Metabolism of 1,25-Dihydroxyvitamin D_3: Evidence for Side-Chain Oxidation†

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ABSTRACT: Approximately 7% of a 650-pmol dose of 25-hydroxy[26,27-^{14}C]vitamin D_3 and 25% of a 325-pmol dose of 1,25-dihydroxy[26,27-^{14}C]vitamin D_3 are metabolized to $^{14}CO_2$ by vitamin D deficient rats. Nephrectomy prevents the metabolism of 25-hydroxy[26,27-^{14}C]vitamin D_3 to $^{14}CO_2$ but not that of 1,25-dihydroxy[26,27-^{14}C]vitamin D_3. Less than 5% of the ^{14}C from 24,25-dihydroxy[26,27-^{14}C]vitamin D_3 is metabolized to $^{14}CO_2$. Feeding diets high in calcium and supplemented with vitamin D_3 markedly diminishes the amount of $^{14}CO_2$ formed from 25-hydroxy[26,27-^{14}C]vitamin D_3 but not that from 1,25-dihydroxy[26,27-^{14}C]vitamin D_3. These results provide strong evidence that only 1-hydroxylated vitamin D compounds and especially 1,25-dihydroxyvitamin D_3 undergo side-chain oxidation and cleavage to yield an unknown metabolite and CO_2.

Conclusive evidence has been presented that 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$)[1] or a further metabolite is the metabolically active form of vitamin D in intestinal calcium transport (Boyle et al., 1972; Wong et al., 1972), bone calcium mobilization (Holick et al., 1972), and intestinal phosphate transport (Chen et al., 1974). In an attempt to determine if 1,25-$(OH)_2D_3$ is further metabolized before it functions, investigations were carried out with 1,25-$(OH)_2$[26,27-^{3}H]D_3 which showed that, at the time the intestine and bone respond, the only ^{3}H-labeled metabolite found in the lipid extracts of these tissues is 1,25-$(OH)_2D_3$ (Frolik and DeLuca, 1971, 1972; Tsai et al., 1972). This was further supported by experiments in which 1,25-$(OH)_2D_3$ was labeled in the 2 position with ^{3}H (Frolik and DeLuca, 1971, 1972). However, only 80% of the total tissue radioactivity was accounted for in these studies and, furthermore, considerably less than 100% of the ^{3}H administered could be detected in the animal shortly after administration of the 1,25-$(OH)_2$[^{3}H]D_3. These results suggested that the complete functional metabolism of vitamin D might not be fully understood, and that the 26,27-^{3}H positions may not survive metabolism in vivo. To examine this question we prepared 25-OH-[26,27-^{14}C]D_3 chemically by methods previously described using [^{14}C]methylmagnesium bromide (Suda et al., 1971). We further prepared 1,25-$(OH)_2$[26,27-^{14}C]D_3 enzymatically (Gray et al., 1972). Using these tools we can demonstrate that both compounds undergo metabolism to yield $^{14}CO_2$, revealing a new pathway of vitamin D metabolism involving loss of at least one carbon from the side chain. Additional evidence strongly suggests that this pathway involves the conversion of 25-OH-D_3 to 1,25-$(OH)_2D_3$ followed by side-chain oxidation of the 1,25-$(OH)_2D_3$. Inasmuch as 24,25-$(OH)_2$[26,27-^{14}C]D_3 also gives some $^{14}CO_2$, it is likely that the side-chain oxidation reaction is not restricted to 1,25-$(OH)_2D_3$ but may involve other 1-hydroxylated forms of vitamin D such as 1,24,25-$(OH)_3D_3$.

Materials and Methods

Isotopically Labeled Compounds. The 26,27-^{14}C was chemically synthesized by the method of Suda et al. (1971). From this compound, 1,25-$(OH)_2$[26,27-^{14}C]D_3 and 24,25-$(OH)_2$[26,27-^{14}C]D_3 were prepared enzymatically as previously described (Gray et al., 1972) using appropriate chick kidney mitochondria. Radiochemical purity was established by cochromatography with crystalline 25-OH-D_3, 1,25-$(OH)_2D_3$, and 24,25-$(OH)_2D_3$ on Sephadex LH-20 columns (Holick and DeLuca, 1971) and on high-pressure liquid chromatography (Jones and DeLuca, 1975).

