Identification of 24,25,26,27-Tetranor-23-hydroxyvitamin D_3 as a Product of the Renal Metabolism of 24,25-Dihydroxyvitamin D_3†

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ABSTRACT: By cochromatography, mass spectrometry, and chemical derivatization, we have shown that a metabolite isolated from the perfused rat kidney incubated with 24-(*R*),25-dihydroxyvitamin D_3 is indistinguishable from chemically synthesized 24,25,26,27-tetranor-23-hydroxyvitamin D_3. The new metabolite is also produced from 24-oxo-25-hydroxyvitamin D_3 but not from 23(*S*),25-dihydroxyvitamin D_3. Enzymes required for the synthesis of the new metabolite are absent in the vitamin D deplete animal but are induced along with the 25-hydroxyvitamin-D_3 24-hydroxylase by vitamin D repletion. The pathway of 24,25-dihydroxyvitamin D_3 metabolism in the perfused kidney is stimulated by pretreatment of the rat with large doses of vitamin D_3, suggesting that the pathway is a degradative one.

Previously, it has been shown with chick kidney homogenates in vitro that 24(*R*),25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] is metabolized into several compounds including 24-oxo-25-hydroxyvitamin D_3, 23,24,25-trihydroxyvitamin D_3, and 24-oxo-23,25-dihydroxyvitamin D_3 [24-oxo-25-(OH)D_3, 23,24,25-$(OH)_3D_3$, and 24-oxo-23,25-$(OH)_2D_3$, respectively] (Takasaki et al., 1978, 1981; Yamada et al., 1983). This was followed by unequivocal identification of biosynthetically generated 24-oxo-25-(OH)D_3 by comparison to chemically synthesized material (Takasaki et al., 1982). Further work by Wichmann et al. (1981) in rats given large doses of vitamin D_3 showed that the formation of 24-oxo-25-(OH)D_3 is not confined to avian species but is carried out in mammals too. Recent work has confirmed that indeed the pathway 24,25-$(OH)_2D_3$ → 24-oxo-25-(OH)D_3 → 24-oxo-23,25-$(OH)_2D_3$ not only exists in the mammalian kidney (Jones et al., 1983; Mayer et al., 1983) but also may continue on with the formation of 24,25,26,27-tetranor-23-(OH)D_3 (Jones et al., 1983). Unequivocal identification of this metabolite, however, was hampered by the lack of a synthetic standard. This paper provides confirmation that the renal product is 24,25,26,27-tetranor-23-(OH)D_3, that it is derived from 24,25-$(OH)_2D_3$ or 24-oxo-25-(OH)D_3 and not from 23(*S*),25-$(OH)_2D_3$, and that its synthesis is stimulated by pretreatment of the rat with large doses of vitamin D.

Experimental Procedures

Materials

Vitamin D Compounds. Crystalline 25-(OH)D_3, 24-(*R*),25-$(OH)_2D_3$, and 23(*S*),25-$(OH)_2D_3$ were generous gifts of Drs. John Partridge and Milan Uskokovic of Hoffmann-La Roche Inc., Nutley, NJ. 24-Oxo-25-(OH)D_3 and 25-(OH)D_3-26,23-lactone were synthesized chemically as described in Yamada et al. (1981b) and Yamada et al. (1981a), respectively.

Solvents. All solvents were "distilled-in-glass" spectroscopic grade from Burdick-Jackson Laboratories.

Animals. Adult, male Wistar rats (~300 g), designated vitamin D deplete, were kept in a dark room and fed a vitamin D deficient low-calcium (0.02%), low-phosphorus (0.18%) diet (Teklad, Madison, WI) for a minimum period of 6 weeks. Male Wistar rats (250 g), designated vitamin D replete (Camm Laboratories, Wayne, NJ), were fed a standard rodent diet (Masterfeed Laboratory, Toronto, Ontario Canada), containing nutritionally adequate amounts of vitamin D, calcium, and phosphorus. In some preparative experiments in which kidneys were perfused with 24(*R*),25-$(OH)_2D_3$, rats were pretreated with five daily oral doses of 50 000 IU of vitamin D_3 in 0.1 mL of ethanol.

