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Isolation and Identification of 23,25-Dihydroxyvitamin D_3, an in Vivo Metabolite of Vitamin D_3†

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ABSTRACT: Vitamin D supplemented rats produce a metabolite of 25-hydroxy[3α-^{3}H]vitamin D_3 that is easily separated from known metabolites by using high-performance liquid chromatography. The production of this metabolite in vivo as well as 1,25-dihydroxyvitamin D_3, 24(*R*),25-dihydroxyvitamin D_3, and 25-hydroxyvitamin D_3 26,23-lactone is largely if not totally eliminated by nephrectomy. Kidney homogenates from vitamin D supplemented chickens incubated with 25-hydroxyvitamin D_3 produce significant quantities of the new, unknown metabolite. This metabolite was isolated in pure form from such incubation mixtures by using both straight-phase and reversed-phase high-performance liquid chromatography. This metabolite has been positively identified as 23,25-dihydroxyvitamin D_3 by ultraviolet absorption spectrophotometry, mass spectrometry, and derivatization. This structure was confirmed by chemical synthesis of both C-23 stereoisomers. Although the natural product exactly comigrates with one of the synthetic isomers, the exact stereochemistry of the natural product remains unknown. It is possible that this new metabolite is an intermediate in the biosynthesis of 25-hydroxyvitamin D_3 26,23-lactone.

In the past 15 years significant progress has been made in our understanding of the metabolism of vitamin D_3 (DeLuca, 1979, 1980). However, much remains to be learned in this area since it is not yet possible to describe the total pathway of metabolism of this important vitamin. Recently, a major metabolite of vitamin D_3 has been found in the plasma of animals and man (Horst et al., 1979; Shepard & DeLuca, 1980). This compound has been isolated in pure form and its structure unequivocally demonstrated to be 25-hydroxyvitamin D_3 26,23-lactone (25-OH-D_3-26,23-lactone)[1] (Wichmann et al., 1979). This structure has recently been confirmed by chemical synthesis (Wichmann et al., 1980). The unexpected structure of this compound has fostered a significant effort to elucidate its pathway of biosynthesis (Tanaka et al., 1980; Littledike et al., 1980). Nephrectomy eliminates biosynthesis of the 25-OH-D_3-26,23-lactone (Tanaka et al., 1980; Littledike et al., 1980). Homogenates of kidney tissue prepared from chickens given large doses of vitamin D_3 are able to convert 25-hydroxyvitamin D_3 (25-OH-D_3) to the 25-OH-D_3-26,23-

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[1] Abbreviations used: 25-OH-D_3-26,23-lactone, 25-hydroxyvitamin D_3 26,23-lactone; HPLC, high-performance liquid chromatography; 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24(*R*),25-$(OH)_2D_3$, 24(*R*),25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; 23,25-$(OH)_2D_3$, 23,25-dihydroxyvitamin D_3; ATP, adenosine 5′-triphosphate; NADP, nicotinamide adenine dinucleotide.

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lactone (Tanaka et al., 1980; Littledike et al., 1980). Thus the kidney appears to be an obligatory site for the reactions responsible for the production of the 25-OH-D_3-26,23-lactone. During the course of these in vitro experiments a major metabolite of 25-OH-D_3 appeared in the high-performance liquid chromatographic (HPLC) profiles of incubation mixtures containing homogenates from chickens given large doses of vitamin D. This compound has now been isolated in pure form, its structure unequivocally identified as 23,25-dihydroxyvitamin D_3 (23,25-$(OH)_2D_3$), its in vivo existence demonstrated, and its structure confirmed by chemical synthesis.

Experimental Procedures

25-OH-D_3 as its monohydrate was a generous gift from the Upjohn Company (Kalamazoo, MI). 1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and 24(*R*),25-dihydroxyvitamin D_3 [24-(*R*),25-$(OH)_2D_3$] were generous gifts of the Hoffmann-La Roche Company (Nutley, NJ). 25,26-Dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$] and 25-OH-[3α-^{3}H]D_3 were prepared in this laboratory (Lam et al., 1975; S. Yamada, H. K. Schnoes, and H. F. DeLuca, unpublished results).

