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Mechanism of 25-Hydroxycholecalciferol 1α-Hydroxylation. Incorporation of Oxygen-18 into the 1α Position of 25-Hydroxycholecalciferol†

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ABSTRACT: All of the oxygen enzymatically inserted as a hydroxyl function by chick kidney mitochondria into the 1α position of 25-hydroxycholecalciferol to give 1,25-dihydroxycholecalciferol is derived from $^{18}O_2$. None could be detected as arising from water demonstrating that the 25-hydroxycholecalciferol-1α-hydroxylase system is a "mixed-function oxidase."

It is now well established that vitamin D_3 must be metabolically activated before it can manifest its physiologic functions (DeLuca, 1969). The activation of vitamin D_3, primarily by the liver (Horsting and DeLuca, 1969), involves a hydroxylation reaction in the 25 position to produce 25-hydroxycholecalciferol (25-OH-D_3).[1] The hydroxylated derivative, 25-OH-D_3, represents the major metabolite of vitamin D_3 circulating in blood plasma, not only of experimental rats (Lund and DeLuca, 1966) and chickens (Lawson *et al.*, 1969), but also of man (Mawer *et al.*, 1969). The 25-OH-D_3 is subsequently converted to 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$) in the kidney (Fraser and Kodicek, 1970; Holick *et al.*, 1971). This hydroxylation takes place in the mitochondrion of the renal cell at least in the chick (Fraser and Kodicek, 1970; Gray *et al.*, 1972). This system has been studied extensively (Gray *et al.*, 1972) and in many ways is similar to the mixed-function oxidases of beef adrenal cortex mitochondria concerned with steroidogenesis (Sih, 1969). Both the kidney and adrenal enzymes require magnesium, molecular oxygen, and a source of reduced pyridine nucleotide. NADPH, rather than NADH, is the specific electron donor for both the kidney and adrenal hydroxylation systems.

Of considerable interest is the molecular mechanism of the 1-hydroxylation reaction. Chemical synthesis of 1α,25-$(OH)_2D_3$ utilizing known stereochemical reactions provides strong evidence that the natural metabolite is 1α,25-$(OH)_2D_3$ (Semmler *et al.*, 1972). The enzymatic insertion of the oxygen could either be by dehydrogenation and subsequent hydration or it could take place by a mixed-function oxidase mechanism. The latter mechanism requires that the 1-hydroxyl oxygen arises from molecular oxygen, while the former requires that the oxygen arises from H_2O.

In this paper we wish to report the finding that in the presence of $^{18}O_2$, all the oxygen enzymatically introduced as a hydroxyl function into the 1α position of 25-OH-D_3 to yield 1,25-$(OH)_2D_3$ is derived from $^{18}O_2$. None was incorporated from water. The results clearly demonstrate that the 25-OH-D_3 hydroxylating system is a "mixed-function oxidase" rather than a dehydrogenase, and no hydration reaction is involved.

Methods

Animals. One-day old white Leghorn cockeral chicks were obtained from Northern Hatcheries (Beaver Dam, Wis.). They were maintained on a vitamin D deficient purified soy protein diet for 4 weeks before use (Omdahl *et al.*, 1971).

Preparation of Mitochondria. The chicks were killed by cervical dislocation, and the kidneys were immediately removed and carefully separated from the adhering connective tissue. The kidneys were rinsed with chilled buffer (0.25 M sucrose–15 mM Tris-acetate, pH 7.4, which was also 1 mM in dithiothreitol and 10^{-4} M in EDTA), then transferred to an ice-cold Potter-Elvehjem homogenizer, fitted with a Teflon pestle, and homogenized in 4 vol of the buffer. The homogenate was centrifuged at 500*g* at 4° for 10 min. The resultant pellet was washed once with buffer. The mitochondria were sedimented by centrifuging the combined supernatant frac-

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[1] Abbreviations used are: 25-OH-D_3, 25-hydroxycholecalciferol; 1,25-$(OH)_2D_3$, 1,25-dihydroxycholecalciferol.

