Synthesis of 1α-Hydroxy[6-3H]vitamin D₃ and Its Metabolism to 1α,25-Dihydroxy[6-3H]vitamin D₃ in the Rat*

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1α-Hydroxy[6-3H]vitamin D₃ has been synthesized with a specific activity of 4 Ci/mmol, and its metabolism in rats has been studied. It is rapidly converted to 1α,25-dihydroxy[6-3H]vitamin D₃ in vivo. Following an intravenous or oral dose, a maximal concentration of 1α,25-dihydroxy[6-3H]vitamin D₃ is found 2 and 4 hours, respectively, before the maximal intestinal calcium transport response is observed. Similarly, 1α,25-dihydroxy[6-3H]vitamin D₃ accumulation in bone precedes the bone calcium mobilization response. It appears, therefore, that the biological activity of 1α-hydroxyvitamin D₃ is largely, if not exclusively, due to its conversion to 1α,25-dihydroxy[6-3H]vitamin D₃ and 1α,25-dihydroxy[6-3H]vitamin D₃ appear in intestine equally well after an oral or an intravenous dose of 1α-hydroxyvitamin D₃. However, much less of both 1α-hydroxy[6-3H]vitamin D₃ and 1α,25-dihydroxy[6-3H]vitamin D₃ appears in bone and blood after an oral than after an intravenous dose. A much reduced bone calcium mobilization response is also noted following an oral dose as compared to an intravenous dose of 1α-hydroxyvitamin D₃, suggesting that oral 1α-hydroxyvitamin D₃ is not utilized as well as intravenously administered material.

The demonstration that 1α,25-dihydroxyvitamin D₃ is probably the metabolically active form of vitamin D₃ in intestinal calcium transport and bone calcium mobilization has engendered great interest in its chemical synthesis and in its use in clinical medicine. The fact that this form of vitamin D₃ is synthesized exclusively in the kidney brought forth the belief that renal osteodystrophy results at least in part from a failure in the 1α-hydroxylation of 25-hydroxyvitamin D₃ (1, 2). The utility of this hormonal form of vitamin D₃ in this and other disease states seems well on its way to being established (3-5), providing an additional impetus for synthetic efforts. The first chemical synthesis of 1α,25-(OH)₂D₃, accomplished in 1972 (6), was followed by other synthetic procedures (7-9), but it was not until efficient methods for the preparation of 25-hydroxycholesterol and its conversion to 1α,25-dihydroxycholesterol were developed (10) that the supply problem could be surmounted. Meanwhile, another possible solution to the problem appeared with the preparation of an analog, 1α-hydroxyvitamin D₃ (11) for which efficient methods of synthesis subsequently appeared (12-16). This compound proved to be almost as biologically active as 1α,25-(OH)₂D₃ (17), and, as might be expected, effective in stimulating both intestinal calcium transport and bone calcium mobilization in anephric rats (11). It was assumed that this synthetic analog would be hydroxylated on C-25 before it functioned. However, the 1α-OH-D₃ appeared to act just as rapidly as 1α,25-(OH)₂D₃ (11, 18) and is equal to 1α,25-(OH)₂D₃ in biological activity in the chick (18). These findings seemed inconsistent with the necessity for 25-hydroxylation. Recently, Zerwekh et al. (19) reported that 1α-OH-D₃ is converted to 1α,25-(OH)₂D₃ in vivo and in vitro, based on a chromatin receptor assay for 1α,25-(OH)₂D₃. Nevertheless, the question of 1α-OH-D₃ metabolism remains largely unsettled. In an effort to resolve this question and to provide information on the metabolism of 1α-OH-D₃, we have synthesized 1α-OH-[6-3H]D₃ and shown it to be converted to 1α,25-(OH)₂[6-3H]D₃ in the rat. Furthermore, this conversion occurs rapidly enough to account for all its reported biological activity. A preliminary report of this work has been communicated (20).