Animals. White Leghorn 1-day-old chicks were obtained from Northern Hatcheries (Beaver Dam, WI). They were fed an adequate commercially available broiler mash diet (Ralston Purina, St. Louis, MO) for a period of 6 weeks. At that time they were given, intramuscularly, 10^5 IU of vitamin D_3 in 50 μL of ethanol per day for 3 days followed by 10^7 IU of vitamin D_3 in four 100-μL doses of ethanol intramuscularly. Four days after the last injection the chickens were sacrificed by decapitation.

In Vitro Incubations. Immediately following decapitation of the chickens, kidneys were removed and placed in 0.25 M sucrose at 0 °C. A 20% homogenate was prepared in 0.25 M sucrose. Incubations were carried out in 125-mL Erlenmeyer flasks containing 600 mg of tissue, 75 mM phosphate buffer, pH 7.4, 22.4 mM glucose 6-phosphate, 20 mM ATP, 160 mM nicotinamide, 0.4 mM NADP, 5 mM $MgCl_2$, 25 mM sodium succinate, 0.1 M KCl, 0.5 unit of glucose-6-phosphate dehydrogenase in a total volume of 10 mL, and 10 μg of diphenyl-*p*-phenylenediamine (DPPD). The reaction was initiated by addition of 80 μg of 25-OH-D_3 or 0.4 μCi of 25-OH-[3α-^{3}H]D_3 dissolved in 20 μL of 95% ethanol. The mixtures were incubated at 37 °C with shaking at 100 oscillations/min for 2 h under an atmosphere of air. The reaction was stopped by the addition of 30 mL of a methanol–chloroform mixture (2:1). The metabolites were extracted by a modification of the Bligh and Dyer procedure as described by Lund & DeLuca (1966). The aqueous phase was extracted 1 time more with chloroform, the chloroform extracts were combined, and the solvent was removed by rotary evaporation.

Chromatography. The residue was dissolved in chloroform–hexane (65:35) and applied to a Sephadex LH-20 column (0.7 × 14 cm) packed and eluted in the same solvent. The first 11 mL of eluant was discarded and the next 20 mL collected. These fractions were evaporated to dryness, and the residue was dissolved in 6% 2-propanol in hexane. The sample was injected into an HPLC apparatus onto a Zorbax-Sil column (4.6 mm × 25 cm, 7–8 μm, Du Pont Instruments, Wilmington, DE). The column was eluted under a pressure of 1000 psi at a flow rate of 2 mL/min with the 6% 2-propanol in hexane solvent mixture. The desired product was eluted at 12–16 mL. The collected product was then chromatographed on a reversed-phase HPLC column by 10 μm, Merck Company, Darmstadt, West Germany) and 25% water in methanol as the eluting solvent. The desired product eluting at 40–45 mL was collected and reapplied to the same straight-phase HPLC system described above. The purified product was then homogeneous and subjected to physical characterization.

In Vivo Production of 23,25-Dihydroxyvitamin D_3. Male weanling rats were purchased from the Holtzman Company, Madison, WI, and fed an adequate calcium and adequate phosphorus diet (Suda et al., 1970a) supplemented with 25 units of vitamin D_3/day for 6 weeks. Two days prior to the conclusion of the experiment the rats were given 0.5 μg of 25-OH-D_3/day dissolved in 0.1 mL of 1,2-propanediol subcutaneously. Twenty-four hours prior to sacrifice the rats were either nephrectomized or sham operated and administered intrajugularly 10 μCi of 25-OH-[3α-^{3}H]D_3 (28 Ci/mmol) dissolved in 0.05 mL of 95% ethanol. Twenty-four hours after the administration of the tritiated 25-OH-D_3 the rats were killed by decapitation, and their blood was collected. The blood was centrifuged to yield serum; 7 mL of serum was pooled from three rats in either the nephrectomized or the sham operated group. The serum was mixed with authentic 1,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, and 24(*R*),25-$(OH)_2D_3$ at a level of 200 ng each. In addition, 100 ng of 25-OH-D_3-26,23-lactone (Wichmann et al., 1980) was added. After 1 h of incubation at room temperature the extractions of the sera were carried out as described above. The chloroform-soluble extract was evaporated to dryness and the residue dissolved in a solvent of 65% chloroform in hexane. This was applied to a Sephadex LH-20 column (0.7 × 14 cm) packed in 65:35 chloroform–hexane and developed as described by Holick & DeLuca (1971). The first 11 mL of effluent was discarded, while the next 25 mL of effluent containing the desired compounds was collected. The elution position of 25-OH-D_3 and 24,25-$(OH)_2D_3$ was established with authentic compounds. The fraction taken from the Sephadex LH-20 column was subjected to HPLC by using a Zorbax-Sil column (4.6 mm × 25 cm) at 1000 psi and a flow rate of 2 mL/min in a solvent of 8% 2-propanol in hexane. The fractions of 0.8 mL were collected and counted.