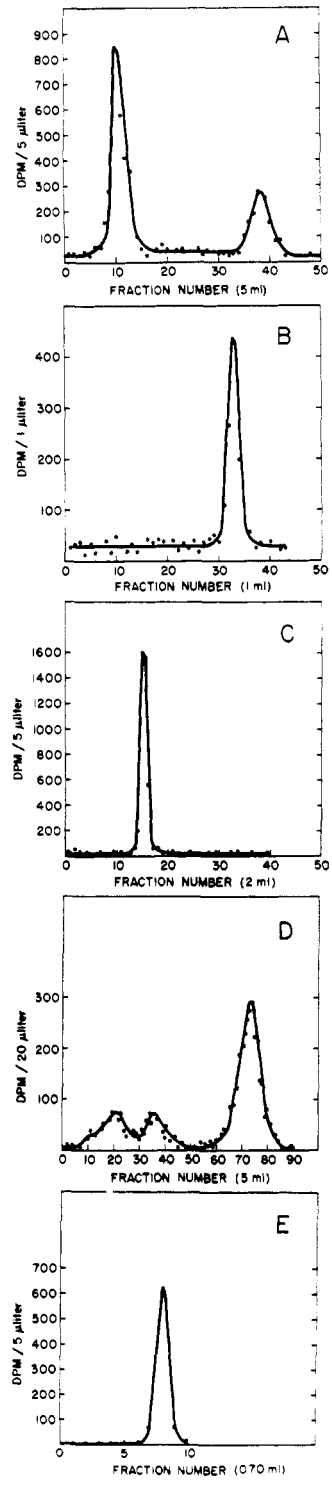

FIGURE 1: Chromatographic profiles during the isolation of the 1α-^{18}O-1,25-[^{3}26,27-H](OH)$_2$D$_3$. (A) Sephadex LH-20 column (1 × 60 cm packed in and eluted with 65:35 $CHCl_3$–Skellysolve B) profile of lipid extract from mitochondrial incubations of 25-[26,27-^{3}H]OH-D$_3$. (B) Sephadex LH-20 column (1 × 60 cm packed in and eluted with MeOH) profile of 1α-^{18}O-1,25-(OH)$_2$D$_3$ region isolated from the Sephadex LH-20 column (65:35) $CHCl_3$–Skellysolve B) shown in A. (C) Bio-Beads S-X8 column (1 × 100 cm packed in and eluted with 65:35 $CHCl_3$–Skellysolve B) profile of 1α-^{18}O-1,25-(OH)$_2$D$_3$ region isolated from the Sephadex LH-20 column (MeOH) shown in B. (D) Sephadex LH-20 column (1 × 60 cm packed in and eluted with 1:1 $CHCl_3$–Skellysolve B) profile of 1α-^{18}O-1,25-(OH)$_2$D$_3$ region isolated from Bio-Beads S-X8 column (65:35 $CHCl_3$–Skellysolve B) shown in C. (E) Sephadex LH-20 column (1 × 10 cm packed in and eluted with 65:35 $CHCl_3$–Skellysolve B) profile of tris(trimethylsilyl ether) derivative of 1α-^{18}O-1,25-(OH)$_2$D$_3$ prepared as described under Methods.

tions at 3600*g* at 4° for 10 min. The mitochondrial pellet was washed once with buffer and suspended in the buffer to give a mitochondrial protein concentration of 8 mg/ml as determined by the method of Lowry *et al.* (1951).

Incubation Conditions and Extraction of Samples. Five incubations, each 1.5-ml total volume, were performed in 20-ml serum bottles containing 8 mg of mitochondrial protein, 5 mM magnesium acetate, and 5 mM succinate. The cofactor additions were made in the buffer previously described. The bottles were sealed with serum bottle caps and connected through needle outlets to a vacuum train. The bottles were then evacuated repeatedly followed by flushings with purified N_2, passed over heated copper, and finally filled with N_2 at 1 atm. To each of the five incubation bottles was introduced (with a micro-Hamilton syringe) 1.2 μg of 25-[26,27-^{3}H]-OH-D$_3$[2] in 10 μl of 95% ethanol with a specific radioactivity of 778,000 dpm/μg (Suda *et al.*, 1971). The hydroxylation reactions were initiated by replacing (with a syringe) 10 ml of the N_2 in each bottle with an equivalent volume of 99.3 atom % $^{18}O_2$ (Bio-Rad Laboratories, Richmond, Calif.). After 60 min of incubation at 37° with gentle agitation, the reactions were terminated by the addition of 10 ml of 2:1 MeOH–$CHCl_3$. A two-phase system at 4° was produced by the addition of 2.5 ml of water and 5 ml of $CHCl_3$. The lower phases were removed and the upper phases were each reextracted twice with 5-ml portions of $CHCl_3$. The combined chloroform extracts contained a total of 4.70 × 10^6 dpm or approximately 100% recovery of added radioactivity.

