The Stimulation of 1,25-Dihydroxycholecalciferol Metabolism in Vitamin D-deficient Rats by 1,25-Dihydroxycholecalciferol Treatment

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ABSTRACT Daily oral administration of 1,25-dihydroxycholecalciferol to vitamin D-deficient rats increases the rate of disappearance of [^{3}H]1,25-dihydroxycholecalciferol and increases the rate of appearance of metabolites both less polar and more polar than 1,25-dihydroxycholecalciferol in the intestine, bone, liver, kidney, plasma, and muscle. Since 1,25-dihydroxycholecalciferol is believed to be the metabolically active form of vitamin D in the stimulation of intestinal calcium transport and bone calcium mobilization, these results provide an explanation for the fact that daily oral administration of 1,25-dihydroxycholecalciferol is relatively ineffective in the maintenance of serum calcium and in the calcification of bone in rats.

INTRODUCTION

It is now generally accepted that vitamin D must be converted to more active forms in order to carry out its physiological actions in intestine and bone. The existence of these active metabolites of vitamin D was originally demonstrated by Lund and DeLuca in 1966 (1). Two years later, the first of these metabolites was isolated from plasma and identified as 25-hydroxycholecalciferol (25-OHD$_3$)[1] (2). This compound, which is produced in the liver from vitamin D$_3$ (3, 4), is not the final active form of vitamin D$_3$. A more polar metabolite appears in the intestine from 25-OHD$_3$ (5–7) and has recently been isolated and identified as 1,25-dihydroxycholecalciferol (1, 25-[OH]$_2$D$_3$) (8, 9). Confirmation of this structure using a partially purified preparation from kidney homogenate was independently achieved by Lawson, Fraser, Kodicek, Morris, and Williams (10).

When given intravenously as a single-pulse dose, 1,25-(OH)$_2$D$_3$ has been shown to act more rapidly than 25-OHD$_3$ in stimulating intestinal calcium transport (11, 12) and in activating the bone calcium mobilization system (12, 13). Although more active than 25-OHD$_3$ in the intestinal calcium transport system (11–13), 1,25-(OH)$_2$D$_3$ was equally as active as 25-OHD$_3$ in the bone calcium mobilization response and was much less active in the cure of rickets in rats (12).

That it is indeed 1,25-(OH)$_2$D$_3$ that is responsible for the observed physiological effects has been shown in a number of experiments. Fraser and Kodicek (14) have demonstrated that the 1,25-(OH)$_2$D$_3$ is produced from 25-OHD$_3$ in the kidney, an observation confirmed by Gray, Boyle, and DeLuca (15). It therefore logically follows that in nephrectomized rats, physiological doses of 1,25-(OH)$_2$D$_3$ but not 25-OHD$_3$ will stimulate intestinal absorption of calcium (16) and the mobilization of calcium from bone (17). Similarly, since the conversion of 25-OHD$_3$ to 1,25-(OH)$_2$D$_3$ is blocked by previous treatment with actinomycin D (18), rats treated with actinomycin D show a normal intestinal response to 1,25-(OH)$_2$D$_3$ but not to 25-OHD$_3$ (19). In fetal bone tissue culture, 1,25-(OH)$_2$D$_3$ will cause bone calcium mobilization at much lower levels than 25-OHD$_3$ (20). Finally, at the time when 1,25-(OH)$_2$D$_3$ is producing its maximal intestinal calcium transport response, it is the only detectable metabolite in the intestine of chicks (21) and the major metabolite in the intestine of rats (22). Similar results have been obtained with the bone calcium mobilization system (22). It therefore seems likely that it is 1,25-(OH)$_2$D$_3$ itself and not a further metabolite that is responsible for the intestinal calcium transport and bone calcium mobilization responses to vitamin D.

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[1] *Abbreviations used in this paper:* 25-OHD$_3$, 25-hydroxycholecalciferol; 1,25-(OH)$_2$D$_3$, 1,25-dihydroxycholecalciferol.