Trimethylsilyl (Me_3Si) Derivitization. The putative 23,25-$(OH)_2D_3$ (1 μg) isolated from the in vitro experiment was dissolved in 20 μL of pyridine and reacted at 50 °C for 45 min with 15 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylsilyl chloride. Solvent was removed under N_2 and the product chromatographed on HPLC using a 4.6 mm × 25 cm Zorbax-Sil column eluted with 0.1% 2-propanol in hexane. This revealed a major 254-nm absorbing peak eluting at 24 mL; it was collected and subjected to mass spectrometry.

General Methods. Ultraviolet absorption spectra were recorded with a Beckman Model 24 spectrophotometer. Radioactivity was measured with a Packard Model 3255 liquid scintillation counter, and electron impact mass spectra were taken with an Associated Electric Industries MS9 mass spectrometer with a direct probe inlet system having a probe temperature of 140 °C above ambient.

Chemical Synthesis of 23,25-$(OH)_2D_3$. The two C-23 stereoisomers of 27-nor-23-hydroxy-25-ketocholesta-5,7-diene were prepared and separated as described previously (Wichmann et al., 1980). To 5 mg of each hydroxy ketone isomer in 5 mL of diethyl ether was added a 10-fold excess of methylmagnesium bromide. The reactions were stirred at room temperature for 15 min and then extracted with 50 mL of CH_2Cl_2 against 25 mL each of 1 N HCl, 10% $NaHCO_3$, and saturated NaCl. The crude products were chromatographed on HPLC using a 0.79 × 30 cm μPorasil column (Waters Associates, Milford, MA) eluted with 3.5% 2-propanol in

CH_2Cl_2. 23,25-Dihydroxycholesta-5,7-diene isomer A (2.4 mg) was recovered, eluting at 39 mL, and isomer B (1.2 mg) was recovered, eluting at 58 mL: mass spectra (isomer A) *m/e* 416 (100, M^+), 398 (10, $M^+ - H_2O$), 383 (60, $M^+ - H_2O - CH_3$), 357 (21, $M^+ - C_3H_7O$), 342 (23, $M^+ - C_3H_7O - CH_3$), 271 (31, M^+ – side chain), 143 (88, $C_{11}H_{11}^+$); (isomer B) *m/e* 416 (83, M^+), 398 (11, $M^+ - H_2O$), 383 (80, $M^+ - H_2O - CH_3$), 357 (33, $M^+ - C_3H_7O$), 342 (22, $M^+ - C_3H_7O - CH_3$), 271 (23, M^+ – side chain), 143 (100, $C_{11}H_{11}^+$).

A 500-μg sample of each isomer was irradiated for 15 min in 150 mL of 20% benzene in diethyl ether by using a quartz immersion well and Hanovia 608A36 lamp fitted with a Corex filter. 23,25-Dihydroxyprevitamin D_3 isomer A was purified by HPLC using a 0.62 × 25 cm Zorbax-Sil column eluted with 3.5% 2-propanol in CH_2Cl_2. The desired product eluted at 51 mL in this system. Previtamin isomer B was chromatographed on HPLC using a 0.79 × 30 cm μPorasil column eluted with 7% 2-propanol in hexane. The desired product was collected at 38 mL.