Animals. Male, albino, weanling rats (Holtzman Co., Madison, Wis.) were maintained in overhanging wire cages. They were fed a purified vitamin D deficient diet with high, low, or normal concentrations of calcium (Suda et al., 1970) and normal or low concentrations of phosphorus (Tanaka and DeLuca, 1974) ad libitum for at least 3 weeks for vitamin D_3 depletion. When indicated, 25 ng of vitamin D_3 or 25-OH-D_3 was administered each day orally in 0.1 ml of cottonseed (Wesson) oil for a period of two weeks prior to experimentation.

To prepare the 1,25-$(OH)_2$[26,27-^{14}C]D_3, 1-day-old white leghorn cockerels (Northern Hatcheries, Beaver Dam, Wis.) were fed a rachitogenic diet for 4 weeks (Omdahl et al., 1971), at which time they were used to prepare kidney mitochondria (Gray et al., 1972). Other chicks from the same source were fed a high calcium diet plus vitamin D for the preparation of 24,25-$(OH)_2$[26,27-^{14}C]D_3 as previously described (Knutson and DeLuca, 1974).

Metabolism Studies and Collection of $^{14}CO_2$. Rats prepared as described above were given the appropriately labeled material (either 650 pmol for 25-OH-D_3 or 325 pmol for 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$) in 0.05 ml of ethanol intrajugularly. They were immediately placed in a sealed metabolism glass apparatus for collection of $^{14}CO_2$ (Delmar Scientific Co., Maywood, Ill.), and were supplied food and distilled water ad libitum during the collection period. The apparatus was operated under slight negative pressure brought about by water pump vacuum. Incoming air was dried over $CaSO_4$ and passed over a column of Ascarite (Arthur Thomas Co., Philadelphia, Pa.) to remove all CO_2 before entering the cage. The $^{14}CO_2$ and unlabeled CO_2 generated in the cage

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[1] Abbreviations used are: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3.

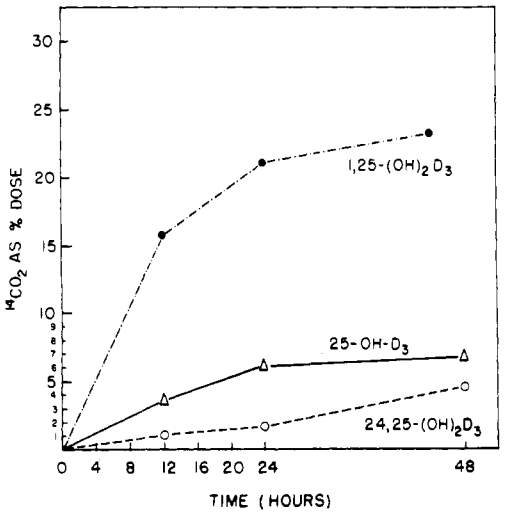

FIGURE 1: Cumulative $^{14}CO_2$ formation after the injection of 25-OH-[26,27-^{14}C]D_3, 1,25-$(OH)_2$[26,27-^{14}C]D_3, and 24,25-$(OH)_2$[26,27-^{14}C]D_3 into rats raised on a vitamin D deficient low calcium diet.