MATERIALS AND METHODS

Compounds—Crystalline synthetic 1α-OH-D₃ was kindly supplied by Leo Pharmaceuticals, Inc. (Copenhagen, Denmark) and the Upjohn Company (Kalamazoo, Mich.), while crystalline 1α,25-(OH)₂-D₃ was kindly supplied by Hoffmann-LaRoche (Nutley, N.J.). Sodium borotritide was generously supplied by New England Nuclear (Boston, Mass.).


Radioactivity Measurements—Radioactive determinations were carried out with a Packard Tri-Carb model 3075 liquid scintillation counter equipped with an automatic external standardization system. Samples were dried in glass vials inserted (14 x 45 mm) with a stream of air, and dissolved in 4 ml of toluene counting solution (2 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)-benzene]/liter of toluene).

Spectroscopy and Chromatography—Ultraviolet absorption spectra were recorded with a Beckman DBG recording spectrophotometer while mass spectrophotometric determinations were carried out with an AEI MS-9 mass spectrometer using direct probe inlet at temperatures of 120-150° above ambient. High pressure liquid chromatography was performed on a Du Pont 830 LC (Du Pont Instruments, Wilmington, Del.) fitted with two Zorbax SL columns (25 cm x 0.1 mm i.d.) in series and using a 2-propanol/Skellysolve B (1/9) solvent system at a pressure of 2,000 p.s.i., which gave a low rate of 0.25 ml/min (21).

Synthesis of 1α-OH-[6-3H]D₃—In order to determine the feasibility of introducing tritium via a reduction of 1α,3β-diacetoxy-5α-cholestan-6-one (Fig. 1, Compound 1), we used sodium borodeuteride and monitored the incorporation of deuterium into the steroid molecule by mass spectrometry. Compound 1 (prepared in nine steps from cholesterol by the procedure used in our earlier synthesis of 1α-OH-D₃ (11)) was reduced with sodium borodeuteride in isopropanol for 3 days, and the C-6-deuterated alcohol was collected and purified for mass spectral analysis. The mass spectrum of the product showed a molecular ion at m/z 505, and fragments at m/z 455 (M⁺-HOAc) and 385 (M⁺-2HOAc). This product was dehydrated with phosphorus oxychloride to yield 1α,3β-diacetoxy-5α-cholestan-6-one, which gave a flow rate of 0.25 ml/mm (21). This was dissolved in 3 ml of pyridine and stirred in an ice bath.

Fractions (2 ml) were collected, and 180 µg of 1α-OH-[6-3H]D₃ (λmax, 265, 228 nm, Fig. 2) was recovered in tubes 40 to 52. The final product (4 Ci/mmol) was co-chromatographed with crystalline 1α-OH-D₃ on the Sephadex LH-20 column (1 x 60 cm), slurried, and developed as described above. Two hundred forty micrograms of 1α-hydroxy- previtamin D₃ diacetate (Compound 5) with a λmax, 265, 228 nm was recovered in the 4% ether/Skellysolve B eluant. This derivative was quantified with methanol/KOH at 80° for 2 hours. The reaction mixture was diluted with 50 ml of water (pH 4) and extracted with 50 ml of ether. The ether phase was collected, and the water phase was re-extracted 3 times with 20 ml of ether. The ether phases were collected and flash evaporated, and the residue was dissolved in 0.2 ml of 65/35 chloroform/Skellysolve B and applied to a Sephadex LH-20 column (1 x 60 cm) packed and developed in the same solvent. Fractions (2 ml) were collected, and 180 µg of 1α-OH-[6-3H]D₃ (λmax, 265, 228 nm, Fig. 2) was recovered in tubes 49 to 52. The final product (4 Ci/mmol) was co-chromatographed with crystalline 1α-OH-D₃ on the Sephadex LH-20 column (1 x 60 cm), slurried, and developed as described above. Two hundred forty micrograms of 1α-hydroxy- previtamin D₃ diacetate (Compound 5) with a λmax, 265, 228 nm was recovered in the 4% ether/Skellysolve B eluant.

