)

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**ABSTRACT** Tritiated 1,25-dihydroxycholecalciferol accumulates in several tissues, to an extent that varies with dietary calcium content, 12 hr after the administration of 325 pmoles of tritiated 25-hydroxycholecalciferol to rats. As the dietary and serum calcium concentrations increase, the amount of 1,25-dihydroxycholecalciferol is diminished and the concentration of 21,25-dihydroxycholecalciferol increases. This correlation is especially evident in rats given vitamin D<sub>3</sub>. In vitamin D-deficient rats, the repression of 1,25-dihydroxycholecalciferol formation occurs with a diet containing 3% calcium and 20% lactose. The results suggest that the production of 1,25-dihydroxycholecalciferol, believed to be the metabolically active form of vitamin D in the intestine, is responsible for the adaptation of calcium absorption to low dietary concentrations of calcium.

A metabolite of vitamin D believed to be the metabolically active form in the intestine has recently been isolated in pure form from intestine and unequivocally identified in this laboratory as 1,25-dihydroxycholecalciferol (1,25-(OH)<sub>2</sub>D<sub>3</sub>) (1, 2). Simultaneously, Lawson *et al.* provided evidence for this structure with a partially purified material from kidney homogenates (3). This metabolite acts more rapidly than 25-(OH)D<sub>3</sub> to initiate intestinal calcium transport (4-6). The kidney is the site of synthesis of 1,25-(OH)<sub>2</sub>D<sub>3</sub> from 25-(OH)D<sub>3</sub> (7); this observation was confirmed by Gray *et al.* (8). Other experiments have provided strong evidence that 1,25-(OH)<sub>2</sub>D<sub>3</sub> is the metabolically active form of vitamin D in the intestine (9-11). It, therefore, seemed possible that the concentration of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in the serum and intestinal mucosa plays an important role in the adaptation of calcium absorption to the concentration of calcium in the diet (12).

This report demonstrates that dietary calcium concentration has a profound effect on the *in vivo* production of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and 21,25-(OH)<sub>2</sub>D<sub>3</sub>. Furthermore, these alterations in metabolite balance correlate with changes in serum calcium, but not serum phosphate concentration. An independent study has also shown that dietary strontium has an equally marked effect on the production of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and 21,25-(OH)<sub>2</sub>D<sub>3</sub> from 25-(OH)D<sub>3</sub> (J. Omdahl and H. F. DeLuca, manuscript in preparation)

Abbreviations: 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxycholecalciferol; 21,25-(OH)<sub>2</sub>D<sub>3</sub>, 21,25-dihydroxycholecalciferol; 25-(OH)D<sub>3</sub>, 25-hydroxycholecalciferol.

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### MATERIALS AND METHODS

#### Preparation of rats and diets

Weanling, male albino rats were obtained from the Holtzman Co., Madison, Wis., and were housed individually in hanging wire cages. They were given food and water *ad libitum*. The diet provided was the vitamin D-deficient diet described by Suda *et al.* (13) containing 0.47% Ca and 0.30% P.

The calcium content was varied by addition or deletion of CaCO<sub>3</sub>, with compensatory changes in the sugar (glucose monohydrate) content. Similarly, where indicated, lactose was added at the expense of the glucose. Diets containing Ca at 0.02%, 0.30%, 0.47%, 2.0%, and 3.0%—and 3.0% Ca plus 20% lactose—were used. Rats were fed the diets for 3 weeks and were then killed to obtain tissue samples.

A daily oral dose of 1 unit of vitamin D<sub>3</sub> in 0.05 ml of cottonseed oil-soybean oil (Wesson) was given to half the animals after 1 week on their vitamin D-deficient diet and was continued for the remaining 2 weeks.

