and an unfolded nucleosome. The H2B–H4 interaction could well provide this pivotal influence as a modal contact.

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Isolation and Identification of 24,25-Dihydroxyvitamin D_2 Using the Perfused Rat Kidney†

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ABSTRACT: 24,25-Dihydroxyvitamin D_2 was biologically generated from synthetic 25-hydroxyvitamin D_2 using an isolated perfused rat kidney incubated under normocalcemic and normophosphatemic conditions. 24(*R*),25-Dihydroxyvitamin D_2 and 24(*S*),25-dihydroxyvitamin D_2 were chemically synthesized starting with stigmasterol and their configurations determined by X-ray diffraction analysis. The biosynthetic metabolite proved to be identical with the synthetic 24(*R*) epimer in its chromatographic mobility, mass spectrometry, and derivative synthesis. Significant quantities of [3α-^{3}H]-24(*R*),25-dihydroxyvitamin D_2 were found to be present in the plasma of vitamin D replete rats 24 h after receiving a physiological dose of [3α-^{3}H]vitamin D_2.

24,25-Dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$)[1] is a major product of renal 25-OH-D_3 metabolism (Suda et al., 1970; Holick et al., 1972) in normocalcemic and normophosphatemic animals receiving adequate intakes of vitamin D (Boyle et al., 1971; Knutson & DeLuca, 1974; Friedlander et al., 1977). Chemical synthesis of the two possible epimers of 24,25-$(OH)_2D_3$ (24(*R*)-hydroxy and 24(*S*)-hydroxy) has led to identification of the natural metabolite with a 24(*R*) configuration (Ikekawa et al., 1977). The role of 24(*R*),25-$(OH)_2D_3$ remains obscure, although its rapid excretion in the

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[1] Abbreviations used: 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_2$; 24,25-dihydroxyvitamin D_2; 24-OH-D_2, 24-hydroxyvitamin D_2; 25-OH-D_3, 25-hydroxyvitamin D_3; 25-OH-D_2, 25-hydroxyvitamin D_2; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1,25-$(OH)_2D_2$, 1,25-dihydroxyvitamin D_2; LC, high performance liquid chromatography; PTH, parathyroid hormone.

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chick has led to the suggestion that 24(*R*),25-$(OH)_2D_3$ represents an inactive metabolite on the degradative pathway of vitamin D_3 (Holick et al., 1976). However, more recent work tends to suggest that 24-hydroxylation is not required for excretion of 1,25-$(OH)_2D_3$ (Kumar et al., 1976), thought to be the most active metabolite of vitamin D. Furthermore, evidence is accumulating to suggest that 24(*R*),25-$(OH)_2D_3$ may have a specific biological activity of its own. 24(*R*),-25-$(OH)_2D_3$ stimulates the incorporation of $^{35}SO_4$ into proteoglycans by cultured growth plate chondrocytes (Corvol et al., 1978); it causes regression of chick parathyroid glands when administered in vivo together with 1,25-$(OH)_2D_3$ (Capen et al., 1977; Henry et al., 1977); it causes diminution of the secretion of immunoreactive PTH when administered in vivo as a bolus into the carotid artery of normal dogs (Canterbury et al., 1978); it is required for normal egg hatchability in the fertile adult hen (Henry & Norman, 1978); and it stimulates the intestinal absorption of calcium in normal and anephric human patients in clinical studies (Kanis et al., 1977). The further potential clinical importance of this metabolite has been indicated by the finding that the hypercalcemia resulting from hypervitaminosis D is not caused by elevated plasma concentrations of 1,25-$(OH)_2D$ but presumably by some other metabolite of vitamin D (Hughes et al., 1977). Additional interest in this metabolite has been generated by the development of new assays for this measurement of 24,25-$(OH)_2D_3$ in human plasma (Taylor et al., 1976; Haddad et al., 1976).

In view of this interest in 24,25-$(OH)_2D_3$ and of the widespread clinical use of vitamin D_2 for therapy and prophylaxis in North America, we perceived the need to establish the existence of the vitamin D_2 analogue of 24,25-$(OH)_2D_3$. Doubts as to the formation of 24,25-$(OH)_2D_2$, because of the presence of a 24(*S*)-methyl group in the side chain of vitamin D_2, were dispelled by the demonstration of the stereospecificity of the 24-hydroxylase for the 24(*R*) position (Tanaka et al., 1975) and by the generation of a metabolite from [^{3}H]-25-OH-D_2 with chromatographic properties similar to 24,25-$(OH)_2D_3$ (Jones et al., 1975, 1976). Such a metabolite, made under conditions ideal for the introduction of the 24-hydroxyl into the vitamin D molecule by the kidney, was never formally identified as 24,25-$(OH)_2D_2$.

This paper reports the first chemical synthesis of the two epimeric forms of 24,25-$(OH)_2D_2$, the separation of 24-(*R*),25-$(OH)_2D_2$ and its use to identify the natural metabolite of vitamin D_2 prepared by a novel perfused kidney system. In addition we present evidence for the formation of this metabolite in vivo in vitamin D replete rats.

Materials and Methods

Solvents

All solvents were from Burdick & Jackson Laboratories, Muskegon, Michigan, "distilled-in-glass" spectroscopic grade.

Vitamin D Metabolites

Crystalline 25-OH-D_2 was a generous gift of Drs. J. A. Campbell, J. Hinman, and J. Babcock of the Upjohn Co., Kalamazoo, MI. Synthetic 24(*R*),25-$(OH)_2D_3$ was kindly donated by Dr. M. R. Uskokovic of Hoffmann-La Roche Inc., Nutley, NJ. [3α-^{3}H]Vitamin D_2 (3.5 Ci/mmol) was synthesized by the sodium [^{3}H]borohydride (11.25 Ci/mmol) reduction of 3α-acetoxyergosta-3,5,7,22-tetraene (Jones et al., 1975). [3α-^{3}H]-25-OH-D_2 (3.5 Ci/mmol) was generated in vivo by the administration of [3α-^{3}H]vitamin D_2 to rats receiving a normal vitamin D sufficient diet. The purity of all metabolites was checked by high performance liquid chromatography (LC) before use (Jones & DeLuca, 1975).