Methods

Kidney Perfusion. Preparation of animals, surgical cannulation of the kidney, and isolation of the organ are described in Rosenthal et al. (1980). Perfusate composition, perfusion apparatus, and perfusion techniques are described in Reddy et al. (1982). Vitamin D compounds, 24(*R*),25-$(OH)_2D_3$, 24-oxo-25-$(OH)_2D_3$, or 23(*S*),25-$(OH)_2D_3$ (40–50 μg), were introduced into 100 mL of perfusate in 100 μL of ethanol after 5 min of stabilization. Aliquots of perfusate (4 mL) were taken hourly for 6 h, and at the termination of each experiment approximately 80 mL of perfusate remained.

Lipid extraction of perfusate samples was based upon the procedure of Bligh & Dyer (1959), except that methylene chloride was substituted for chloroform. Residues were redissolved in 250 μL of hexane–2-propanol–methanol (94:5:1 or 91:7:2).

Analytical Chromatography of Perfusate Extracts. Four-milliliter samples of perfusate yielded extracts that could be run directly on a conventional high-pressure liquid chromatograph (HPLC) without overloading problems. Samples were subjected to HPLC on a 25 cm × 6.2 mm Zorbax-SIL column in the solvent hexane–2-propanol–methanol (94:5:1) at a flow rate of 1.5 mL/min (Jones, 1980). Alternatively, samples dissolved in hexane–2-propanol–methanol (94:5:1) were subjected to HPLC on a 25 cm × 4.6 mm Zorbax-CN column (Du Pont Instruments, Wilmington, DE) in the same solvent at a flow rate of 1.3 mL/min (Jones, 1983).

Preparative Chromatography of Perfusate Extracts. Bulk extracts of perfusate (80 mL) gave lipid residues that were chromatographed on a larger 25 cm × 9.4 mm column of Zorbax-SIL in the solvent hexane–2-propanol–methanol (91:7:2) (Jones, 1980). Fractions containing metabolites of 24(*R*),25-$(OH)_2D_3$ or 24-oxo-25-(OH)D_3 were chromatographed on Zorbax-CN as described above for analytical-sized samples or on Zorbax-CN in the solvent methylene chlo-

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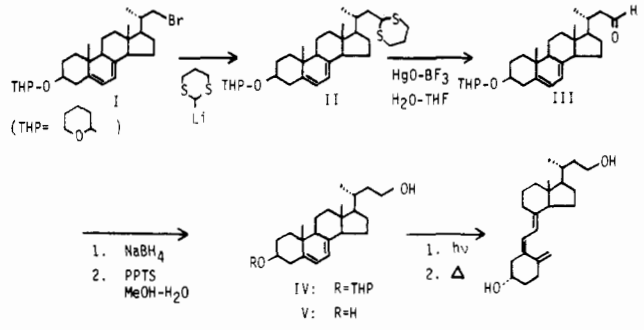

FIGURE 1: Chemical synthesis of 24,25,26,27-tetranor-23-(OH)D_3.

ride–2-propanol–methanol (96.5:3.5:1).

Purified renal metabolites were subjected to ultraviolet spectrophotometric analysis with a diode-array spectrophotometer (HP8450, Hewlett-Packard, Palo Alto, CA) as a HPLC detector in the scanning mode or with cuvettes in the conventional spectrophotometric mode. Renal metabolites and synthetic vitamin D compounds were subjected to electron-impact mass spectrometry with a direct-insertion probe (either HP5985, Hewlett-Packard, Palo Alto, Ca, or JEOL JMS-D300). Ionization voltage in all cases was 70 eV and the source temperature programmed in the range of 25–400 °C.

Derivatization of the Vitamin D Metabolites. Metabolites were trimethylsilylated by treating a sample in 15 μL of pyridine with 10 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylsilyl chloride (Pierce Chemical Co., Rockford, IL). After 45 min at 55 °C, reagents were removed under a stream of N_2, and the sample was rechromatographed on a Zorbax-SIL 6.2 mm × 25 cm column with the solvent 0.25% 2-propanol in hexane.