#### Administration of [<sup>3</sup>H]25-(OH)D<sub>3</sub> and preparation of extracts

325 pmoles of tritiated 25-(OH)D<sub>3</sub> was administered intrajugularly in 0.05 ml of 95% ethanol with mild ether anesthesia. The rats were fasted at this time, but water continued to be available *ad libitum*. 12 hr later the rats were killed by decapitation and blood serum was collected. The upper 50 cm of small intestine was quickly removed, flushed with ice-cold saline, slit open, and the mucosa was scraped off with a glass slide. The kidneys and livers were removed. The fore- and hind-limbs were stripped of muscle, the long bones were split, and the marrow was discarded. All tissues were stored at -16°C until they were extracted with chloroform and methanol (14). In early experiments, the tissue from 3 to 4 animals fed the same diet was combined before extraction, while in later experiments the serum calcium concentrations of the rats were analyzed for similarity before pooling of tissues. In other cases, the tissues from each animal were analyzed individually.

Radioactive metabolites were separated on a 2 × 15 cm column containing 10 g of Sephadex LH-20 equilibrated with 35% Skellysolve B (petroleum ether, b.p. 67-68°C) in 65% chloroform (15). The tissue extracts were applied to the column in 1 ml of the same solvent mixture and eluted with a further 190 ml. Recovery of radioactivity from the columns varied from 90 to 105%.

The elution position of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on this column has been established (2, 8). The less-polar metabolite was identified as 21,25-(OH)<sub>2</sub>D<sub>3</sub> by cochromatography. A sample of the

TABLE 1. Regulation by calcium of the metabolism *in vivo* of [<sup>3</sup>H]25-(OH)D<sub>3</sub> to polar metabolites

	Diet calcium (%)	No. of animals	Average weight (g)	Average serum calcium (mg/100 ml)		% Recovered <sup>3</sup> H in serum as	
				Before 25-(OH)D <sub>3</sub>	12 hr after 25-(OH)D <sub>3</sub>	1,25-(OH) <sub>2</sub> D <sub>3</sub>	21,25-(OH) <sub>2</sub> D <sub>3</sub>
Vitamin D-deficient	0.02	4	87	3.8	5.2	15.8	1.6
	0.02	4	83	(3.9)*	5.5	16.5	0.9
	0.47	3	147	(5.2)	7.5	18.0	0.5
	2.0	4	153	(8.3)	8.7	14.0	0.7
	3.0	3	163	9.1	9.3	15.6	1.0
	3.0 + 20% lactose	1	145	9.6	9.4	3.1	7.7
	3.0 + 20% lactose	1	112	9.8	9.4	1.0	7.1
	3.0 + 20% lactose	1	115	9.6	9.8	0.7	10.5
1 Unit of vitamin D daily	0.02	4	97	(4.5)	5.4	14.0	1.0
	0.02	4	101	4.4	5.2	14.5	1.4
	0.3	3	165	7.0	8.7	15.7	1.0
	0.47	4	187	9.3	9.6	5.0	5.1
	0.47	4	167	(9.2)	9.5	2.5	8.6
	2.0	3	141	...	9.7	0.8	10.1
	3.0 + 20% lactose	1	120	9.8	8.9	1.1	11.2
	3.0 + 20% lactose	1	110	9.9	8.6	1.5	13.3

\* Figures in brackets are representative serum calcium concentrations of animals on identical diets from another series of experiments.

metabolite was obtained by column chromatography on Sephadex LH-20 of a kidney extract obtained from rats fed the 3% calcium-20% lactose plus vitamin D diet (see Fig. 2A). The rats had received an injection of [<sup>3</sup>H]25-(OH)D<sub>3</sub> 12 hr before they were killed. Authentic [<sup>3</sup>H]21,25-(OH)<sub>2</sub>D<sub>3</sub>, isolated and identified as described (16), was mixed with this metabolite and the mixture was chromatographed on a Celite liquid-liquid partition column specifically designed for 21,25-(OH)<sub>2</sub>D<sub>3</sub> isolation and capable of completely resolving the polar metabolites of 25-(OH)D<sub>3</sub> (ref. 16, see Fig. 2B).