The question then arises, if 1,25-$(OH)_2D_3$ is the active form of vitamin D in both the intestine and bone, why is it relatively inactive in the cure of rickets (12)? In recent experiments by Tanaka, Frank, and DeLuca (23) it has been demonstrated that when 1,25-$(OH)_2D_3$ is administered orally over a period of days to vitamin D-deficient rats (the method normally used to test for the cure of rickets) it is much less effective in the calcification of bone and in the maintenance of serum calcium than either 25-OHD_3 or vitamin D_3 administered in the same manner. However, intravenous daily administration of 1,25-$(OH)_2D_3$ to rats gives the expected results where 1,25-$(OH)_2D_3$ is at least as effective and probably more effective than 25-OHD_3. It therefore was suggested that 1,25-$(OH)_2D_3$ may be rapidly destroyed by the intestine and that this destruction may be induced by 1,25-$(OH)_2D_3$ itself. It is the purpose of this report to demonstrate that the metabolism of 1,25-$(OH)_2D_3$ is indeed faster in rats given daily oral doses of 1,25-$(OH)_2D_3$ than in untreated, vitamin D-deficient rats.

METHODS

Animals. Male, weanling, albino rats (Holtzman Co., Madison, Wis.) were fed ad libitum a low-calcium (0.02%), vitamin D-deficient diet described earlier (24). At the end of 2–3 wk the rats were severely hypocalcemic and were then used for the following experiments.

[26,27-^{3}H]1,25-$(OH)_2D_3$. The high specific activity 1,25-$(OH)_2D_3$ (247,000 dpm/62.5 pmol) was synthesized from [26,27-^{3}H]25-OHD_3 (25) (247,000 dpm/62.5 pmol) using the kidney homogenate method originally described by Fraser and Kodicek (14) and modified by Boyle and coworkers (16) with the exception that a smaller incubation volume and substrate concentration were used. To produce low specific activity 1,25-$(OH)_2D_3$ (170 dpm/60 pmol) nonradioactive 25-OHD_3 (a gift from the Philips-Duphar Company of The Netherlands) was mixed with the radioactive 25-OHD_3 to the given specific activity. This was used as the substrate for the kidney homogenate incubation. Before injection of the high specific activity 1,25-$(OH)_2D_3$ into animals for a metabolism study, it was rechromatographed on a Sephadex LH-20 column (chloroform: Skellysolve B [petroleum ether redistilled at bp 67–68°C]: methanol, 75:23:2, vol/vol). In all cases only one peak was observed which accounted for at least 90% of the applied radioactivity.

Experimental procedure. The rats were divided into three groups. To each of the four rats in one group 72 pmol of high specific activity [26,27-^{3}H]1,25-$(OH)_2D_3$ in 0.05 ml of 95% ethanol was given intravenously. A second group of four rats received via stomach tube a single oral dose of 59 pmol of the high specific activity 1,25-$(OH)_2D_3$ in 0.1 ml of cottonseed-soybean oil (Hunt Wesson Foods, Inc., Fullerton, Calif.). An oral dose consisting of 56 pmol of 1,25-$(OH)_2D_3$ (170 dpm/60 pmol) every 12 h for 6 days was administered in 0.1 ml of the oil by stomach tube to each of the nine rats in the third group. On the 7th day, three of the rats in this group received orally 0.1 ml of oil as a control while the remaining six rats were given 58 pmol of high specific activity [^{3}H]1,25-$(OH)_2D_3$ in 0.1 ml of oil. All of the rats in the three groups were killed 7 h after their last dose. The plasma, intestine, bone, liver, kidney, and muscle of all the rats but those in the control group were collected. The individual tissues from all rats in a group were pooled. These tissues were then counted, extracted with chloroform-methanol, and the lipid extracts chromatographed on Sephadex LH-20 columns (chloroform: Skellysolve B: methanol, 75:23:2, vol/vol) as described previously (22). For the control group, the radioactivity present in the tissues was determined by combustion in a Packard Tri-Carb Sample Oxidizer, (model 305, Packard Instrument Co., Inc., Downers Grove, Ill.) before counting the sample.

To determine the biological activity of 1,25-$(OH)_2D_3$ under these conditions rats were again divided into three groups. To each of five rats in one group was given intravenously 60 pmol of low specific activity 1,25-$(OH)_2D_3$. Five additional rats received intravenously 0.05 ml 95% ethanol only. A second group of six rats received an oral dose of 64 pmol of 1,25-$(OH)_2D_3$ in 0.1 mol of oil every 12 h for 7 days. Similarly, an oral dose consisting of 0.1 ml of oil only was given to each of 10 rats every 12 h for 6 days. On the 7th day five of these rats were orally given 64 pmol of 1,25-$(OH)_2D_3$ in 0.1 ml of oil while the remaining five rats received 0.1 ml of oil only. All the rats in the three groups were killed 7 h after their last treatment. Their serum calcium and intestinal calcium transport ratios were determined as described previously (22).