Chemical Synthesis of 24,25,26,27-Tetranor-23-(OH)D_3. For confirmation of the assigned structure, chemical synthesis of 23-hydroxy-24,25,26,27-tetranorvitamin D_3 was carried out starting with 22-bromo-23,24-dinor-5,7-choladien-3β-yl tetrahydropyranyl ether (I) (Figure 1), which is readily obtained from commercially available ergosterol. Reaction of the bromide I with 2-lithiodithiane gave the dithioacetal II (95%), which upon removal of the dithioacetal group afforded the aldehyde III (55%). The aldehyde III was converted to the desired provitamin D V (77%) by reduction followed by deprotection. UV irradiation and subsequent thermal isomerization of the provitamin D V afforded the tetranorvitamin D_3. The spectral properties [UV λ_{max} (95% ethanol) 265 nm; mass spectrum, see Figure 4B and below; ^{1}H NMR ($CDCl_3$) δ 0.55 (3 H, s, H-18), 0.95 (3 H, d, J = 6 Hz, H-21), 3.62 (2 H, m, H-23), 3.86 (1 H, m, H-3), 4.75 (1 H, br s, H-19), 4.95 (1 H, br s, H-19), 5.90 (1 H, d, J = 11 Hz, H-7), 6.12 (1 H, d, J = 11 Hz, H-6)] confirmed the structure of the vitamin D thus synthesized.

1,3-Propylenedithioacetal of 3β-(Tetrahydropyranoxy)-24-nor-5,7-choladien-23-al (II). A 1.7 M hexane solution of *n*-butyllithium (0.8 mL, 1.36 mmol) was added to a solution of 1,3-dithiane (156 mg, 1.3 mmol) in tetrahydrofuran (THF) (1 mL) at 0 °C. The mixture was stirred for 5 min at that temperature, and then a solution of 23,24-dinor-5,7-choladien-3β-yl tetrahydropyranyl ether (I) (115 mg, 0.24 mmol) and hexamethylphosphoric triamide (43 mg, 0.24 mmol) in THF (1 mL) was added. The mixture was stirred at 0 °C for 3 min and then quenched with water. The mixture was extracted with ethyl acetate; the extracts were washed with water, dried over Na_2SO_4, and evaporated. The residue was chromatographed on silica gel (Wakogel C-200, Wako Pure Chemical Industries, Ltd., Osaka, Japan; 10 g) with 5% ethyl acetate in *n*-hexane as the eluent to afford the dithioacetal II (118 mg, 95%): mass spectrum, m/z 516 (M^+), 432, 414; ^{1}H NMR ($CDCl_3$) δ 0.66 (3 H, s, H-18), 0.95 (3 H, s, H-19), 1.05 (3 H, d, J = 6 Hz, H-21), 2.85 (4 H, m, CH_2S), 5.45 (1 H, m, H-7), 5.60 (1 H, m, H-6).

3β-(Tetrahydropyranoxy)-24-nor-5,7-choladien-23-al (III). A solution of the dithioacetal II (22 mg, 40 μmol) in THF (200 μL) was added to a stirring mixture of red mercuric oxide (18.5 mg, 80 μmol) of boron trifluoride etherate (12.1 mg, 80 μmol) in 15% aqueous THF (220 μL) at room temperature during a period of 10 min. The mixture was stirred at room temperature for 3 h. The reaction was extracted with ethyl acetate; the extracts were washed with water, dried over Na_2SO_4, and evaporated. The residue was chromatographed on silica gel (15 g) with 5% ethyl acetate in *n*-hexane as the eluent to give the aldehyde III (12 mg, 55%): mass spectrum, m/z 426 (M^+), 342, 324; IR ($CHCl_3$) 1720 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.66 (3 H, s, H-18), 0.95 (3 H, s, H-19), 1.05 (3 H, d, J = 6 Hz, H-21), 5.45 (1 H, m, H-7), 5.60 (1 H, m, H-6), 9.80 (1 H, m, H-23).

24-Nor-5,7-choladiene-3β,23-diol 3-(Tetrahydropyranyl ether) (IV). A solution of sodium borohydride ($NaBH_4$) (350 μg, 9.3 μmol) in ethanol (2.5 mL) was added to a solution of the aldehyde III (8 mg, 19 μmol) in ethanol (2.5 mL) at room temperature, and the mixture was stirred for 20 min at that temperature. After evaporation of the solvent, the residue was dissolved in dichloromethane, washed with water, dried over Na_2SO_4, and evaporated. The residue was chromatographed on silica gel (2 g) with 5% ethyl acetate in benzene as the eluent to give the alcohol IV (7.2 mg, 90%): mass spectrum, m/z 428 (M^+), 410, 344, 326; IR ($CHCl_3$) 3400 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.65 (3 H, s, H-18), 0.95 (3 H, s, H-19), 1.00 (3 H, d, J = 6 Hz, H-21), 3.40–4.1 (5 H, m, OCH and OCH_2), 5.45 (1 H, m, H-7), 5.60 (1 H, m, H-6).