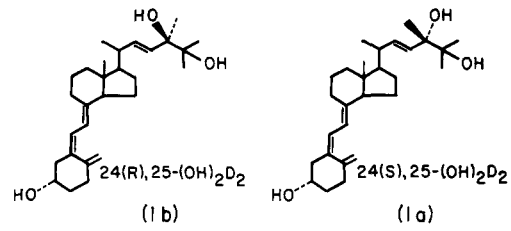

FIGURE 1: Structures of 24(*R*),25-$(OH)_2D_2$ and 24(*S*),25-$(OH)_2D_2$.

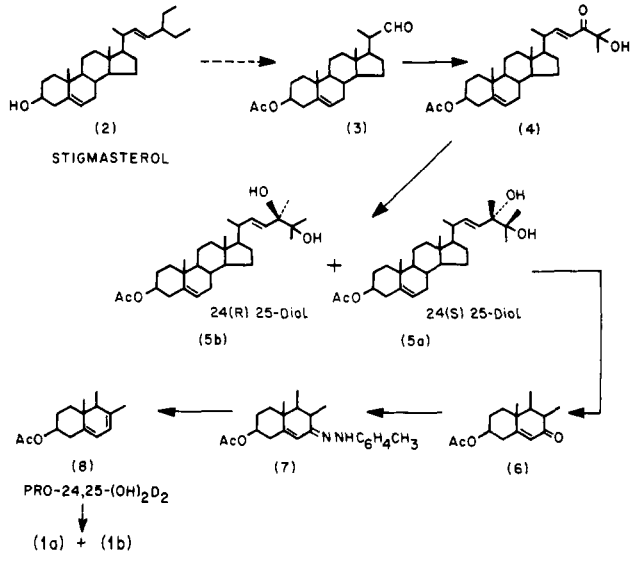

FIGURE 2: The scheme for the synthesis of 24(*R*)- and 24(*S*),25-$(OH)_2D_2$.

General Procedures

LC was performed on a Model LC 204 fitted with two Model 6000A pumping systems, Model 660 Solvent Programmer, U-6-K injection valve, and a Model 440 ultraviolet fixed wavelength (254 nm) detector (all from Waters Associates, Milford, MA). Stainless steel columns (22 cm × 6.2 mm i.d.) prepacked with microparticulate Zorbax-SIL or Zorbax-ODS were purchased from Du Pont Instruments, Wilmington, DE. Vitamin D metabolites were eluted from Zorbax-SIL columns using mixtures of 5–25% isopropyl alcohol in hexane (v/v) and required flow rates of 2 mL/min and operating pressures of 700–1200 psi. Elution from Zorbax-ODS was achieved using mixtures of 88–95% methanol in water (v/v) and required flow rates of 1.5 mL/min and pressures of 1000–1200 psi.

Mass spectra were obtained with a multiple scanning MAT CH-5 mass spectrometer coupled to a Model 620 i computer (both from Varian Instruments, Palo Alto, CA). Samples of purified metabolites (300–500 ng) were introduced by a direct-insertion probe, temperature-programmed from 50 to 300 °C over 500 s. Ionization voltage was 70 eV and background was subtracted by using a Varian module subtractor. Scintillation counting was performed on a Model LS355 (Beckman Instruments, Palo Alto, CA) ambient-temperature scintillation counter fitted with external standardization.

*Synthesis of Epimeric Mixture of 24(R,S),25-$(OH)_2D_2$ (**1a**) and (**1b**) (Figures 1 and 2)*

3β-Acetoxy-25-hydroxycholesta-5,22-dien-24-one (**4**). *n*-Butyllithium (1.5 mmol) in hexane (9.2 mL) was added to a solution of diisopropylamine (1.38 g, 1.38 mmol) in dry tetrahydrofuran at 0 °C with stirring. This solution was then treated dropwise with a solution of 3-methyl-3-(tetrahydropyran-2-oxy)butan-2-one (2.54 g, 12.6 mmol) at 0 °C for 30

min. After an additional 1.5 h at 0 °C, it was cooled to −70 °C and treated with a solution of the aldehyde **3** (Fryberg et al., 1971) (4 g, 10.6 mmol) in 60 mL of tetrahydrofuran. After being warmed to −20 °C, the reaction mixture was left at this temperature for 3 h and then treated with acetic acid (1 mL) and warmed to room temperature. The material was extracted with ether and washed with a 5% HCl, saturated $NaHCO_3$, and saturated NaCl solutions. Evaporation of the ether solution resulted in oily material (6.7 g) which was dissolved in tetrahydrofuran (80 mL) containing HCl (9 mL, 1.5 N) and left for 18 h at room temperature. The material isolated from ether was chromatographed on silica gel column (ethyl acetate:hexane, 3:7) to give the enone **4**: 3.45 g (67% yield); mp 84–85 °C; UV (MeOH) λ_{max} 227 nm (ϵ 12000); NMR ($CDCl_3$) 7.07 (1, dd, J = 15 and 8 Hz, 22-H), 6.4 (1 d, J = 15 Hz, 23-H), 5.30 (1 d, J = 4 Hz, 6-H), 4.9 (1, m, 3-H), 2.02 (3 s, 3β-OAc), 1.44 (6, s, 26 and 27-H), 1.22 (d, J = 6 Hz, 21-H), 1.14 (3, s, 19-H), and 0.87 (3, s, 18-H). Anal. Calcd for $C_{30}H_{48}O_4 \cdot H_2O$: C, 70.43; H, 10.27. Found: C, 73.32; H, 10.29.

24-Methyl-3β-acetoxy-24(R,S),25-dihydroxycholesta-5,22-diene (**5a** *and* **5b**). A solution of methyllithium (80 mmol) in ether (50 mL, 1.6 mmol) was added with stirring to a solution of the enone **4** (4 g, 8.87 mmol) in tetrahydrofuran (100 mL) at 0 °C under argon. After being left at 0 °C for 3 h the reaction mixture was treated with a solution of HCl (5 mL, 5%). The material was isolated from ethyl acetate and then treated with acetic anhydride (30 mL) and pyridine (30 mL) and left at room temperature overnight. The product was isolated from ethyl acetate and purified by chromatography on a column of silica gel (ethyl acetate) to give a mixture of the two 24-epimeric ketols (**5a** and **5b**) (3.3 g, 80%): mp 121–122 °C; NMR ($CDCl_3$) 5.55 (2, d, J = 4 Hz, H-22 and 23), 5.38 (1, m, H-6), 4.57 (1, m, 3-H), 2.02 (3, s, 3-OAc), 1.27 (3, s, 28-H), 1.2 (6, 2 s, 26 and 27-H), 1.04 (3, d, J = 6 Hz, 21-H), 1.01 (3, s, 19-H), and 0.70 (3, s, 18-H) ppm.