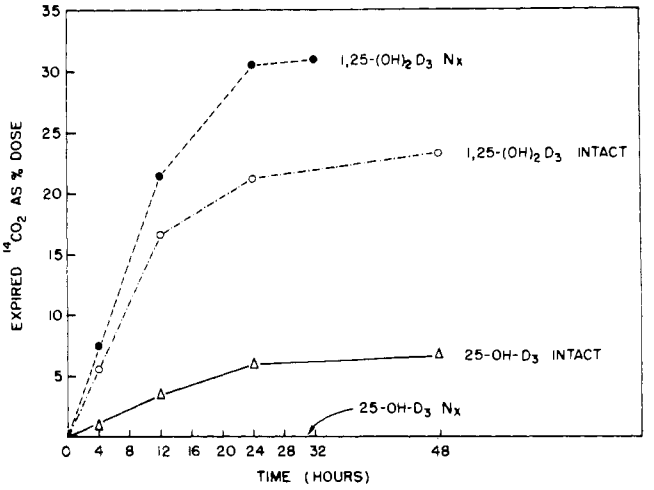

FIGURE 2: Cumulative $^{14}CO_2$ formation after the injection of 25-OH-[26,27-^{14}C]D_3 or 1,25-$(OH)_2$[26,27-^{14}C]D_3 into intact or nephrectomized rats.

were trapped in an ethanolamine–methyl Cellusolve solution (Jeffay and Alvarez, 1961) which was changed at 4, 8, 12, 24, 48, and 72 h or 4, 8, 12, 24, 28, and 32 h.

Scintillation Counting. The $^{14}CO_2$ trapped in the ethanolamine methyl Cellusolve solution was counted in scintillation fluid containing toluene:methyl Cellusolve (2:1 v/v) and 5.5 g of 2,5-diphenoxazole per l. (Jeffay and Alvarez, 1961). Radioactive metabolites were counted in a scintillation solution containing 2 g of 2,5-diphenoxazole, 100 g of 1,4-bis[2-(4-methyl-5-phenoxazolyl)]benzene per l. of toluene. Radioactivity was determined using a Packard TriCarb liquid scintillation counter, Model 3375, equipped with automatic external standardization.

Animals injected with the ethanol solution with isotopically labeled material produced no detectable $^{14}CO_2$, while standard $^{14}CO_2$ produced by acidification of $Ba^{14}CO_3$ was collected with at least a 95% efficiency.

Measurement of Calcium and Phosphorus. Calcium was measured in the presence of 0.1% $LaCl_3$ using a Perkin-Elmer atomic absorption spectrophotometer, Model 402. Phosphate was measured using the method of Chen et al. (1956).

Results

Vitamin D deficient rats on a low calcium diet (serum calcium concentration of approximately 4 mg/100 ml) clearly produce $^{14}CO_2$ from 25-OH-[26,27-^{14}C]D_3, 1,25-$(OH)_2$[26,27-^{14}C]D_3, and 24,25-$(OH)_2$[26,27-^{14}C]D_3 (Table I, Figure 1). Of these metabolites the most effectively metabolized to $^{14}CO_2$ is 1,25-$(OH)_2$[26,27-^{14}C]D_3, with the 24,25-$(OH)_2$[26,27-^{14}C]D_3 yielding the smallest percent of an injected dose as $^{14}CO_2$. Of considerable interest is that $^{14}CO_2$ appears in the expired air within 4 h after dose, illustrating metabolism early enough to be of significance in function (Figure 2).

Inasmuch as the kidney is the sole site of 1,25-$(OH)_2D_3$ biosynthesis (Fraser and Kodicek, 1970; Gray et al., 1971), experiments were carried out with the nephrectomized vitamin D deficient animals in an attempt to assess the essentiality of 1-hydroxylation to the metabolism of 25-OH-[26,27-^{14}C]D_3 to $^{14}CO_2$. The results shown in Figure 2 and Table I demonstrate that nephrectomy abolishes the metabolism of 25-OH-[26,27-^{14}C]D_3 but does not appreciably affect the metabolism of 1,25-$(OH)_2$[26,27-^{14}C]D_3 to $^{14}CO_2$.