24-Nor-5,7-choladiene-3β,23-diol (V). A solution of the tetrahydropyranyl ether IV (7 mg, 16 μmol) and pyridinium-*p*-toluenesulfonate (PPTS) (8 mg, 32 μmol) in ethanol (500 μL) was stirred at 43 °C for 5 h under argon. The solvent was evaporated, the residue was dissolved in dichloromethane, washed with water, dried over Na_2SO_4, and evaporated. The residue was chromatographed on silica gel (4 g) with ethyl acetate–*n*-hexane (1:2) as the eluent to give the previtamin D V (4.8 mg, 85%): mass spectrum, m/z 344 (M^+), 326, 308; ^{1}H NMR ($CDCl_3$) δ 0.65 (3 H, s, H-18), 0.95 (3 H, s, H-19), 1.00 (3 H, d, J = 6 Hz, H-21), 3.60–3.90 (3 H, m, H-3 and H-23), 5.45 (1 H, m, H-7), 5.60 (1 H, m, H-6).

23-Hydroxy-24,25,26,27-tetranorvitamin D_3. A solution of the previtamin D V (4.6 mg, 13 μmol) in freshly distilled ether (200 mL) was irradiated with a high-pressure mercury lamp (200 W. Shigemi Standard, Co.) through Vycor filter at 0 °C for 5.5 min. After evaporation of the solvent, the residue was chromatographed on Sephadex LH-20 (24 g) with *n*-hexane–$CHCl_3$ (1:1) as the eluent to yield 23-hydroxy-24,25,26,27-tetranorprevitamin D_3 (1.4 mg, 29%): UV (95% ethanol) 260 nm. A solution of the previtamin (1.4 mg) in ethanol (30 mL) was stored in the dark for 2 weeks at room temperature. The solvent was evaporated, and the residue was chromatographed on Sephadex LH-20 (24 g) with *n*-hexane–$CHCl_3$ (1:1) as the eluent to give the vitamin D (933 μg): high-resolution mass for $C_{23}H_{36}O_2$ requires m/z 344.2715; found m/z 344.2697.

Results

Identification of 24,25,26,27-Tetranor-23-(OH)D_3. After 6 h of perfusion of kidneys from vitamin D replete rats pretreated with large doses of vitamin D_3 and using 24(*R*),25-$(OH)_2D_3$ as a substrate, we obtained the chromatographic