24-Methyl-3,24(R,S),25-trihydroxycholesta-5,22-dien-7-one 3-Acetate (**6**). A solution of the 24,25-diols (**5a** and **5b**) (1 g, 2 mmol) in *tert*-butyl alcohol (70 mL) was treated with mercuric bromide (1 g, 2.7 mmol) and dry sodium acetate (0.5 g, 6.4 mmol). This mixture was irradiated in an open quartz tube with an outside light source emitting at 254 nm (Rayonet) for 15 h. The material was extracted with ether washed with water. The dry residue was chromatographed on a silica gel column using a mixture of hexane, ethyl acetate (7:3) to give the 5-en-7-one **6** (0.45 g): UV (MeOH) λ_{max} 234.5 nm (ϵ 12000); NMR ($CDCl_3$) 5.69 (1 m, 6-H), 5.55 (2, d, J = 4 Hz, 22 and 23-H), 2.04 (3, s, 3-OAc), 1.26 (3, s, 28-H), 1.21 (9, s, 19, 26, and 27-H), 1.04 (3 s, d, J = 7 Hz, 7-H), and 0.70 (3, s, 18-H) ppm.

24-Methyl-3β,24(R,S),25-trihydroxycholesta-5,7,22-triene 3-Acetate (**8**). A solution of the 5-en-7-one **6** (0.2 g, 0.4 mmol) and tosylhydrazide (0.2 g, 1 mmol) in methanol (60 mL) was heated under reflux with stirring for 1.5 h and then was evaporated to dryness. The residue was chromatographed on a column of Sephadex LH-20 using a mixture of chloroform:hexane (6.5:3.5) resulting in the oily tosylhydrazone **7**: UV ($C_{16}H_{12}$) 225, 270 nm; NMR ($CDCl_3$) 7.37, 7.93 (4, 2 d, J = 8 Hz), 6.25 (1, s, 6-H), 4.6 (1, m, 3-H), 2.04 (3, s, 3-OAc), 1.29 (3, s, 28-H), 1.24 (6, s, 26,27-H), 1.00 (3, s, 19-H, OAc), 0.95 (3, d, J = 8 Hz, 21-H), and 0.67 (3, s, H-18).

A solution of the tosylhydrazone **7** (0.2 g, 0.3 mmol) in toluene (50 mL) was treated with lithium hydride (0.6 g, 75 mmol) and was heated under reflux for 1.5 h in an atmosphere of argon. Methanol (2 mL) was added to the cooled reaction mixture and then the material was extracted with ethyl acetate and washed with water to give the triene **8** (0.035 g): 28%; UV λ_{max} (C_6H_{12}) 294, 282, 272, 265 nm (ϵ 7900, 13300, 12200, and 8300).

24(R,S),25-(OH_2)D_2 (**1a** *and* **1b**). A solution of the triene **8** (0.035 g, 0.08 mmol) in a 9:1 mixture of ether:ethanol (200 mL) was irradiated with light emitting at 300 nm at 0 °C through $NaNO_3$ filter for 20 min. The solvent was then evaporated to dryness and the residue was dissolved in ethanol (5 mL) and heated at reflux for 1 hr. The solvent was then evaporated to dryness and the residue was chromatographed on silica gel plate using a 10:90 mixture of methanol:methylene chloride, to give 24(*R,S*),25-$(OH)_2D_2$ (**1a** and **1b**) (5 mg): UV λ_{max} 265 nm (ϵ 17000).

Synthesis of 24R,25-(OH)$_2D_2$ (**1a**). The mixture of the two epimers (**5a** and **5b**) (3 g) was separated by chromatography on a column packed with silica gel type H (3 × 150 cm) using 2 atm of pressure and a mixture of methylene chloride–methanol (95:5). The first eluted material epimer, A (**5a**) (1.95 g), had mp 157–158 °C and $[\alpha]_D$ ($CHCl_3$) −35.5°, and the second eluted compound, epimer B (**5b**) (1.05 g), had mp 164–165 °C and $[\alpha]_D$ ($CHCl_3$) −51.5°; NMR spectrum of each epimer was identical with that of the mixture. The epimer B (**5b**) was oxidized as described above for the mixture of **5a** and **5b** to give 5-en-7-one (**6b**), mp 189–190 °C; UV and NMR were identical with the corresponding epimeric mixture (**6a** and **6b**). This material was further converted to 24*R*,-25-$(OH)_2D_2$ (**1a**) using identical reaction conditions as described for the conversion of the 24(*R,S*),25-diols (**5a** and **5b**) to the respective epimeric mixture of 24(*RS*),25-$(OH)_2D_2$ (**1a** and **1b**).

Biosynthesis of 24,25-(OH)$_2D_2$ by the Perfused Rat Kidney. The perfused rat kidney system reported here represents the model developed for the study of 25-OH-D_3 metabolism to 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ (Rosenthal et al., 1977, and manuscript in preparation). Methodology is based upon the work of Little & Cohen (1974). Animals (350 g) reared on regular diet (normal Ca and PO_4; vitamin D sufficient) were anaesthetized with inactin (100 mg/kg of body weight; Promonta, Hamburg, West Germany). Surgery involved the introduction of catheters into the inferior vena cava below the renal vein, the ureter, and lastly the renal artery. Perfusion was started whilst the kidney was still in the visceral cavity and at no time was the oxygen supply to the kidney cut off. The kidney was carefully excised from the animal while perfusion was continuing and the organ placed into a perfusion apparatus (Little & Cohen, 1974) for continuous recycling of perfusion medium. Perfusion medium consisted of bicarbonate buffer (Krebs-Henseleit, 1932) containing, in addition, 6% bovine serum albumin (fraction v; Miles Labs, Elkhart, Ind.). The medium contained normal physiological concentrations of calcium (2.5 mM) and phosphate (2 mM). Recycled perfusate passed first through a 1.2-μm filter (Millipore Corp., Bedford, MA) and then through a glass-bulb oxygenator before being recirculated through the kidney.