RESULTS

The biological activity of 1,25-$(OH)_2D_3$ after various methods of administration of the metabolite to vitamin D-deficient rats is shown in Table I. When examining the bone calcium mobilization response, as determined by the serum calcium values, a single intravenous dose is more effective than a single oral dose, which in turn is more effective than repeated oral doses. The intestinal calcium transport response shows no difference after a single intravenous or oral dose of 1,25-$(OH)_2D_3$. With repeated oral doses of 1,25-$(OH)_2D_3$ a slightly lower response is noted; however, this decreased response is not significantly different from the response observed after a single oral dose of 1,25-$(OH)_2D_3$ to the rats.

The distribution of radioactivity among the various tissues studied after administration of the high specific activity 1,25-$(OH)_2D_3$ to vitamin D-deficient rats or deficient rats pretreated orally with 1,25-$(OH)_2D_3$ is shown in Table II. It is immediately apparent that, except for there being a slightly higher concentration of radioactivity in the plasma of the intravenously dosed rats, the animals given the single intravenous and the single oral dose show little difference in the tissue distribution of radioactivity. However, the animals given 1,25-$(OH)_2D_3$ daily show a significant decrease in the percent of the total administered dose per gram of tissue. It is interesting to note, however, that the concentration of radioactivity in the bone and muscle remains at the levels observed for the single oral and intravenously dosed animals. That the radioactivity observed in the tissues of the pretreated animals is due to the last administered dose of high specific activity 1,25-$(OH)_2D_3$ and not to the previously administered low specific activity 1,25-$(OH)_2D_3$ is shown in the last column of Table II. It is

TABLE I

Biological Activity of 1,25-$(OH)_2D_3$ 7 h after Administration of 60–64 pmol of 1,25-$(OH)_2D_3$ to Vitamin D-deficient and 1,25-$(OH)_2D_3$-supplemented Rats

	I.v. dose	Single oral dose	Oral dose after pretreatment with 1,25-$(OH)_2D_3$	I.v. control	Oral control
Serum calcium (*mg/100 ml*)	5.8±*0.2‡ (5)	5.2±0.1§ (5)	4.8±0.4‖ (12)	4.3±0.4 (5)	4.4±0.2 (5)
Intestinal calcium transport (serosal/mucosal)	5.0±0.9 (5)	5.2±1.8 (5)	4.3±1.2¶ (6)	2.0±0.5 (5)	1.7±0.3 (5)

10 vitamin D-deficient rats on a low calcium diet were administered an i.v. 60 pmol dose of 1,25-$(OH)_2D_3$ in 0.05 ml of 95% ethanol or the ethanol vehicle only. Six additional rats received an oral dose of 64 pmol 1,25-$(OH)_2D_3$ in 0.1 ml oil every 12 h for 7 days. Similarly 10 rats received 0.1 ml of oil only every 12 h for 6 days. On the 7th day, five of these rats received 64 pmol of 1,25-$(OH)_2D_3$ orally while the remaining five again received oil only. All rats were killed 7 h after the last treatment and serum calcium and intestinal calcium transport were determined as described previously (22). The serum calcium values of the six rats receiving the pretreatment with 1,25-$(OH)_2D_3$ used in the metabolite studies were combined with the serum calcium values of the similarly treated animals used in the biological activity studies. The numbers in parentheses represent the number of rats in each group.

* Standard deviation.

‡ Different from the single orally dosed rats at the 0.001 level.

§ Different from the pretreated rats at the 0.05 level.

‖ Different from the oral control at the 0.10 level.

¶ Not significantly different from the single orally dosed rats at the 0.20 level.

apparent that there is essentially no radioactivity in the rats due to the pretreatment with the low specific activity 1,25-$(OH)_2D_3$.