TABLE I: Summary of $^{14}CO_2$ Production from 26,27-^{14}C-Labeled Vitamin D Metabolites by Vitamin D Deficient Rats.[a]

Compound	Intact[b] (% dose)	Nephrectomized[b] (% dose)
25-OH-D_3	7.0 ± 1.4	0
1,25-$(OH)_2D_3$	23.2 ± 11.4	31.8 ± 1.3
24,25-$(OH)_2D_3$	4.6 ± 3.3	Not determined

[a] Vitamin D deficient rats were injected intrajugularly with 650 pmol of 25-OH-[26,27-^{14}C]D_3 or 325 pmol of either 1,25-$(OH)_2$[26,27-^{14}C]D_3 or 24,25-$(OH)_2$[26,27-^{14}C]D_3. The $^{14}CO_2$ was collected for the next 48 h period. [b] Rats were either sham operated or bilaterally nephrectomized prior to the injection of the metabolites in 0.05 ml of 95% ethanol.

Boyle et al. (1971) demonstrated that rats given a small daily dose of 25 ng of vitamin D_3/day vary in their ability to produce 1,25-$(OH)_2D_3$. Such rats on a low calcium diet produce large amounts of 1,25-$(OH)_2D_3$ while those on a high calcium diet produce little 1,25-$(OH)_2D_3$. Furthermore, this regulation in chicks has been demonstrated by measurement of the 1-hydroxylase in vitro (Omdahl et al., 1972; Henry et al., 1974). The results in Table II show that rats on the low calcium diet produce $^{14}CO_2$ from 25-OH-[26,27-^{14}C]D_3 in greater amounts than those rats on a normal or high calcium diet when supplemented with vitamin D_3. Although phosphate deprivation also stimulates 1-hydroxylation (Tanaka and DeLuca, 1973; DeLuca et al., 1976), the low phosphorus diet did not appreciably stimulate $^{14}CO_2$ production from 25-OH-[26,27-^{14}C]D_3. In agreement with Boyle et al. (1971), vitamin D deficient rats produce large amounts of $^{14}CO_2$ from 25-OH-[26,27-^{14}C]D_3 regardless of dietary levels of calcium and phosphorus. Note that to reduce variability due to difference in body weight among groups the data are expressed as percent dose/100 g body weight. Table III demonstrates that vitamin D status and dietary levels of calcium do not influence $^{14}CO_2$ production from 1,25-$(OH)_2$[26,27-^{14}C]D_3.

Discussion

The present report demonstrates clearly that vitamin D undergoes side-chain oxidation with the loss of at least one carbon and possibly more as CO_2, revealing a heretofore un-

TABLE II: $^{14}CO_2$ Formation After the Injection of 650 pmol of 25-OH-[26,27-^{14}C]D_3 to Rats on Different Calcium and Phosphorus Diets with or without Vitamin D_3.

Diet [a]	Time (h) after Dose	+D Diet [b]	Serum Ca/P [c]	−D Diet [b]	Serum Ca/P [c]	P Value
1	12	7.0±2.4	4.4/9.5	4.3±1.1	4.37/10	NS
	24	12.2±3.6		7.1±1.4		NS
	48	14.0±3.8		8.0±1.7		NS
2	12	1.9±0.3	9.1/9.5	2.8±0.5	5.5/9.5	0.10
	24	2.4±0.4		3.9±0.3		0.05
	48	2.5±0.5		4.8±0.9		0.05
3	12	1.9±0.3	9.5/−	5.3±0.9	8.3/−	0.01
	24	2.3±0.3		7.5±1.0		0.01
	48	2.3±0.5		8.2±0.9		0.01
4	12	2.1±0.4	11.3/3.5	3.5±0.5	9.9/2.4	0.05
	24	2.3±0.4		5.2±0.9		0.01
	48	3.1±0.4		7.8±1.9		0.05

[a] The diets contained: (1) 0.02% Ca, 0.3% P; (2) 0.47% Ca, 0.3% P; (3) 3.0% Ca, 0.3% P; and (4) 1.2% Ca, 0.1% P. [b] Rats in the +D group received 1 IU vitamin D_3 orally for 2 weeks, while those in the −D group received no vitamin D supplementation. The results are expressed as % dose/100 g body weight. [c] Expressed as mg/100 ml.