Crystalline 25-OH-D_2 (60 μg) mixed with [3α-^{3}H]-25-OH-D_2 (400000 dpm) in 500 μL of ethanol was incubated with 10 mL of perfusion medium containing 100 μL of plasma from a vitamin D deficient rat at 37 °C for 2 h prior to perfusion. Approximately 15 min after the perfusion was begun, the 10 mL of perfusion medium containing [^{3}H]-25-OH-D_2 was added to that circulating through the kidney perfusion system by gradual addition to one of the collection

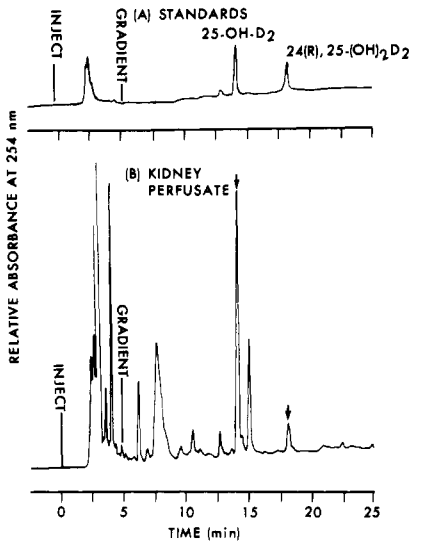

FIGURE 3: UV_{254} profile of the gradient LC of (A) standard 25-OH-D_2 and 24(*R*),25-$(OH)_2D_2$ and (B) extract of kidney perfusate containing [^{3}H]-25-OH-D_2 and ^{3}H-labeled kidney metabolite indicated by arrows. Gradient LC on Zorbax-SIL. Solvent system: isopropyl alcohol/hexane (1/99 to 25/75 in 15 min). Flow rate: 2 mL/min.

reservoirs. Perfusion continued for 3–4 h at which time perfusate (~100 mL) was collected and extracted with methanol/chloroform (2:1) according to the procedure of Bligh & Dyer (1957). Chloroform extracts were rotary evaporated to dryness, redissolved in 2 mL of isopropyl alcohol/hexane (5.5/94.5, v/v) and filtered through a syringe fitted with a Swinney filter holder containing a Millipore 0.45-μm Teflon filter (organic sample clarification kit; Waters Associates). The resultant filtrate was evaporated to dryness under a stream of nitrogen gas and redissolved in 250 μL of isopropyl alcohol/hexane (1/99, v/v).

Purification of the Kidney Metabolite. LC of this extract on Zorbax-SIL, using a linear gradient of isopropyl alcohol in hexane from 1/99 to 25/75 in 15 min, resulted in separation of the substrate 25-OH-D_2 and product 24,25-$(OH)_2D_2$ from other lipids present in the kidney perfusate (Figure 3). The effluent containing kidney product, from 17.5 to 19 min, was collected and further purified by isocratic HPLC on Zorbax-ODS using methanol–water (91/9 v/v). The metabolite eluted from this column as a single radioactive peak between 8.0 and 9.2 min. Finally the partially purified product was subjected to rechromatography on Zorbax-SIL, this time employing isocratic conditions based upon a solvent strength of 2-propanol/hexane (5.5/94.5, v/v). Only kidney metabolite purified to this degree was used in the mass spectral and derivitization studies reported below.

Periodate Cleavage of 24(R),25-$(OH)_2D_2$ and the Kidney Metabolite. For ultraviolet absorption analysis, aliquots (500 ng) of 24(*R*),25-$(OH)_2D_3$, synthetic 24(*R*),25-$(OH)_2D_2$, or kidney metabolite were dissolved in 40 mL of methanol and treated with 20 μL of a 5% (w/v) solution of sodium metaperiodate. Reaction mixtures were incubated in the dark at 22 °C for times ranging from 30 min to 2 days. Solvent was evaporated under a stream of nitrogen and the residue redissolved in isopropyl alcohol/hexane (17.5/82.5, v/v). LC was performed on Zorbax-SIL using a solvent system of similar composition. Larger quantities (~5 μg) of 24(*R*),25-$(OH)_2D_2$ and 24(*R*),25-$(OH)_2D_3$ were used for mass spectral analysis of periodate cleavage products.

In Vivo Production of 24,25-$(OH)_2D_2$ in the Rat. Ten normal rats fed a diet sufficient in vitamin D were each given 2.5 μg of [3α-^{3}H]vitamin D_2 in 50 μL of 95% ethanol by intrajugular injection. Thirty-six hours later, the animals were sacrificed by exsanguination whilst under ether anaesthesia. Plasma obtained from these animals was pooled and extracted according to the method of Bligh & Dyer (1957). The chloroform layer was concentrated, filtered, and prepared for LC as described above. LC was carried out using Zorbax-SIL eluted with isopropyl alcohol/hexane (5.5/94.5, v/v).

Results

Chemical Synthesis of 24(R,S)25-$(OH)_2D_2$ and 24R,-25-$(OH)_2D_2$ and Cochromatography with Kidney Metabolite. The chemical synthesis of 24,25-$(OH)_2D_2$ (**1a** and **1b**) (Figure 1) was performed starting from stigmasterol (**2**) (Figure 2). This plant sterol was converted to the known aldehyde **3** (Fryberg et al., 1971), which on condensation with 3-methyl-3-(tetrahydropyranyl-2-oxy)butan-2-one (Eyley & Williams, 1976) gave the ketol **4**. The latter, on reaction with methyllithium afforded a mixture of 24-epimeric 24,25-diols **5a** and **5b** which, without separation, was oxidized to the 6-en-7-ones (**6**) and then deoxygenated to the respective 5,7-dienes (**8**) (Edelstein et al., 1979). Irradiation, followed by heating and hydrolysis, results in the epimeric mixture of 24(*S*)- and 24(*R*)-25-$(OH)_2D_2$ (**1a** and **1b**). This mixture was separated by LC with Zorbax-SIL, using the 5.5%/94.5% isopropyl alcohol/hexane solvent system into two peaks which were eluted with retention times of 19.6 and 20.7 min, and whose area ratio was 65:35, respectively (Figure 4, upper frame).