Each previtamin isomer was dissolved in 2 mL of ethanol and heated at 70 °C for 2.5 h. Isomer A was chromatographed on HPLC using a 0.62 × 25 cm Zorbax-Sil column and 4% 2-propanol in CH_2Cl_2 solvent system. 23,25-Dihydroxyvitamin D_3 isomer A (240 μg) was collected, eluting at 28 mL. 23,25-$(OH)_2D_3$ isomer B (60 mg) was collected at 35 mL with 7% 2-propanol in hexane as eluant by using the same column. Spectral data for isomer A: UV λ_{max} 265 nm, λ_{min} 228 nm; mass spectra *m/e* 416.3319 (calcd, 416.3290) (27, M^+), 398 (2.5, $M^+ - H_2O$), 383 (13, $M^+ - H_2O - CH_3$), 271 (8.5, M^+ – side chain), 253 (9.7, M^+ – side chain – H_2O), 136 (100, A ring + C_6 + C_7^+), 118 (96, 136 – H_2O); NMR ($CDCl_3$) δ 6.23 (d, *J* = 11 Hz, 1 H, C-6), 6.03 (d, *J* = 11 Hz, 1 H, C-7), 5.05 [m, 1 H, C-19(*E*)], 4.81 [m, 1 H, C19(*Z*)], 4.13 (m, 1 H, C-23), 3.94 (m, 1 H, C-3), 1.33 (s, 3 H, C-26), 1.26 (s, 3 H, C-27), 1.00 (d, *J* = 7 Hz, 3 H, C-21), 0.58 (s, 3 H, C-18). Spectra data for isomer B: UV λ_{max} 265 nm, λ_{min} 228 nm; mass spectrum *m/e* 416.3273 (calcd, 416.3290) (18, M^+), 398 (2, $M^+ - H_2O$), 383 (10, $M^+ - H_2O - CH_3$), 271 (7, M^+ – side chain), 253 (8, M^+ – side chain – H_2O), 136 (100, A ring + C_6 + C_7^+), 118 (97, A ring + C_6 + $C_7^+ - H_2O$); NMR ($CDCl_3$) δ 6.23 (d, *J* = 10.5 Hz, 1 H, C-6), 6.03 (d, *J* = 10.5 Hz, 1 H, C-7), 5.05 [m, 1 H, C-19(*E*)], 4.82 [m, 1 H, C-19(*Z*)], 4.10 (m, 1 H, C-23), 3.95 (m, 1 H, C-3), 1.32 (s, 3 H, C-26), 1.28 (s, 3 H, C-27), 0.98 (d, *J* = 6 Hz, 3 H, C-21), 0.56 (s, 3 H, C-18).

Comigration of Synthetic and Natural Products. Synthetic 23,25-$(OH)_2D_3$ (1 μg) was chromatographed on a 0.79 × 0.62 cm μPorasil column eluted with 4% 2-propanol in CH_2Cl_2. Isomer A elutes at 33 mL and isomer B at 52 mL. Synthetic isomer B (500 ng) and the natural product (500 ng) were cochromatographed on this system to yield a single 265-nm absorbing peak eluting at 52 mL.

Results

During the course of our investigation of the site of production of the 25-OH-D_3-26,23-lactone (Tanaka et al., 1980), we became aware of a radioactive metabolite of 25-OH-D_3 that appeared in the profiles of rats given radioactive 25-OH-D_3. Figure 1 illustrates the radioactive profile obtained when the serum from rats given 25-OH-[3α-^{3}H]D_3 was extracted and chromatographed on HPLC. Radioactive peaks in these profiles corresponding to 1α,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, and 24(*R*),25-$(OH)_2D_3$ and a peak eluting just prior to 24(*R*),25-$(OH)_2D_3$ and corresponding to 25-OH-D_3-26,23-lactone could be located. However, in addition, there appeared a peak of equal size that preceded the 25-OH-

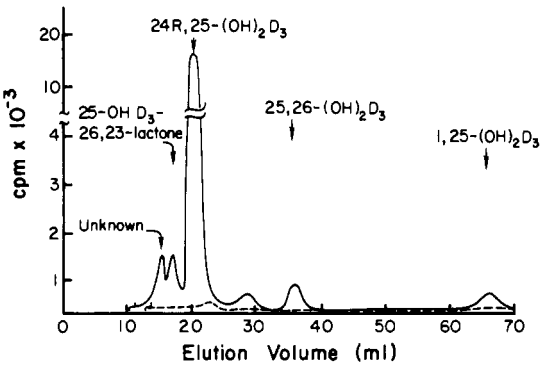

FIGURE 1: In vivo production of a new metabolite of 25-OH-D_3 and its elimination by nephrectomy. Vitamin D treated rats were either sham operated (—) or nephrectomized (---) and given 25-OH-[3α-^{3}H]D_3. 24 h later they were killed, and their serum was collected, extracted, and chromatographed as described in the text. Arrows indicate elution positions of authentic compounds.