TABLE III: Cumulative $^{14}CO_2$ Formation After the Injection of 325 pmol of 1,25-$(OH)_2$[26,27-^{14}C]D_3 in Rats Raised on Different Diets with or without Vitamin D_3 in the Diet.

Diet [a]	Time (h) after Dose	$^{14}CO_2$ % Dose/100 g Body wt
1	12	10.9±4.5
	24	13.4±5.5
	48	14.8±4.9
2	12	10.3±3.0
	24	13.9±4.5
	48	14.1±4.8
3	12	19.8±4.4
	24	23.5±4.0
	48	25.8±4.9
4	12	16.8±4.2
	24	19.8±4.7
	48	20.2±4.8

[a] The diets contained: (1) 1.2% Ca, 0.3% P, and 1 IU of vitamin D_3 orally for 2 weeks; (2) 1.2% Ca, 0.3% P, and 1 IU of 25-(OH)-D_3 for 2 weeks; (3) 0.02% Ca, 0.3% P, no vitamin D_3; and (4) 1.2% Ca, 0.1% P, and no vitamin D_3. There was no significant difference between vitamin D deficient and vitamin D fed groups.

known pathway of vitamin D metabolism. The most actively metabolized form of vitamin D by this pathway is 1,25-$(OH)_2D_3$, followed by 25-OH-D_3 and 24,25-$(OH)_2D_3$. Inasmuch as nephrectomy prevents this metabolism in the case of 25-OH-D_3 but not 1,25-$(OH)_2D_3$, it is apparent that the pathway involves the conversion of vitamin D_3 to 25-OH-D_3 and subsequently to 1,25-$(OH)_2D_3$ before it undergoes the side-chain metabolism described in this report. It is of some interest that 24,25-$(OH)_2$[26,27-^{14}C]D_3 is also metabolized to $^{14}CO_2$. Unfortunately, the small amount of $^{14}CO_2$ produced from this substrate in 24 h (survival time of nephrectomized animals) precluded an experiment to determine if it must be 1-hydroxylated before it is metabolized to $^{14}CO_2$. Thus, it is probable, but not proved, that 1,24,25-$(OH)_3D_3$ undergoes similar side-chain metabolism to the 1,25-$(OH)_2D_3$.

In strong support of the idea that the side-chain metabolism occurs only on 1-hydroxylated vitamin D compounds, it was demonstrated that conditions (low dietary calcium and vitamin D deficiency) which favor 1-hydroxylation (see Boyle et al., 1971; Omdahl et al., 1972; Tanaka and DeLuca, 1971) also favor $^{14}CO_2$ production from 25-OH-[26,27-^{14}C]D_3, while conditions which favor 24-hydroxylation (high calcium, vitamin D supplementation) do not. Furthermore, the metabolism of 1,25-$(OH)_2$[26,27-^{14}C]D_3 to $^{14}CO_2$ is not affected by such dietary manipulation. A discrepancy is that phosphate deprivation does not stimulate $^{14}CO_2$ production from 25-OH-[26,27-^{14}C]D_3 despite increased production of 1,25-$(OH)_2D_3$ by this condition. The reason for this remains unknown at the present time.

The nature of the side-chain metabolism is unknown, but it is certain that at least one and probably more carbons are cleaved from the side chain of 1,25-$(OH)_2D_3$. Failure to detect metabolites as a result of this metabolism is probably because of the use of 1,25-$(OH)_2$[26,27-^{3}H]D_3, the only abundant form of labeled 1,25-$(OH)_2D_3$ available. Metabolism of this compound probably results in total loss of the label. Thus, it appears that further progress with this pathway will require synthesis of 1,25-$(OH)_2D_3$ labeled in non-side-chain positions. This is currently in progress in our laboratory.