The principal peak from the partially purified kidney metabolite (which was also the only radioactive component) had a retention time of 20.7 min (Figure 4, lower frame), suggesting that the natural metabolite is identical with the minor peak of the synthetic material. Cochromatography of the synthetic mixture and the natural metabolite established this identity.

In order to determine the configuration at C-24 of the natural metabolite, we have undertaken to separate the 24-epimeric mixture of the 24,25-diols **5a** and **5b**.

This separation was achieved by chromatography on silica gel type H resulting in the epimer A **5a** and the epimer B **5b** in a 65:35 ratio. The minor constituent of this mixture, the epimer B, was converted to the respective 24,25-$(OH)_2D_2$ (**1b**) by repeating the reaction sequence described above. This material had the retention time of 20.7 min on LC (under identical conditions as above), and cochromatographed with both the minor peak from the synthetic mixture and the partially purified kidney metabolite (Figure 4).

X-Ray Diffraction Analysis of Synthetic 24R,25-Diol (**5b**). The structure of the epimer B (**5b**) was determined by single crystal X-ray analysis, from diffractometer data.

Space group is *C*2 with 8 molecules in the cell. The cell dimensions are: $a = 73.6$, $b = 7.6$, $c = 10.3$ Å; $\beta = 92.0°$.

The two independent molecules display two different conformations about the 24,25 bond, both having the 24*R* configuration (Figure 5).

This result proves unequivocally that the synthetic 24,25-$(OH)_2D_2$, which cochromatographed with the kidney metabolite of 25-OH-D_2, has also the 24*R* configuration (**1b**) as in Figure 1.

Mass Spectrometry of Synthetic and Kidney Metabolites. The mass spectra of synthetic epimers of 24,25-$(OH)_2D_2$ were found to be identical and indistinguishable from 24(*R*),25-$(OH)_2D_2$. Figure 6A depicts the fragmentation pattern of the 24(*R*),25-$(OH)_2D_2$ compound synthesized from the separated 24(*R*) intermediate. The expected molecular ion at *m/e* 428 generates the usual fragments at 271 (loss of side chain), 253

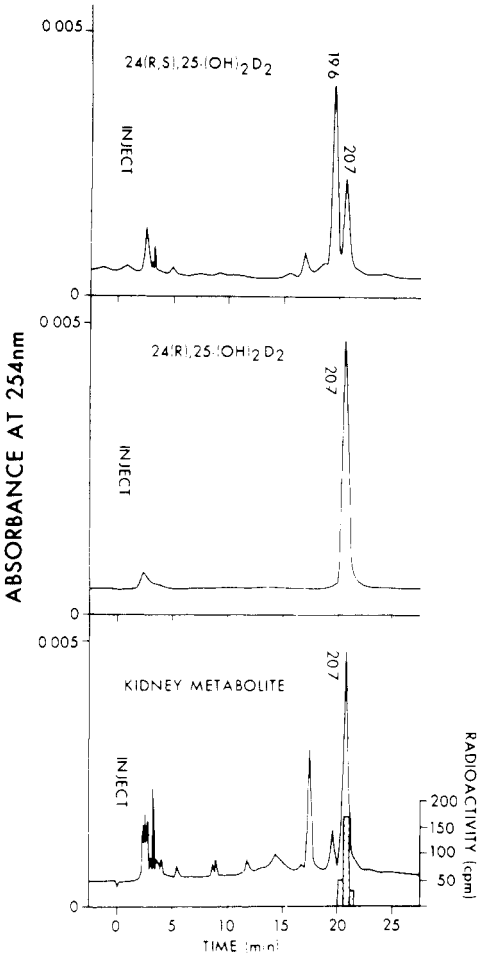

FIGURE 4: UV_{254} profile of the isocratic LC of (upper frame) epimeric mixture of 24(*R*)- and 24(*S*),25-$(OH)_2D_2$, (middle frame) pure 24(*R*),25-$(OH)_2D_2$ made from 24-methyl-3β-acetoxy-24(*R*),25-dihydroxycholesta-5,22-diene, (lower frame) partially purified kidney metabolite previously purified by gradient LC on Zorbax-SIL (as depicted in Figure 3B) and isocratic LC on Zorbax-ODS. Histogram represents radioactivity in 0.5-min fractions of collected effluent. Isocratic LC on Zorbax-SIL. Solvent system: isopropyl alcohol/hexane (5.5/94.5). Flow rate: 2 mL/min.

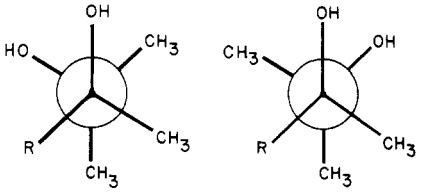

FIGURE 5: The Newman projection of the two rotameric conformations of 24(*R*),25-$(OH)_2D_2$.

(271 – H_2O), 136 (*cis*-triene cleavage) and 118 (136 – H_2O) observed for other vitamin D compounds. In addition 24-(*R*),25-$(OH)_2D_2$ gives rise to a significant peak at *m/e* 370 corresponding to loss of the fragment *m/e* 59 due to C_{24}–C_{25} cleavage. Fragments *m/e* 395 and 337 can be attributed to loss of 33 ($-H_2O$ and $-CH_3$) from the molecular ion and the C_{24}–C_{25} cleavage product, respectively. A comparison of the relative intensity of the assigned fragments (428, 395, 370, 337, 271, 253, 136, and 118) with scan number showed that maximum intensities of fragments were synchronized. This suggests that fragments are from a single compound in the sample loaded on to the direct probe.