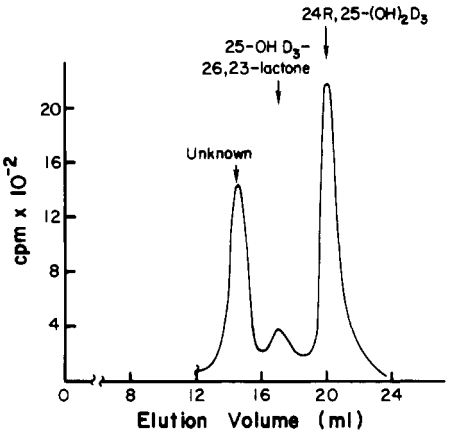

FIGURE 2: In vitro production of a new metabolite of 25-OH-D_3. Chickens given large doses of vitamin D_3 were sacrificed, homogenates of their kidneys were incubated with 25-OH-[3α-^{3}H]D_3, and the incubation mixture was extracted and chromatographed as described in text. Arrows indicate elution positions of authentic compounds.

D_3-26,23-lactone. Nephrectomy largely eliminated the radioactivity appearing in all three metabolites including the unknown (Figure 1). There results indicated that the unknown metabolite as well as the 25-OH-D_3-26,23-lactone is produced at least in part in the kidney. We therefore designed experiments in an attempt to generate this metabolite in vitro by using kidney preparations. We utilized homogenates of kidneys taken from chickens given large doses of vitamin D since these had previously been demonstrated to produce large amounts of 25-OH-D_3-26,23-lactone (Wichmann et al., 1979; Tanaka et al., 1980). As shown in Figure 2, these preparations are fully capable of producing a metabolite that migrates on Zorbax-Sil HPLC prior to the 25-OH-D_3-26,23-lactone.

We then generated sufficient quantities of this material and subjected it to purification. The material eluting between fractions at 12 and 16 mL was collected and chromatographed on a reversed-phase Licrosorb RP-18 column (profile not shown), and the desired product was eluted at 40–44 mL. It was collected and reapplied to the Zorbax-Sil straight-phase HPLC column previously described. The purified product exhibited an ultraviolet absorption maximum at 265 nm and a minimum of 228 nm with an absorption ratio ($\epsilon_{265}/\epsilon_{228}$) of 1.8. The ultraviolet absorption spectrum suggested that the metabolite retained the 5,6-*cis*-triene chromophore of vitamin D. Figure 3 provides the mass spectrum of the isolated compound. It exhibited a molecular ion at *m/e* of 416 as expected for a derivative of 25-OH-D having an additional hydroxyl function. The presence of the additional hydroxyl on the side

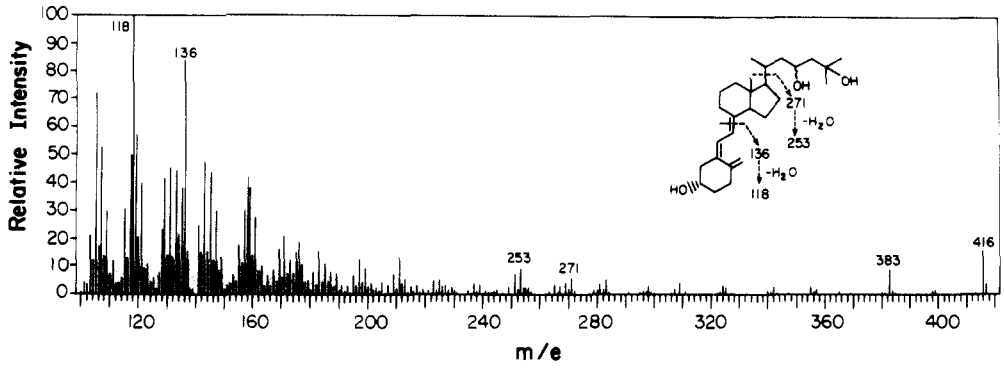

FIGURE 3: Mass spectrum of putative 23,25-$(OH)_2D_3$.