Preparation of Rats—Weanling male rats obtained from the Holtzman Co. (Madison, Wis.) were fed an adequate calcium and phosphorus diet and saponified with methanol/KOH at 80° for 2 hours. The reaction mixture mixture was diluted with 50 ml of water (pH 4) and extracted with 50 ml of ether. The ether phase was collected, and the water phase was re-extracted 3 times with 20 ml of ether. The ether phases were collected and flash evaporated, and the residue was dissolved in 0.2 ml of 65/35 chloroform/Skellysolve B and applied to a Sephadex LH-20 column (1 x 60 cm), slurried, and developed as described above. Two hundred forty micrograms of 1α-hydroxy- previtamin D₃ diacetate (Compound 5) with a λmax, 265, 228 nm was recovered in the 4% ether/Skellysolve B eluant. Two hundred forty micrograms of 1α-hydroxy- previtamin D₃ diacetate (Compound 5) with a λmax, 265, 228 nm was recovered in the 4% ether/Skellysolve B eluant. This derivative was quantified with methanol/KOH at 80° for 2 hours.

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chloroform/Skellysolve B and applied to a glass column (1 x 60 cm) containing 12 g of Sephadex LH-20 slurried and developed in the same solvent.

Identification of 1a-OH-D3 and 1,25-(OH)2D3 on Chromatography—The 1a,25-(OH)2[6-3H]D3 and 1a-OH-[6-3H]D3 were identified (a) by elution position on the chromatogram as revealed by standards and (b) by co-chromatography with crystalline 1a-OH-D3 (Upjohn Co.) and crystalline 1a,25-(OH)2D3 (Hoffmann-LaRoche Co.) on Sephadex LH-20 and high pressure liquid chromatography as previously reported (20).

Intestinal Calcium Transport Assay—Groups of six rats received 0.125 μg of crystalline synthetic 1a-OH-D3 intrajugularly in 0.06 ml of 95% ethanol or orally via stomach tube in 0.1 ml of propylene glycol. At the desired time after administration, the animals were decapitated and their blood and duodenas were collected. The duodenas were prepared according to the procedure of Martin and DeLuca (24) for measurement of intestinal calcium transport activity by the everted gut sac technique. Samples (100 μl) from both the inside and outside of the intestinal sac were spotted on filter paper discs, dried, and placed in 20-ml counting vials containing 10 ml of the scintillation counting solution.

Bone Calcium Mobilization—The blood from the rats treated as described above for the intestinal calcium transport assay was centrifuged, and 0.1 ml of serum was mixed with 1.9 ml of 0.1% LaCl3 solution. Serum calcium concentration was determined with a Perkin-Elmer atomic absorption spectrometer model 406. The rise in serum calcium of rats on a low calcium diet in response to vitamin D is considered an in vivo measurement of bone calcium mobilization.

RESULTS

The response of intestinal calcium transport to a single dose of 0.125 μg of 1a-OH-D3 given intrajugularly or orally is illustrated in Fig. 3A. Animals dosed intravenously (Fig. 3A) failed to respond by 4 hours, but responded maximally by 6 hours. The high level of transport achieved was maintained for up to 96 hours. Orally dosed animals (Fig. 3B) showed a significant intestinal calcium transport response by 4 hours and a maximum response at 12 hours, which remained high for up to 96 hours. The concentration of 1a,25-(OH)2[6-3H]D3 in the intestine of animals that received 0.125 μg of 1a-OH-[6-3H]D3 is also plotted. As early as 2 hours after a single dose of 1a-OH-[6-3H]D3 orally or intravenously, 1a,25-(OH)2[6-3H]D3 reached a concentration of 240 and 330 pg/g of tissue, respectively, and by 4 hours, the intestine had a maximum concentration of 1a,25-(OH)2[6-3H]D3 of 310 and 250 pg/g, respectively. After 4 hours, the intestinal tissue concentration of 1a,25-(OH)2[6-3H]D3 rapidly declined to 50 pg/g and 108 pg/g, respectively, in the orally and intravenously dosed animals at 96 hours. Fig. 4 demonstrates the bone calcium mobilization response (rise in serum calcium concentration) and the appearance of 1a,25-(OH)2[6-3H]D3 in bone as a function of time after the dose of 1a-OH-[6-3H]D3. There was a maximum concentration of 1a,25-(OH)2[6-3H]D3 in bone (367 pg/g) at 4 hours after an intravenous dose of 1a-OH-[6-3H]D3, and it quickly declined to 40 pg/g of tissue at 96 hours (Fig. 4A). A significant bone calcium mobilization response was also noted at 4 hours, which reached a maximum at 12 hours continuing to 48 hours. At 96 hours, the serum calcium concentration returned to control levels. The maximum tissue concentration of 1a,25-(OH)2[6-3H]D3 in the orally dosed animals was not reached until 12 hours after the dose of 1a-OH-[6-3H]D3. This concentration (64 pg/g) is only 20% of the maximum concentration achieved in the intravenously dosed animals. Only a minimal bone calcium mobilization response was observed at 6 hours, with a weak maximum response observed between 12 and 24 hours after the oral dose of 1a-OH-D3. The levels of 1a-OH-[1H]D3 and 1a,25-(OH)2[1H]D3 in intestine, bone, and blood after oral and intravenous doses are shown in Table I. It is immediately obvious that, although the intestinal levels of 1a,25-(OH)2D3 are the same for orally and intravenously administered 1a-OH-[6-3H]D3, the levels in bone and blood are markedly reduced in the orally dosed rats. Note also that at 96 hours virtually all of the 1a-OH-[6-3H]D3 has disappeared from these tissues.