Purified kidney metabolite yielded a mass spectrum (Figure 6B) virtually identical with that of 24(*R*),25-$(OH)_2D_2$. It

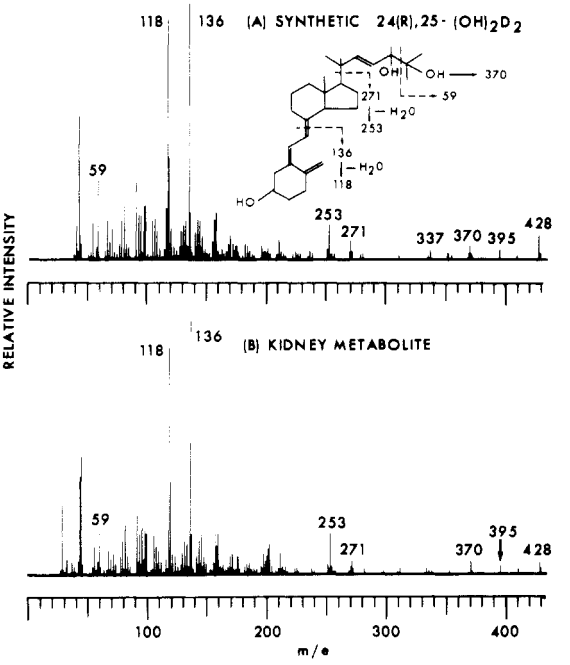

FIGURE 6: Mass spectra of (A) synthetic 24(*R*),25-$(OH)_2D_2$; (B) purified kidney metabolite.

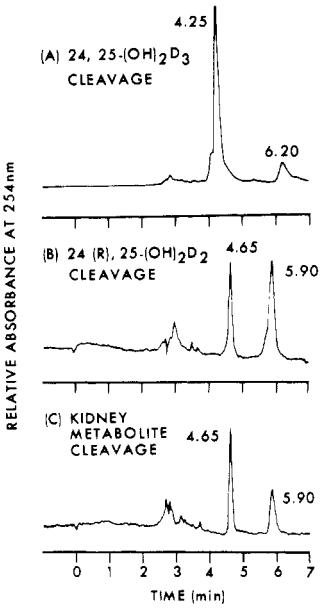

FIGURE 7: UV_{254} profile of the isocratic LC of (A) reaction product of synthetic 24,25-$(OH)_2D_3$ treated with sodium metaperiodate for 30 min, (B) reaction product of synthetic 24(*R*),25-$(OH)_2D_2$ treated with sodium metaperiodate for 24 h, (C) reaction product of purified kidney metabolite treated with sodium metaperiodate for 24 h. Isocratic LC on Zorbax-SIL. Solvent system: isopropyl alcohol/hexane (17.5/82.5). Flow rate: 2 mL/min.

showed that molecular ion and fragments attributed to the pure synthetic compound and in addition the relative intensities of fragments were also very similar. Furthermore, comparison of relative intensity of expected fragments with scan number again showed that maximum intensities of fragments were reached simultaneously. Mass spectral evidence is thus consistent with the hypothesis that the metabolite made by the rat kidney perfusion system is 24(*R*),25-$(OH)_2D_2$.

Periodate Cleavage of Synthetic and Kidney Metabolites. 24(*R*),25-$(OH)_2D_3$, used in these studies as a control, was cleaved by sodium metaperiodate in approximately 30 min. Figure 7A represents LC of the reaction mixture at the end

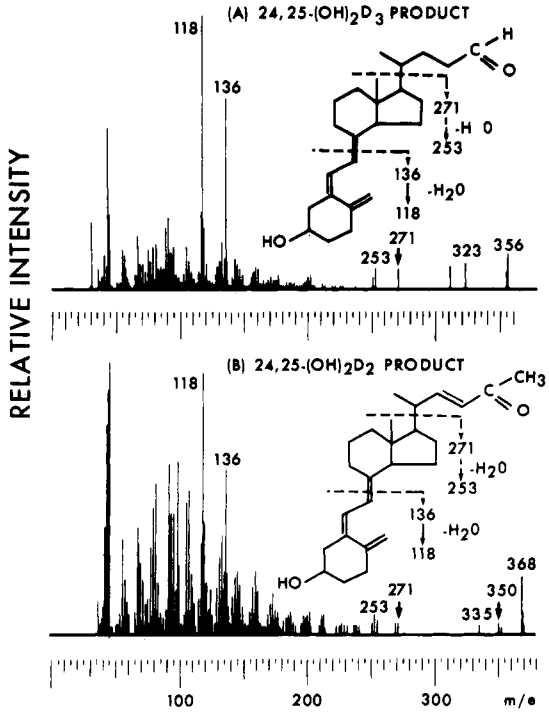

FIGURE 8: Mass spectra of periodate cleavage products of (A) synthetic 24(*R*),25-$(OH)_2D_3$, (B) synthetic 24(*R*),25-$(OH)_2D_2$.

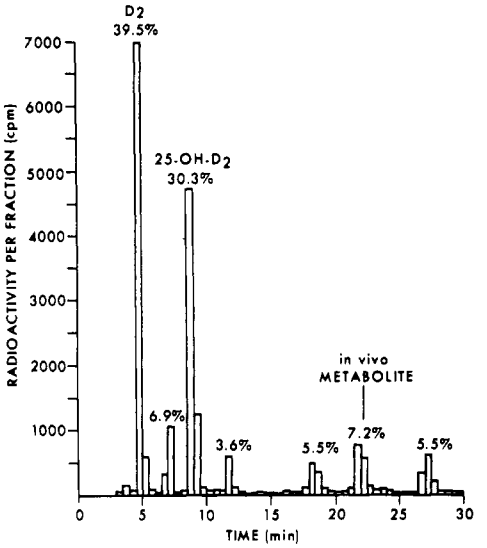

FIGURE 9: Histogram of radioactivity collected during LC of a plasma lipid extract of rats given 2.5 μg of [^{3}H]D_2 and killed 24 h later. Percentages represent fractions of total recovered radioactivity. Isocratic LC conditions as in Figure 4.

of 30 min showing a large product peak at 4.25 min and a small peak of the original compound at 6.20 min. The retention time of the product is that expected for a compound with a vitamin D nucleus with a single hydroxyl function and a single aldehyde function (vitamin D_3 (1-hydroxyl), 3.3 min; 25-OH-D_3 (2-hydroxyls), 4.45 min; 24,25-$(OH)_2D_3$ (3-hydroxyls), 6.20 min). Identity was confirmed as 25,-26,27-trisnorcholecalcifer-24-al by mass spectrometry of the collected peak at 4.25 min (Figure 8A). This periodate cleavage product had a large molecular ion at *m/e* 356 and the usual fragments corresponding to loss of H_2O and $-CH_3$ at 323, and a vitamin D nucleus at 271, 253 (271 – H_2O), 136 (*cis*-triene cleavage), and 118 (136 – H_2O), reported by Holick et al. (1972). It was observed that the yield of cleavage product was somewhat lower than the 60–80% observed at 30 min if the reaction mixture was left for 24 h before termination of the reaction (reduced to about 25%). There was a concomitant appearance of another reaction product, with a retention time of 9.0 min, after 24 h. Though we were unable to identify this compound, we believe it represents a further reaction of the original periodate cleavage product with one of the other components of the reaction mixture.