Chromatography. The combined chloroform extracts were evaporated to dryness under N_2 at room temperature. The residue was dissolved in 500 μl of 65:35 $CHCl_3$–Skellysolve B and applied to a 1 × 60 cm glass column containing 18 g of Sephadex LH-20 (Pharmacia Corp., Piscataway, N. J.) packed and eluted in the same solvent system (Holick and DeLuca, 1971). A total of 50 fractions (5 ml at a flow rate of 1 ml/min) were collected and 5 μl of each fraction was used for tritium counting (Figure 1A) in a Packard Model 3375 liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). The efficiency of counting was 42%. The relative position of the metabolites has been determined previously by chromatography of the pure metabolites under identical experimental conditions (Holick and DeLuca, 1971). The product peak region, fractions 35–42, was combined and evaporated to dryness under N_2 at room temperature to yield a total radioactivity of 1.21 × 10^6 dpm equivalent to 1.55 μg of 1,25-(OH)$_2$D$_3$ (based on 778,000 dpm/μg, the specific radioactivity of the substrate 25-OH-D$_3$). The residue was dissolved in 100 μl of MeOH and applied to a 1 × 60 cm glass column packed with 18 g of Sephadex LH-20 in MeOH (Suda *et al.*, 1970). The column was eluted with MeOH. A total of 40 fractions (1 ml at a flow rate of 0.5 ml/min) was collected and 1 μl of each fraction was used for tritium counting (Figure 1B). The product peak region, fractions 31–35, was combined and evaporated to dryness under N_2 at room temperature to yield a total radioactivity of 1.07 × 10^6 dpm equivalent to 1.38 μg of product. The residue was dissolved in 100 μl of 65:35 $CHCl_3$–Skellysolve B and applied to a 1 × 100 cm glass column packed with Bio-Beads S-X8 (Bio-Rad Corp., Richmond, Calif.) in the same solvent. The column was eluted with the same solvent. A total of 40 fractions (2 ml at a flow rate of 0.5 ml/min) were collected and 5 μl of each fraction was

[2] Calculated from the ultraviolet spectrum in 95% EtOH using a value for ϵ_{264} of 18,200 M^{-1} cm^{-1}.

used for tritium counting (Figure 1C). The peak tubes (14–16) were combined and dried under N_2 at room temperature to yield a total radioactivity of 1.04×10^6 dpm equivalent to 1.34 μg of product. The residue was dissolved in 100 μl of 1:1 $CHCl_3$–Skellysolve B and applied to a 1×60 cm glass column packed with Sephadex LH-20 in the same solvent. The column was eluted with the same solvent. A total of 90 fractions (5 ml at a flow rate of 0.75 ml/min) were collected and 20 μl of each fraction was used for tritium counting (Figure 1D). The total radioactivity in the region of 1,25-$(OH)_2D_3$ (fractions 66–81) was 6.6×10^5 dpm equivalent to 850 ng of product. Fractions 72–74 were combined and dried under N_2 at room temperature to yield a total radioactivity of 2.05×10^5 dpm equivalent to 264 ng of 1,25-$(OH)_2D_3$. This residue was dissolved in 100 μl of MeOH and applied to a 1×60 cm glass column packed with Sephadex LH-20 in MeOH and eluted as previously described. A total of 40 fractions was collected and 10 μl of each fraction was used for tritium counting. The elution profile was identical with that shown in Figure 1B. The peak fractions 33 and 34 were combined and dried under N_2 to yield a total radioactivity of 1.19×10^5 dpm equivalent to 153 ng of 1,25-$(OH)_2D_3$. This residue was analyzed in the mass spectrometer (Associated Electrical Industries, Ltd., Manchester, England, Model MS-902) by direct probe inlet at 130° and 70-eV ionization energy.