#### Chemicals

Crystalline vitamin D<sub>3</sub> was a gift of the N. V. Philips-Duphar Co., Weesp, The Netherlands. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. [26,27-<sup>3</sup>H](OH)D<sub>3</sub> was synthesized by the method of Suda *et al.* (17) and had a specific activity of 1.3 Ci/mmol.

#### Analytical procedures

Radioactivity was measured with a Packard Tri-Carb liquid-scintillation spectrometer, model 3375, equipped with auto-

matic external standardization (Packard Instrument Corp., LaGrange, Ill.).

In all but the first group of experiments, a small sample of blood was obtained from the tail vein of each animal just before it was injected with [<sup>3</sup>H]25-(OH)D<sub>3</sub>. This, as well as a sample of the blood obtained at the end of the experiment, was analyzed for calcium, in the presence of 0.1% LaCl<sub>3</sub>, by atomic absorption spectrometry.

Serum inorganic phosphorus was measured with a Technicon autoanalyzer by the method of Fiske and Subbarow (18).

#### RESULTS

Fig. 1 shows the profiles of radioactive metabolites separated on Sephadex LH-20 from serum extracts obtained from rats fed the various diets, with and without vitamin D. As expected, the daily supplement of one unit of vitamin D failed to prevent vitamin D deficiency (Table 1); this low dose was chosen to minimize dilution of radioactive metabolites and to provide a small amount of 25-(OH)D<sub>3</sub> as a possible inducer for the 1-hydroxylase enzyme in the kidney (9, 10). The comparative

TABLE 2. Appearance of polar metabolites of [<sup>3</sup>H]25-(OH)D<sub>3</sub> in various tissues as a function of dietary calcium concentration

		Vitamin D-deficient			Vitamin D-supplemented		
		0.02% Ca	0.47% Ca	2.0% Ca	0.02% Ca	0.47% Ca	2.0% Ca
Intestinal mucosa	1,25-(OH) <sub>2</sub> D <sub>3</sub>	48	60	64	46	8	8
	21,25-(OH) <sub>2</sub> D <sub>3</sub>	2	3	2	3	5	8
Kidney	1,25-(OH) <sub>2</sub> D <sub>3</sub>	29	32	28	30	6	3
	21,25-(OH) <sub>2</sub> D <sub>3</sub>	1	1	1	1	14	14
Bone	1,25-(OH) <sub>2</sub> D <sub>3</sub>	21	25	20	25	5	2
	21,25-(OH) <sub>2</sub> D <sub>3</sub>	1	1	1	2	7	8
Serum	1,25-(OH) <sub>2</sub> D <sub>3</sub>	17	18	14	17	3	1
	21,25-(OH) <sub>2</sub> D <sub>3</sub>	1	1	1	1	9	10

The values represent the percentage of the total radioactivity recovered as the indicated metabolite. The remaining radioactivity in each case was [<sup>3</sup>H]25-(OH)D<sub>3</sub>.

weights of the vitamin D-deficient and vitamin D-supplemented animals (Table 1), however, demonstrate a growth effect of the vitamin even at this low concentration in the diet.

In initial experiments with the vitamin D-deficient rats fed diets with 0.02–2.0% Ca, 14–18% of the total radioactivity in the serum was consistently recovered as 1,25-(OH)<sub>2</sub>D<sub>3</sub>. However, in the rats given vitamin D, the recovered radioactivity as 1,25-(OH)<sub>2</sub>D<sub>3</sub> was 17% with 0.02% dietary calcium; this figure decreased to 2.5% with 0.47% dietary calcium and to 0.8% with 2.0% dietary calcium. It was of great interest that another metabolite, 21,25-(OH)<sub>2</sub>D<sub>3</sub>, appeared in rats fed the vitamin D-supplemented diets. This less polar metabolite was identified by cochromatography on the liquid-liquid partition column (Fig. 2B).