The reaction between 24(*R*),25-$(OH)_2D_2$ and sodium metaperiodate was much slower than with the corresponding D_3 analogue. The reaction mixture after 24 h of incubation (depicted in Figure 7B) showed a cleavage product at 4.65 min and a significantly greater amount of the starting compound at 5.90 min. The retention time of the product was slightly greater than that of 25-OH-D_2 (= 4.30 min) which has two hydroxyl functions. Nevertheless the product was identified as the α,β-unsaturated ketone 25,26,27-trisnorergocalcifer-24-one with a single hydroxyl function and a conjugated keto grouping by mass spectrometry (Figure 8B). The periodate cleavage product, though isolated in only a 20% yield, had a molecular ion at *m/e* 368, the difference of 12 mass units over the D_3 analogue being attributable to the C_{28} methyl group and the C_{22}–C_{23} double bond. Like the D_3 analogue, the 24(*R*),25-$(OH)_2D_2$ periodate cleavage product had fragments corresponding to: loss of H_2O and a $-CH_3$ group (at *m/e* 335); side chain cleavage (271; 253 (271 – H_2O)); and *cis*-trene cleavage (136; 118 (136 – H_2O)).

Purified kidney metabolite treated with sodium metaperiodate underwent the same reaction as its synthetic counterpart (Figure 7C). The periodate cleavage product again had a retention time of 4.65 min, while starting material remained at 5.90 min after 24 h. Lack of sufficient purified kidney metabolite prevented the collection of enough periodate cleavage product to facilitate mass spectrometry. In the case of the kidney metabolite, however, scintillation counting of the fractions from the chromatogram in Figure 7C enabled us to show that the periodate cleavage product was radioactive due to the presence of a 3α-^{3}H in the original 24,25-$(OH)_2D_2$. Unlike the 26,27-$^3H_2$ label commonly used in vitamin D_3 compounds, this 3α-^{3}H label is not on the cleaved portion of the molecule and the sterol nucleus remains radioactive after periodate reaction.

Thus, the evidence suggests that the synthetic and biosynthetic 24,25-$(OH)_2D_2$ compounds undergo identical reactions with sodium metaperiodate to produce 25,26,27-trisnorergocalcifer-24-one.

24,25-$(OH)_2D_2$ Produced in Vivo. The in vivo metabolite picture which was obtained from the administration of [^{3}H]vitamin D_2 to normocalcemic rats is shown in Figure 9. The major part of the recovered radioactivity in the plasma remains unchanged as vitamin D_2 (39.5%), while 30.3% is in the form of the major transport form of the vitamin, 25-OH-D_2 (Jones et al., 1975). Small amounts of five other metabolites can be observed. One metabolite with a retention time between D_2 and 25-OH-D_2 represents 6.9% of the recovered radioactivity. This is identical with the metabolite tentatively identified by G. Jones, H. K. Schnoes, & H. F. DeLuca (unpublished results) as 24-OH-D_2. Peaks at 12.0, 18.0, and 27.5 min remain unidentified. The peak at 22.0 min and representing 7.2% of the radioactivity is identical with the 24,25-$(OH)_2D_2$ metabolite generated by the kidney perfusion system. When we collected the metabolite from rat plasma and rechromatographed it with the epimeric mixture of 24(*R*)- and 24(*S*),25-$(OH)_2D_2$ compounds, the radioactivity ran exclusively with the synthetic 24(*R*) epimer (Figure 10). It is concluded that 24(*R*),25-$(OH)_2D_2$ is a normal product of

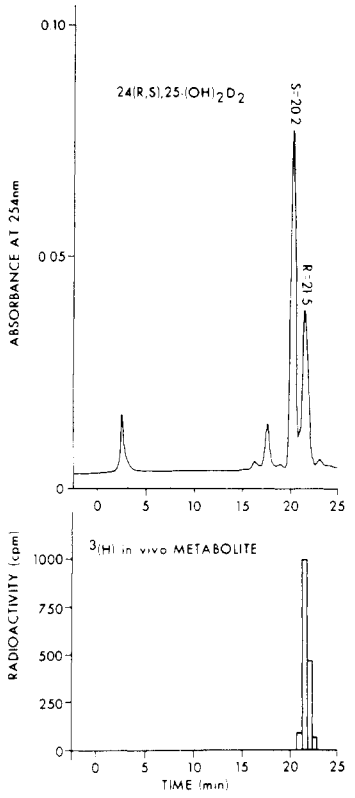

FIGURE 10: (A) UV_{254} profile of the isocratic LC of epimeric mixture of 24(*R*)- and 24(*S*),25-$(OH)_2D_2$ and (B) histogram of radioactivity collected during LC of [3α-^{3}H]-24,25-$(OH)_2D_2$ produced in vivo and purified as in Figure 9. Isocratic LC conditions as in Figure 4.

the metabolism of vitamin D_2 in the rat.

Discussion

It was known from previous work that a metabolite with the likely structure 24,25-$(OH)_2D_2$ could be formed in the pig given massive amounts of vitamin D_2 (Jones et al., 1976). The results presented here prove conclusively that the principal dihydroxylated metabolite of vitamin D_2 made by vitamin D sufficient animals with normo- or hypercalcemia is 24(*R*),-25-$(OH)_2D_2$. The metabolite generated by a perfused kidney system was identified by comparison with authentic 24-(*R*),25-$(OH)_2D_2$ synthesized chemically from stigmasterol. The 24*R* configuration of the synthetic material was unequivocably demonstrated by X-ray diffraction analysis.