When the radioactivity appearing in the lipid extracts of the various tissues was chromatographed on Sephadex LH-20 columns the concentration of 1,25-$(OH)_2D_3$ in these tissues could be calculated (Table III). It is evident that the concentration of 1,25-$(OH)_2D_3$ appearing in the tissues is lower in the rats given the single oral dose when compared with those given the intravenous

TABLE II

Concentration of Radioactivity in Tissues 7 h after Administration of [^3H]1,25-$(OH)_2D_3$ to Vitamin D-deficient and 1,25-$(OH)_2D_3$-supplemented Rats

	I.v. dose	Single oral dose	Oral dose after pretreatment with 1,25-$(OH)_2D_3$	Oil control after pretreatment with 1,25-$(OH)_2D_3$
			% total dose/g tissue	
Intestine	3.0±0.8*	3.2	1.3	0.002
Bone	1.2	0.8	1.5	0.007
Kidney	1.6	1.4	0.08	0.002
Plasma	2.3±0.3	1.3±0.2	0.7±0.1	0.002
Liver	1.5	1.1	0.6	0.002
Muscle	0.5	0.3	0.3	0.001

* Standard deviation.

Vitamin D-deficient rats on a low calcium diet were administered either a single 72 pmol i.v. or 59 pmol oral dose of high specific activity [^3H]1,25-$(OH)_2D_3$ or a 56 pmol oral dose of low specific activity 1,25-$(OH)_2D_3$ every 12 h for 6 days followed on the 7th day by an oral dose of either the oil vehicle only (controls) or 58 pmol of high specific activity [^3H]1,25-$(OH)_2D_3$. All rats were killed 7 h after their last dose and the radioactivity present in the tissues determined as described in the text.

TABLE III

Concentration of 1,25-$(OH)_2D_3$ in Tissues 7 h after Administration of 1,25-$(OH)_2D_3$ to Vitamin D-deficient or 1,25-$(OH)_2D_3$-supplemented Rats

	I.v. dose	Single oral dose	Oral dose after pretreatment with 1,25-$(OH)_2D_3$
	pmol 1,25-$(OH)_2$ D_3/g tissue		
Intestine	1.8	1.5	0.45
Bone	0.7	0.3	0.07
Kidney	0.8	0.3	0.07
Plasma	1.2	0.3	0.07
Liver	0.6	0.1	0.04
Muscle	0.2	0.05	0.01

The rats were treated as described in Table II. The tissues were extracted with chloroform-methanol and the lipid extracts applied to Sephadex LH-20 columns (chloroform: Skellysolve B:methanol, 75:23:2, vol/vol).

dose. This decrease becomes more pronounced in the rats pretreated with 1,25-$(OH)_2D_3$.

When the metabolism of [^{3}H]1,25-$(OH)_2D_3$ is examined (Table IV) a number of interesting observations can be made. First a comparison between the intravenously dosed animals and the animals given the single oral dose shows a decrease in the percent of the homogenate radioactivity appearing as 1,25-$(OH)_2D_3$ and an increase in the water-soluble metabolites in the kidney, plasma, liver, and muscle of the orally dosed rats. This change in the metabolite picture is also noted when the tissues from the pretreated rats are examined. A second striking change in those animals given the pretreatment of 1,25-$(OH)_2D_3$ is found in the bone and muscle. In these tissues 45 and 31% of the homogenate radioactivity appears in two peaks less polar than 1,25-$(OH)_2D_3$ compared with 0–4% in the intravenously and orally dosed rats. Finally, the tissues of the pretreated rats show a peak more polar than 1,25-$(OH)_2D_3$ which accounts for 6–14% of the homogenate radioactivity. In the intravenously and single orally dosed rats this same peak represents only 0–5% of the total homogenate radioactivity.

DISCUSSION

Earlier results of Omdahl et al (12) produced the apparent conflict that after an intravenous dose of 1,25-

TABLE IV

Metabolites Found in the Tissues 7 h after Administration of [^{3}H]1,25-$(OH)_2D_3$ to Vitamin D-deficient and 1,25-$(OH)_2D_3$-supplemented Rats

	Radioactivity in Homogenate					
	Less polar than 1,25-$(OH)_2D_3$	1,25-$(OH)_2D_3$	More polar than 1,25-$(OH)_2D_3$	Column strip	H_2O soluble radioactivity	Total recovery
	%	%	%	%	%	%
Single intravenous dose						
Intestine	—	89	2	0.6	3	95
Bone	1	77	1	1	9	89
Kidney	3	68	2	2	17	92
Plasma	—	73	4	0.4	9	86
Liver	5	49	3	4	33	94
Muscle	4	55	2	2	20	83
Single oral dose						
Intestine	—	80	1	0.5	0	82
Bone	—	61	5	1	14	81
Kidney	3	37	—	—	48	88
Plasma	—	36	5	0.4	35	76
Liver	4	22	5	1	46	78
Muscle	—	31	3	0.8	46	81
Oral dose after pretreatment with 1,25-$(OH)_2D_3$						
Intestine	8	59	6	1	21	94
Bone	45	8	7	20	0	80
Kidney	2	23	11	3	44	83
Plasma	6	19	13	2	39	79
Liver	8	13	14	4	27	66
Muscle	31	9	7	5	33	85