Fractions 68–70 and 76–78 from the 50% $CHCl_3$ column (Figure 1D) were combined and dried under N_2 to yield a total radioactivity of 2.52×10^5 dpm equivalent to 323 ng of 1,25-$(OH)_2D_3$. This residue was dissolved in 100 μl of MeOH and applied to a 1×60 cm glass column packed with Sephadex LH-20 in MeOH and eluted as previously described. Again, a total of 40 fractions was collected and 10 μl of each fraction was used for tritium counting. The peak fractions 33 and 34 were combined and dried under N_2 to yield a total radioactivity of 1.18×10^5 dpm equivalent to 152 ng of 1,25-$(OH)_2D_3$. The dried product (152 ng) was dissolved in 25 μl of pyridine and allowed to react with 10 μl of TBT (a special combination of trimethylsilylimidazole, bis(trimethylsilyl-acetamide), and trimethylchlorosilane, Pierce Chemical Co., Rockford, Ill.) at 60° for 1 hr. The reaction mixture was evaporated under N_2 and the oily residue dissolved in 100 μl of 65:35 $CHCl_3$–Skellysolve B and applied to a 1×10 cm Sephadex LH-20 column packed and eluted in the same mixed solvent system. A total of 10 fractions (0.70 ml each) was collected and 5 μl of each fraction was used for tritium counting (Figure 1E). Fraction 8 containing the tris(trimethylsilyl) ether derivative of 1α-^{18}O-1,25-$(OH)_2D_3$ was dried under N_2 to yield a total radioactivity of 8.66×10^4 dpm equivalent to 110 ng of the trisilyl derivative of the metabolite. This derivative was also analyzed in the mass spectrometer as previously described.

Results

The purification of the ^{18}O metabolite depended heavily on liquid–gel partition chromatography (Holick and DeLuca, 1971) using Sephadex LH-20 suspended in double-distilled organic solvents as indicated throughout the procedures. An excellent resolution (Figure 1A) is observed of 1,25-$(OH)_2D_3$ from the unaltered, undegradated substrate 25-OH-D_3. An overall enzymatic conversion of 25% as 1,25-$(OH)_2D_3$ (1.55 μg) is calculated from the initial amount of substrate added (6.0 μg). The recovery of metabolites from the various columns was essentially quantitative. Minor impurities were successfully removed using the Sephadex LH-20 column developed in 50% $CHCl_3$–Skellysolve B (Figure 1D). Hence, the product obtained was sufficiently pure to allow mass spectral analysis.

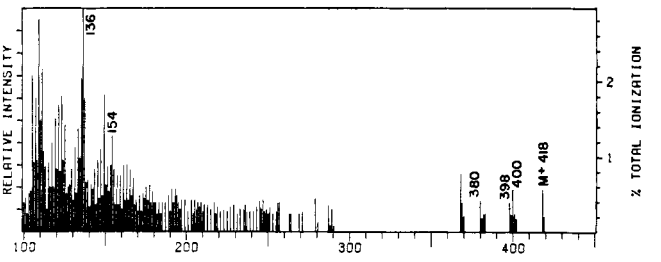

FIGURE 2: Mass spectrum of 1α-^{18}O-1,25-$(OH)_2D_3$.

The isolation and identification of the ^{16}O 1α-hydroxy analog of 25-OH-D_3 have been reported by Holick *et al.* (1971). The mass spectrum of the natural dihydroxy metabolite (Holick *et al.*, 1971; Lawson *et al.*, 1971) was shown to exhibit a molecular ion peak at *m/e* 416 and prominent fragment peaks at *m/e* 134, 152, 380, and 398. The tris(trimethylsilyl ether) derivative with a molecular ion peak at *m/e* 632 also exhibited the expected fragment peaks of *m/e* 206 and 542.

The mass spectrum of the ^{18}O 1α-hydroxy analog of 25-OH-D_3 (Figure 2) was compared with that of its natural metabolite (Holick *et al.*, 1971). The molecular ion peak at *m/e* 418 and the fragment peaks at *m/e* 136, 154, 380, 382, and 400 (Figure 3) establish conclusively the mitochondrial incorporation of molecular oxygen as a hydroxyl function into the C-1 position of 25-OH-D_3. Furthermore, the molecular ion peak at *m/e* 634 of the tris(trimethylsilyl) derivative spectrum (not shown) and its prominent fragment peaks at *m/e* 208 and 544 provide additional evidence for the above conclusion. It should be noted that appearing on the mass spectrum is a contaminant exhibiting an ion peak at *m/e* 368. This contaminant has been previously observed in our kidney metabolite studies.