In rats given vitamin D, the suppression by dietary calcium of 1,25-(OH)<sub>2</sub>D<sub>3</sub> production correlates with an increase in serum calcium concentration, whereas the suppression of 21,25-(OH)<sub>2</sub>D<sub>3</sub> production correlates with a decrease in serum calcium content. With very high concentrations of dietary calcium (Table 1, 3% calcium plus lactose; ref 19), the production of 1,25-(OH)<sub>2</sub>D<sub>3</sub> is inhibited in vitamin D-deficient rats as well.

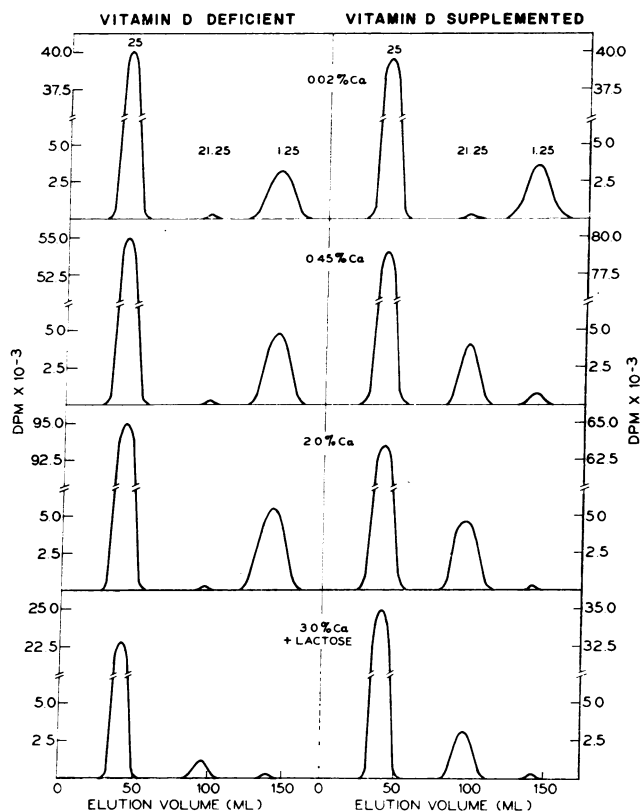


FIG. 1. Sephadex LH-20 chromatographic profiles of serum extracts from rats on diets with different calcium contents. Weanling rats were maintained on the indicated diets for 3 weeks. Half the animals on each diet were given 1 unit of vitamin D<sub>3</sub> each day for the last 2 weeks of the experiment. Rats were given 325 pmoles of [<sup>3</sup>H]25-(OH)<sub>2</sub>D<sub>3</sub> intrajugularly, 12 hr before they were killed. Serum was collected for chromatographic analysis from the different numbers of rats used in each group; this accounts for the apparent differences in total radioactivity shown on the ordinate. 25 = 25-(OH)<sub>2</sub>D<sub>3</sub>; 21.25 = 21,25-(OH)<sub>2</sub>D<sub>3</sub>; 1.25 = 1,25-(OH)<sub>2</sub>D<sub>3</sub>.

The relationship between 1,25-(OH)<sub>2</sub>D<sub>3</sub> accumulation in serum and the serum calcium concentration appears to involve an abrupt rather than a gradual change. At serum calcium concentrations above 9.0–9.6 mg/100 ml, production of 1,25-(OH)<sub>2</sub>D<sub>3</sub> is suppressed and 21,25-(OH)<sub>2</sub>D<sub>3</sub> is enhanced, whereas below this range of concentrations, the synthesis of 1,25-(OH)<sub>2</sub>D<sub>3</sub> appears to be unaffected by calcium concentration and the synthesis of 21,25-(OH)<sub>2</sub>D<sub>3</sub> is suppressed. Similar correlations of metabolite patterns with the serum calcium concentration were found in the other tissues analyzed (Table 2).