The rats were treated as described in Table III

$(OH)_2D_3$ there was little calcification of bone, although a similar dose brought about both a marked stimulation of intestinal calcium transport and a rise in serum calcium due to bone calcium mobilization. It was initially thought that the 1,25-$(OH)_2D_3$ was turning over too rapidly to cause a long-lasting effect on calcification. However, this was not supported by later work where daily oral administration of 1,25-$(OH)_2D_3$ for 8 days also failed to give a calcification response (23). More recent experiments have shown that the failure of the rats to respond to a daily oral dose of 1,25-$(OH)_2D_3$ was due to the mode of metabolite administration (23). If daily intravenous doses of 1,25-$(OH)_2D_3$ were given to rats, a definite and marked bone calcification response was noted. This difference in response in orally and intravenously dosed animals was thought to be due to more rapid metabolism of the orally administered 1,25-$(OH)_2D_3$.

Previous experiments have demonstrated that after administration of 1,25-$(OH)_2D_3$ to rats it is the 1,25-$(OH)_2D_3$ itself which is responsible for the stimulation of the intestinal calcium transport system and the bone calcium mobilization response, both of which are needed to raise the serum calcium and phosphorus to a sufficient level for calcification to occur. If the 1,25-$(OH)_2D_3$ is rapidly degraded in the intestine to other compounds the effective lifetime of 1,25-$(OH)_2D_3$ in the body would be markedly shortened. That such an action may be occurring is suggested by the results in Tables III and IV where after pretreatment of rats with 1,25-$(OH)_2D_3$, the concentration of 1,25-$(OH)_2D_3$ in the tissues due to the last administered dose is decreased and the amount of the metabolites of 1,25-$(OH)_2D_3$ is increased when compared with rats given a single oral or intravenous dose. These data also explain the previous observation that vitamin D-deficient rats treated orally with 1,25-$(OH)_2D_3$ over a period of days behave in many respects like untreated vitamin D-deficient rats (23). In contrast to this, vitamin-D-deficient rats given daily oral doses of either vitamin D_3 or 25-OHD_3 show a marked stimulation in growth, serum calcium levels, and bone calcification. It therefore appears likely that 1,25-$(OH)_2D_3$ stimulates a system or systems in intestine which rapidly metabolizes the 1,25-$(OH)_2D_3$. Thus when oral doses of 1,25-$(OH)_2D_3$ are given to such rats, little of it survives passage through the intestine, thus reducing markedly its physiological effects.

It is interesting to note that the concentration of 1,25-$(OH)_2D_3$ in the intestine of the pretreated animals is still at a high enough level to stimulate the intestinal calcium transport response. However, even with calcium entering the plasma through the intestine, bone calcification still does not occur suggesting perhaps, among other things, the further involvement of 1,25-$(OH)_2D_3$ in the calcification process.

The control of intestinal 1,25-$(OH)_2D_3$ metabolism by 1,25-$(OH)_2D_3$ itself can be visualized to have the important function of inactivating the 1,25-$(OH)_2D_3$ after it has performed its physiological function. If the intestinal 1,25-$(OH)_2D_3$ was allowed to remain active over a long period of time, the postulated control of vitamin D metabolism at the level of the conversion of 25-OHD_3 to 1,25-$(OH)_2D_3$ (26) would be relatively ineffective at least as far as intestine is concerned.

The significance of the large amount of the metabolites less polar than 1,25-$(OH)_2D_3$ in the muscle and bone from rats pretreated with oral 1,25-$(OH)_2D_3$ is not yet clear. Possible explanations for these peaks include a storage form of 1,25-$(OH)_2D_3$ or an excretion form of 1,25-$(OH)_2D_3$, although if it were the latter one might expect to observe more in the kidney and liver, the two major organs of excretion. More work must be done to verify one or both of the explanations or to suggest a third. Experiments also are underway to determine whether the intestine is indeed the site of the modification of the 1,25-$(OH)_2D_3$ in the orally dosed animals as the results in this paper appear to suggest.

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