DISCUSSION

The present results and those reported previously (20) establish that 1a-OH-D3 is rapidly metabolized in rats to 1a,25-(OH)2D3. In fact, the metabolism is so rapid that it is extremely likely that 1a-OH-D3 is first converted to 1a,25-(OH)2D3 before it functions in intestine and bone. There is no doubt that 1a,25-(OH)2D3 derived from 1a-OH-D3 appears in significant amounts long before intestinal calcium transport is initiated in response to 1a-OH-D3 (Fig. 3). A similar conclusion can be drawn for the mobilization of calcium from bone. Since 1a,25-(OH)2D3 is known to act directly on these tissues, it seems likely that it, and not 1a-OH-D3, is responsible for the observed responses. Unfortunately, it is still not possible to completely exclude the possibility that 1a-OH-D3 can act directly on these tissues when present in large amounts. A recent report that both 1a-OH-D3 and 1a,25-(OH)2D3 act within 30 min in rats has appeared (25). We have been unable to reproduce this observation with crystalline preparations of either compound obtained from the Upjohn Co., Leo Phar-
maceuticals, and Hoffmann-LaRoche Co., or with biologically generated 1α,25-(OH)₂D₃. The reason for this apparent discrepancy remains unknown at the present time.

It is apparent that 1α-OH-D₃ is metabolized rapidly inasmuch as it has disappeared from blood, intestine, and bone already by 96 hours. This is much more rapid than vitamin D₃ (25), which suggests that the 25-hydroxylation of 1α-OH-D₃ may not be as limiting as in the case of vitamin D₃.

Of some interest is the fact that after an oral dose of 1α-OH-D₃, it and its product, 1α,25-(OH)₂D₃, appear in bone or blood to a much lesser extent than after an intravenous dose. Furthermore, the bone calcium mobilization response to an oral dose is markedly lower than to an intravenous dose. Yet previously, chronic oral and chronic intravenous doses gave similar biological activity in the mobilization of calcium from bone. Perhaps chronic dosage allows for better intestinal utilization, although this cannot be decided with the presently available information. Obviously more work in this area must be carried out before this disparity can be resolved.

It seems likely that the biological, and hence medical, effectiveness of 1α-OH-D₃ is due to its conversion to 1α,25-(OH)₂D₃, the natural hormone, however, it is of some interest that 1α-OH-D₃ is approximately 20 to 50% as active as 1α,25-(OH)₂D₃ in the rat (11), and about 50% as active in man (27-29). One possible explanation for this is that 1α-OH-D₃ may be metabolized to other products which are devoid of biological activity. Although no other metabolites were detected in the lipid extracts from intestine in the present study, the possibility that other metabolites will be found in other tissues or in the aqueous soluble portions of the extracts cannot be excluded.

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