It also is apparent (Table 1) that when the initial calcium concentration of the serum is low, a distinct rise of from 1 to 2 mg of calcium per 100 ml of serum occurs in response to the 325-pmole dose of 25-(OH)<sub>2</sub>D<sub>3</sub>, whereas when the initial serum calcium concentration is normal, no further rise occurs.

Finally, there is no correlation between inorganic P concentrations of serum and production of the metabolites (Table 3). These results suggest that it is calcium *per se*, or possibly the hormones that control the serum calcium concentration, that regulates the synthesis of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and 21,25-(OH)<sub>2</sub>D<sub>3</sub>.

## DISCUSSION

Our results demonstrate a clear relationship between the amount of 1,25-(OH)<sub>2</sub>D<sub>3</sub> appearing in tissues after the injection of [<sup>3</sup>H]25-(OH)<sub>2</sub>D<sub>3</sub> and the serum calcium concentra-

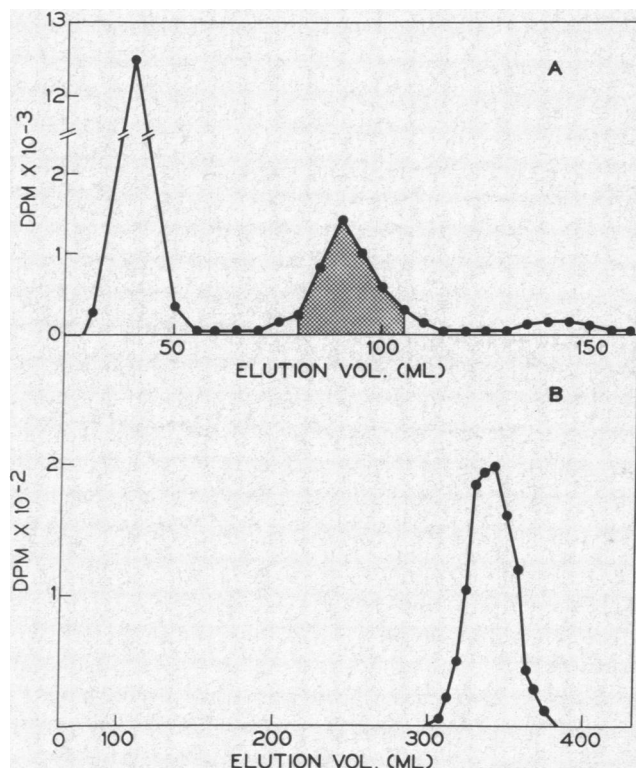


FIG. 2. A. Sephadex LH-20 chromatographic profile of kidney extracts from rats on a diet of 3% calcium plus lactose for 3 weeks. The rats were given 1 unit of vitamin D<sub>3</sub> daily for the last 2 weeks. The fractions in the shaded area were pooled for cochromatography, described below in B.

B. Liquid-liquid partition column cochromatography of 1,000 dpm of the metabolite isolated in A (above) together with 600 dpm of pure 21,25-(OH)<sub>2</sub>D<sub>3</sub> isolated by Suda *et al.* (16). The chromatographic system used is that described by the same investigators.

TABLE 3. Lack of correlation between the concentration of inorganic phosphorus in serum and the production of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and 21,25-(OH)<sub>2</sub>D<sub>3</sub> from [<sup>3</sup>H]25-(OH)D<sub>3</sub>

Diet	Serum calcium (mg/100 ml)	Serum P <sub>i</sub> (mg/100 ml)	% Radioactivity recovered from serum as	
			1,25-(OH) <sub>2</sub> D <sub>3</sub>	21,25-(OH) <sub>2</sub> D <sub>3</sub>
3% Ca	9.1	3.7	15.6	1.0
0.45% Ca + D	9.5	9.8	2.5	8.6
0.3% Ca + D	7.5	10.5	15.7	1.0
0.02% Ca + D	4.4	9.9	14.5	1.4

tion at the time of injection. In young rats, the synthesis of 1,25-(OH)<sub>2</sub>D<sub>3</sub> is maximal in hypocalcemia and is almost completely repressed in the presence of normal or high concentrations of serum calcium. This relationship is evident in the presence or absence of vitamin D. Coincident with the repression of 1,25-(OH)<sub>2</sub>D<sub>3</sub> synthesis in normocalcemic animals is an equally dramatic appearance of 21,25-(OH)<sub>2</sub>D<sub>3</sub>.