The generation of the natural metabolite identified in our studies employed a novel approach to large scale production: the use of a perfused kidney system. We have established that such a perfused kidney will convert microgram quantities of 25-OH-D_3 into either 24,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ depending on the physiological state of the donor rat. Perfused kidneys used here converted 5–10% of the 60 μg of 25-OH-D_2 provided into a metabolite which later proved to be 24(*R*),-25-$(OH)_2D_2$. Certainly this represents the most efficient mammalian model system yet developed, when one considers the problems encountered by other in vitro mammalian kidney hydroxylase systems (Botham et al., 1974). Also since an artificial perfusion medium, low in extractable lipids, is used, lengthy extraction and purification procedures are avoided. The purification procedure used in this study is based exclusively on the different facets of high pressure liquid chromatography. The combination of adsorption chromatography on silica coupled with reverse bonded-phase chromatography on octadecyl-silanized silica, that we have used so successfully in the purification of vitamin D metabolites in human plasma (Jones, 1978), proved important here too. The high degree of purification that we achieved enables the use of chemical derivatization and mass spectrometry techniques which were essential for the successful identification of the kidney metabolite.

The identification of the natural metabolite as 24(*R*),-25-$(OH)_2D_2$ was similar to that put forward for the identification of 24(*R*),25-$(OH)_2D_3$ (Holick et al., 1972; Ikekawa et al., 1977). It rested mainly on three principal pieces of evidence.

Firstly, the metabolite cochromatographed with the 24(*R*) epimer and not the 24(*S*) epimer when run on LC using a solvent system which resolved the two epimers. Secondly, the kidney metabolite had an identical mass spectrum (including the important *m/e* 370 fragment suggestive of C_{24}–C_{25} fragility) to the synthetic 24(*R*),25-$(OH)_2D_2$. Thirdly, the kidney metabolite underwent the same cleavage with sodium metaperiodate exhibited by the synthetic 24(*R*),25-$(OH)_2D_2$ and 24(*R*),25-$(OH)_2D_3$. This reaction is specific for compounds with vicinal hydroxyl groups and the introduction of an extra hydroxyl function into the C_{24} position. The slow reaction of 24(*R*),25-$(OH)_2D_2$ and the kidney metabolite (as compared with 24(*R*),25-$(OH)_2D_3$) with sodium metaperiodate can be reasonably assumed to be due to the extremely hindered side chain of the D_2 analogue with its two tertiary carbon atoms (C_{24} and C_{25}). The low recovery of 25,-26,27-trisnorergocalcifer-24-one from 24(*R*),25-$(OH)_2D_2$ is not surprising when one considers the slow rate of its formation due to steric effects and the fact that it is a α,β-unsaturated ketone and may react with trace impurities in the metaperiodate. It also would explain earlier failure to generate this material from biologically generated material (Jones, Schnoes, & DeLuca, unpublished observations). Together, the three separate pieces of evidence for identification of the kidney product as 24(*R*),25-$(OH)_2D_2$ are overwhelming.

In addition, we also show here that 24(*R*),25-$(OH)_2D_2$ produced by the kidney in vitro is the same metabolite made by the rat in vivo. Since this metabolite must be the principal product of 25-OH-D_2 in mammals using vitamin D_2 as their source of vitamin D, it must be considered as a possible active compound in clinical situations involving high dosage therapy with vitamin D_2 for treatment or prevention of disease. It is also possible that 24,25-$(OH)_2D_2$ represents one of the causes of hypercalcemia during hypervitaminosis D_2.

Acknowledgments

The approach used here depended heavily on ideas generated by Glenville Jones, Heinrich K. Schnoes, and H. F. DeLuca during an earlier attempt to isolate and identify the biological metabolites of vitamin D_2 whilst the first author was a postdoctoral fellow in the laboratory of H. F. DeLuca. Their contribution is gratefully acknowledged.

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Human Liver π-Alcohol Dehydrogenase: Kinetic and Molecular Properties†

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ABSTRACT: A new and distinctive form of human liver alcohol dehydrogenase, π-ADH, has recently been purified to homogeneity [Bosron, W. F., Li, T.-K., Lange, L. G., Dafeldecker, W. P., & Vallee, B. L. (1977) *Biochem. Biophys. Res. Commun. 74*, 85–91]. Its general characteristics, i.e., molecular weight of 78 000, dimeric structure, and zinc content, 4 g-atoms per mol, are all similar to those of other mammalian alcohol dehydrogenases. However, its amino acid composition differs from that of both horse liver ADH and previous preparations of human liver ADH containing a mixture of isoenzymes. The kinetics of π-ADH follow an ordered bibi mechanism with cofactor adding first to form a binary enzyme complex. In contrast to the other molecular forms, π-ADH is less stable in vitro, exhibits a more limited substrate specificity, has higher K_M values for ethanol and acetaldehyde, both approximately 30 mM at pH 7.5, and is much less sensitive to inhibition by pyrazole and 4-methylpyrazole, with K_I values of 30 and 2 mM, respectively. Hence, differentiation of ADH-independent ethanol oxidizing pathways in man cannot be based solely upon the lack of inhibition of alcohol oxidation by pyrazole or 4-methylpyrazole, the inhibitors most commonly employed for such purposes. Significantly, π-ADH exhibits markedly lower K_I values toward other pyrazole derivatives, 4-bromo-, 4-nitro-, or 4-pentylpyrazole ranging from 4 to 27 μM. Hence, these pyrazole derivatives may be suitable for quantitative inhibitor studies of all molecular forms of human liver ADH, including π-ADH.

Alcohol dehydrogenase (ADH)[1] is the principal enzyme responsible for the oxidation of ethanol in liver. Human livers contain multiple molecular enzyme forms (Von Wartburg et al., 1964; Smith et al., 1971; Schenker et al., 1971; Pietruszko et al., 1972), whose number and amount vary, seemingly dependent upon genetic background (Smith et al., 1971), state of health of the individual, and time of storage of the tissue after removal (Azevedo et al., 1974; Li & Magnes, 1975). Since the various pharmacologic, addictive, and pathologic consequences of ethanol consumption must relate directly to the biochemical properties of ethanol and/or its metabolic byproducts, the delineation of those ADH isoenzymes principally responsible for ethanol oxidation is a major objective

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[1] Abbreviations used: ADH, alcohol dehydrogenase; CapGapp, 4-[3-(*N*-6-aminocaproyl)aminopropyl]pyrazole; $NaDodSO_4$, sodium dodecyl sulfate.

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