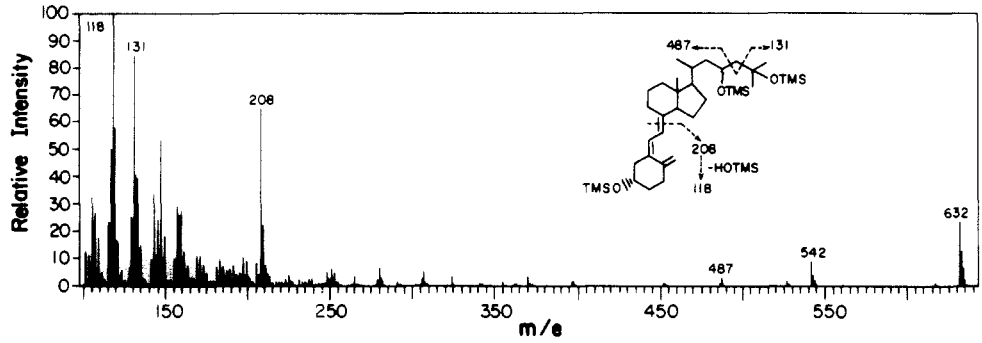

FIGURE 4: Mass spectrum of trimethylsilyl ether derivative of putative 23,25-$(OH)_2D_3$.

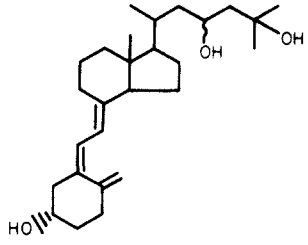

FIGURE 5: Structure of 23,25-$(OH)_2D_3$.

chain was evident from the peaks at *m/e* 271 and 253 (271 – H_2O). That the *cis*-triene structure remained intact and that the additional oxygen was not on the A ring were confirmed by the characteristic ions at *m/e* 136 and 118. The position of the side-chain hydroxy groups was established by the mass spectrum of the tris(trimethylsilyl) ether derivative of the metabolite (Figure 4). The spectrum showed a molecular ion at *m/e* 632 as required for a trisilylated derivative of the 23,25-$(OH)_2D_3$. The spectrum also showed characteristic side-chain cleavage peaks at *m/e* 131 $[(CH_3)_2C{=}OSi(CH_3)_3]^+$ which established the presence of a hydroxyl group at C-25 and a peak at *m/e* 487 ($M^+ - 145$) which proved the presence of a second hydroxyl at carbon 23. The data therefore established the structure of the new vitamin D metabolite as 23,25-$(OH)_2D_3$ (Figure 5).

For confirmation of the assigned structure, chemical synthesis of the metabolite was carried out starting with 27-nor-23-hydroxy-25-ketocholesta-5,7-diene available from a previous synthesis (Wichmann et al., 1980). As illustrated in Figure 6, this substance (I) was subjected to the Grignard reagent methylmagnesium bromide in 10-fold excess. This afforded 2.4 mg of 23,25-$(OH)_2D_3$ cholesta-5,7-diene isomer A and 1.2 mg of isomer B (II in Figure 6). UV irradiation and thermal isomerization yielded upon HPLC purification both 23,25-$(OH)_2D_3$ isomers A and B (III in Figure 6). The product produced by in vitro incubation with chick kidney homogenates comigrated with product B (Figure 7). Furthermore, the mass spectra and cochromatography on HPLC shown in Figure 7 firmly establish the structure of the natural product as 23,25-$(OH)_2D_3$. Inasmuch as the configuration of isomer B has not yet been established, the stereochemical configuration of the natural product cannot be assigned at this time.