It is not known in which tissue side-chain metabolism of 1,25-$(OH)_2D_3$ occurs. Certainly the kidney can be excluded as the sole site since nephrectomy does not prevent the $^{14}CO_2$ from appearing from 1,25-$(OH)_2$[26,27-^{14}C]D_3. Again this must await an appropriately labeled compound, although in vitro experiments using the 1,25-$(OH)_2$[^{14}C]D_3 and $^{14}CO_2$ production using tissue preparations may provide some insight into this question.

The metabolism of 1,25-$(OH)_2$[26,27-^{14}C]D_3 to $^{14}CO_2$ begins within 4 h after administration. Intestinal calcium transport and bone calcium mobilization are significantly elevated at 3 h after dose (Boyle et al., 1972; Omdahl et al., 1972; Tanaka and DeLuca, 1971) in rats. Inasmuch as exhaled CO_2 is being measured, it appears possible that side-chain metabolism occurs in sufficient time to be of significance in the function of 1,25-$(OH)_2D_3$. Alternatively it may represent a degradative pathway. Since degradation is an important aspect of hormonal regulation and 1,25-$(OH)_2D_3$ is an important hormone in calcium and phosphorus metabolism, the pathway reported here must be assigned considerable potential importance physiologically regardless of whether it is of functional importance or of degradative importance.

Acknowledgment

We are grateful to Dr. Michael F. Holick for his efforts in the synthesis of 25-OH-[26,27-^{14}C]D_3.

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Polymorphism of *Sporothrix schenckii* Surface Polysaccharides as a Function of Morphological Differentiation†

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ABSTRACT: The alkali-extractable polysaccharides from different morphological types of two *Sporothrix schenckii* strains (1099.12 and 1099.18) were investigated. Dissociation of morphological phase transition and temperature effects was possible in a synthetic medium which produced cultures with 100% yeast forms either at 25 or at 37 °C. Only rhamnomannans with single-unit α-L-rhamnopyranosyl side chains were formed by the yeast forms irrespective of the incubation temperature. The higher temperature inhibited formation of 4-*O*- and 2,4-di-*O*-substituted α-D-mannopyranose units in the rhamnomannan. An apparently unsporulated mycelium culture of one *S. schenckii* strain (1099.12) synthesized a galactomannan whose structure was partially determined by methylation analysis and by proton and ^{13}C nuclear magnetic resonance spectroscopy. In another strain (1099.18), a mannan was excreted in the medium of an apparently conidia-less mycelial form at 25 °C with short incubation. Its structure was also partially determined. An apparent mixture of this mannan and a rhamnomannan rich in α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranose side chains formed in these cultures on prolonged incubation. The proportion of the excreted rhamnomannan increased as the mycelium sporulated and conidia were more numerous. Mannans or galactomannans may be transient polysaccharides in the young mycelium of *S. schenckii*. As the culture develops, rhamnomannans are formed in amounts usually masking the presence of other mannose-containing polysaccharides. It is suggested that in *S. schenckii* different polysaccharides are formed with side chains containing different proportions of rhamnose, mannose, or galactose, as a function of morphological differentiation.

Surface polysaccharides are important fungal antigens, and their fine chemical structures may differ depending on the microbial strain, species, or genus. Mannans or heteropolymers containing mannose are the usual polysaccharides present in the majority of yeasts (Gorin and Spencer, 1968; Phaff, 1971). Mannose-containing polysaccharides are also found in filamentous fungi including pathogenic organisms (Azuma et al., 1971, 1974; Kanetsuna et al., 1974). Among the latter is *Sporothrix schenckii,* which synthesizes an L-rhamno-D-mannan (Ishizaki, 1970; Lloyd and Bitoon, 1971). All studied strains of *S. schenckii* formed rhamnomannans with similar structures, as inferred by their proton nuclear magnetic resonance (^1H NMR) and ^{13}C nuclear magnetic resonance (^{13}C NMR) spectra (Travassos et al., 1974). Rhamnomannans were also found in several *Ceratocystis* and *Graphium* species and in *Taphrina deformans* (Gorin and Spencer, 1970), but were not found in pathogenic fungi besides *S. schenckii.*

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