Experimental work by Fairbanks and Mitchell (20), Rottensten (21), and Nicolaysen *et al.* (22) in the period from 1935 to 1943 established that young rats absorb calcium more efficiently after a period on a diet low in calcium concentration (0.15–0.2%) than after a similar period on a diet high in calcium concentration (0.8–1.25%). Calcium absorption was measured by these authors either as a difference in carcass calcium content and/or by calcium-balance studies. Serum calcium concentrations were not reported.

Nicolaysen *et al.* proposed that the adaptation mechanism is regulated by some endogenous factor (22). This factor or factors was shown to be vitamin D-dependent and to influence the velocity with which calcium passes through the mucosa of isolated intestinal loops. The calcium-adaptation phenomenon is a feature of young rather than old rats; neither sexual maturity nor the presence of the thymus gland are important (22).

Kimberg *et al.* (23), using an everted gut-sac technique as a measure of calcium absorption, confirmed Nicolaysen's observations regarding the vitamin D-dependence and expanded them to show that the adaptation mechanism remains after removal of the pituitary, thyroid, parathyroid, or adrenal glands. Our data strongly suggest that the serum calcium concentration, or factors that correlate with the serum calcium concentration—such as parathyroid hormone or calcitonin concentration—have a controlling influence on the rate of synthesis *in vivo* and on the tissue concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub>.

In view of the strong evidence that 1,25-(OH)<sub>2</sub>D<sub>3</sub> is the metabolically active form of vitamin D in the intestine (9–11), and that its concentration is regulated by the dietary calcium concentration, we suggest that 1,25-(OH)<sub>2</sub>D<sub>3</sub> is the "endogenous factor" of Nicolaysen *et al.* (22). The presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> is obviously vitamin D-dependent and even slight hypocalcemia stimulates its synthesis. Slight hypocalcemia undoubtedly occurs in the young growing animals that were fed a diet low in calcium concentration, and the hypocalcemia probably causes increased 1,25-(OH)<sub>2</sub>D<sub>3</sub> synthesis. Calcification also may remove calcium from the blood, causing slight hypocalcemia. This tendency to slight hypocalcemia and,

hence, 1,25-(OH)<sub>2</sub>D<sub>3</sub> production would persist until the bones are fully calcified or until adequate calcium intake could overcome these needs. Thus, 1,25-(OH)<sub>2</sub>D<sub>3</sub> meets the criteria of Nicolaysen *et al.* (22) for the endogenous factor.

There is a strong possibility that the concentration of other factors, such as parathyroid hormone or calcitonin, which regulate serum calcium concentration, may be the actual regulators of 1,25-(OH)<sub>2</sub>D<sub>3</sub> synthesis. In this regard, the observation of Kimberg *et al.* (23) that thyroparathyroidectomy does not abolish the adaptation of calcium absorption to dietary calcium concentration may be relevant. However, more direct studies on the interplay of these hormones with 1,25-(OH)<sub>2</sub>D<sub>3</sub> synthesis are in progress.

The 21,25-(OH)<sub>2</sub>D<sub>3</sub> seen in the normocalcemic animals may not be involved directly in the adaptation mechanism. It is notable that this metabolite activates bone mineral mobilization and has slight activity in intestinal calcium transport (16). That the kidney has the highest concentration of 21,25-(OH)<sub>2</sub>D<sub>3</sub> suggests a renal function for this compound (Table 2). For example, it may play a protective role by preventing hypercalcemia by decreasing the tubular reabsorption of calcium, or it may be involved in tubular phosphate reabsorption.

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