Discussion

This report demonstrates the existence of a heretofore unknown metabolite of vitamin D_3. The significance of this metabolite remains unknown and preliminary data indicate it has little biological activity in intestine and bone, target systems known to be responsive to vitamin D and its metabolites. The possibility that 23,25-$(OH)_2D_3$, or metabolites derived from it, may activate other, as yet unrecognized target systems is, of course, not excluded. Its structure suggests a possible role as an intermediate in the biogenesis of the previously discovered 25-OH-D_3-26,23-lactone. However, we cannot at this time draw such a conclusion. It is equally possible that the 25-OH-D_3-26,23-lactone can arise from 25-(*S*),26-$(OH)_2D_3$, a previously identified metabolite of the

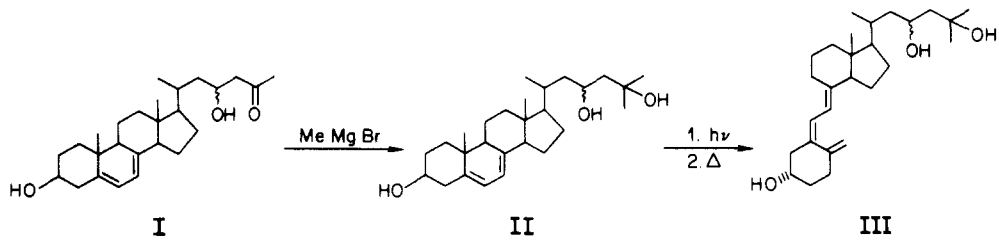

FIGURE 6: Synthesis of 23,25-$(OH)_2D_3$.

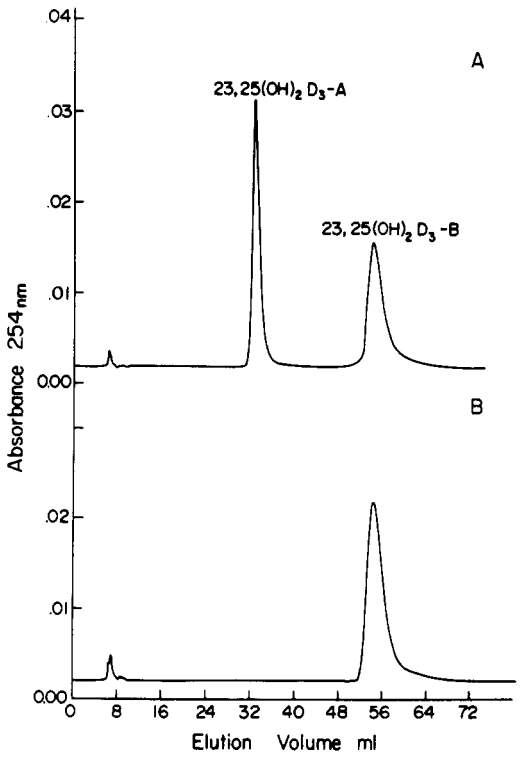

FIGURE 7: Chromatography of the two synthetic stereoisomers and natural product on HPLC. (A) Chromatogram of 1 μg each of synthetic 23,25-$(OH)_2D_3$ isomers A and B. (B) Chromatogram of 500 ng each of the natural product and synthetic 23,25-$(OH)_2D_3$ isomer B.

vitamin (Suda et al., 1970b). In any case, current work has failed to reveal any biological activity of significance for the 25-OH-D_3-26,23-lactone. Therefore, it is quite possible that the 23,25-$(OH)_2D_3$ is an intermediate in the biosynthesis of the lactone.

The identification of the metabolite as the 23,25-$(OH)_2D_3$ relies on several strong lines of evidence. The isolated metabolite has the mass and ultraviolet absorption spectra that can only be accounted for by the 23,25-$(OH)_2D_3$ compound. The peaks at *m/e* 131 and 487 ($M^+ - 145$) in the spectrum of the Me_3Si derivative require a 23,25-dihydroxy-substitution pattern in the side chain, and this information combined with the characteristic UV absorption and mass spectral pattern of the original metabolite defines the structure of this compound as 23,25-$(OH)_2D_3$ (Figure 5).

Chemical synthesis was used to confirm the assigned structure of the metabolite or 23,25-$(OH)_2D_3$. It is obvious that two isomers are possible and both have been synthesized chemically. As expected, only one of the isomers exactly comigrated with the naturally generated metabolite. Unfortunately, although the isomers are easily resolved on HPLC, the stereochemical configuration of each is unknown. Therefore, the configuration about the C-23 of the natural metabolite remains unknown.

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