Examination of the dehydration pattern of the C-7–C-8 cleavage product with a fragment of *m/e* 154 (Figure 3) indicates that the 3β-hydroxyl function of ring A is preferentially eliminated to *m/e* 136 (154 − H_2O) *vs.* *m/e* 134 (154 − $H_2{}^{18}O$). This observation appears to be consistent with that of the cor-

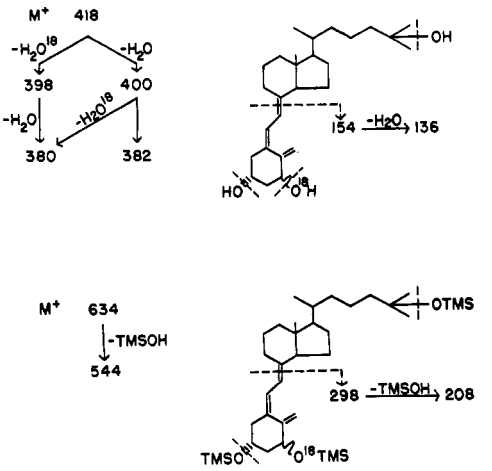

FIGURE 3: Fragmentation pattern leading to prominent ions in the mass spectrum of 1α-^{18}O-1,25-$(OH)_2D_3$ and its tris(trimethylsilyl) derivative. Dashed lines indicate points of cleavage or dehydration.

responding trimethylsilyl derivative fragment. The fragment at *m/e* 298 appears to eliminate the 3β-silyloxy group preferentially to give *m/e* 208 (298 − $HOSiMe_3$) *vs.* *m/e* 206 (298 − $H^{18}OSiMe_3$).

Discussion

Mason (1965) in a comprehensive review of oxidases distinguished "mixed-function oxidases" as those which catalyze the consumption of one molecule of oxygen per molecule of substrate; one atom of the oxygen molecule appears in the product, the other is reduced to water by a hydrogen donor other than the substrate, usually NADH or NADPH.

The results of this investigation have provided additional evidence that chick kidney mitochondria possess the ability to hydroxylate 25-OH-D_3 to its corresponding dihydroxy derivative, 1,25-$(OH)_2D_3$. They also provide evidence that the enzyme system(s) responsible for this hydroxylation reaction may belong to the class of "mixed-function oxidases."

The similarity of 25-OH-D_3–1-hydroxylase to other mixed-function oxidases has been pointed out in recent investigations (Fraser and Kodicek, 1970; Gray *et al.*, 1972). Fraser and Kodicek (1970) have suggested that the tritium loss during 25-[4-^{14}C-1-^{3}H]OH-D_3 conversion to what later proved to be 1,25-$(OH)_2D_3$ indicated oxygenation of C-1. Their finding that the lost tritium was recovered as tritiated water neither supports nor excludes a mixed-function oxidase reaction.

The 1-hydroxylase system is carbon monoxide sensitive (Gray *et al.*, 1972; Wilson *et al.*, 1965) providing additional support for the idea that it is a mixed-function oxidase. On the other hand, the reaction is sensitive to diphenyl-*p*-phenylenediamine (Gray *et al.*, 1972) which is more characteristic of lipid peroxidation systems. The absolute dependency of the 1-hydroxylation reaction upon a source of reducing equivalents (Fraser and Kodicek, 1970; Gray *et al.*, 1972) and its inhibition by several respiratory inhibitors and uncouplers (Gray *et al.*, 1972) have implicated the direct involvement of the respiratory electron transport chain in the overall hydroxylation reaction. Consistent with this conclusion is the observation (J. G. Ghazarian and H. F. DeLuca, unpublished observations) that menadione (an effective electron acceptor) markedly inhibits the 1-hydroxylation reaction. Thus it is evident that although the present results provide evidence for the 25-OH-D_3-1-hydroxylase of chick kidney as being a mixed-function oxidase, it is not possible to ascertain further the biochemical mechanism of the hydroxylation. This must await solubilization and purification of the components of the system as has been done with beef adrenal 11β-hydroxylase (Omura *et al.*, 1965a,b, 1966).

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