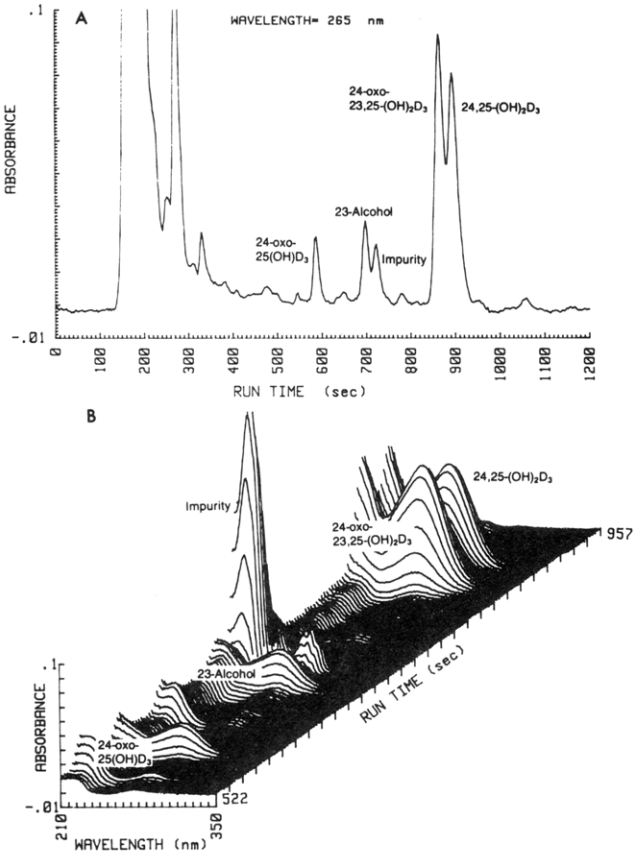

FIGURE 2: HPLC of the extract of perfusate from kidneys perfused in the presence of 1250 nM 24(*R*),25-$(OH)_2D_3$: (A) absorbance at 265 nm vs. run time; (B) absorbance vs. wavelength vs. run time. (A) and (B) represent the same chromatographic run. Note in (A) that the peak marked as an impurity appears minor because the wavelength used for monitoring is 265 nm. The impurity peak absorbs strongly at 225 nm and in the 275–280-nm region. Note also in (B) that all vitamin D metabolites have λ_{max} = 265 nm and λ_{min} = 228 nm. Chromatographic conditions were as follows: Zorbax-SIL 25 cm × 6.2 mm column; solvent of hexane–2-propanol–methanol (94:5:1 v/v), 1.5 mL/min.

profile shown in Figure 2A. Peaks at 587, 698, 860, and 892 S, not present in the lipid extract of the original perfusate, corresponded to 24-oxo-25-$(OH)D_3$, putative 24,25,26,27-tetranor-23-$(OH)D_3$, 24-oxo-23,25-$(OH)_2D_3$, and the substrate 24(*R*),25-$(OH)_2D_3$, respectively. As well illustrated in the three-dimensional plot of the same chromatogram shown in Figure 2B (only 522–957 S of Figure 2A shown), each peak had the typical UV spectral characteristics of vitamin D (λ_{max} = 265 nm, λ_{min} = 228 nm, and $\lambda_{max}/\lambda_{min}$ = 1.8–2.1). Another peak appeared in the profile at 725 S but did not possess the UV spectral characteristics of a vitamin D metabolite. Instead, the impurity peak had intense absorbance at λ_{max} = 225 nm and 275 nm.

The bulk extract of six perfusions with 24(*R*),25-$(OH)_2D_3$ gives a lipid residue that was chromatographed in batches on a 25 cm × 9.4 mm preparative Zorbax-SIL column as described under Experimental Procedures. The three peaks representing: 24-oxo-25-$(OH)D_3$, putative 24,25,26,27-tetranor-23-$(OH)D_3$, and 24-oxo-23,25-$(OH)_2D_3$ were collected separately and purified by further chromatography on Zorbax-CN with methylene chloride–2-propanol–methanol (96.5:3.5:1) as the solvent and finally on Zorbax-SIL again (Figure 3). The yield of pure putative 24,25,26,27-tetranor-23-$(OH)D_3$ was 5 μg.

Mass spectral analysis of the putative 24,25,26,27-tetranor-23-$(OH)D_3$ revealed a mass spectrum (Figure 4A) with

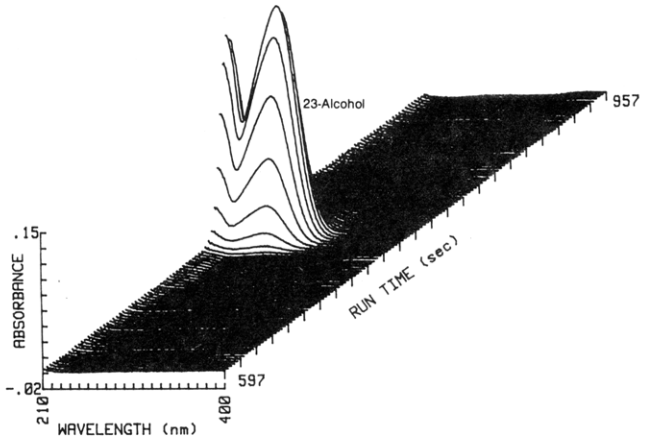

FIGURE 3: Final HPLC of putative 24,25,26,27-tetranor-23-$(OH)D_3$ from kidney perfusate. Chromatographic conditions were as follow: Zorbax-SIL 25 cm × 6.2 mm column; solvent of hexane–2-propanol–methanol (94:5:1 v/v), 1.5 mL/min. Material from this column was used for mass spectrometry.

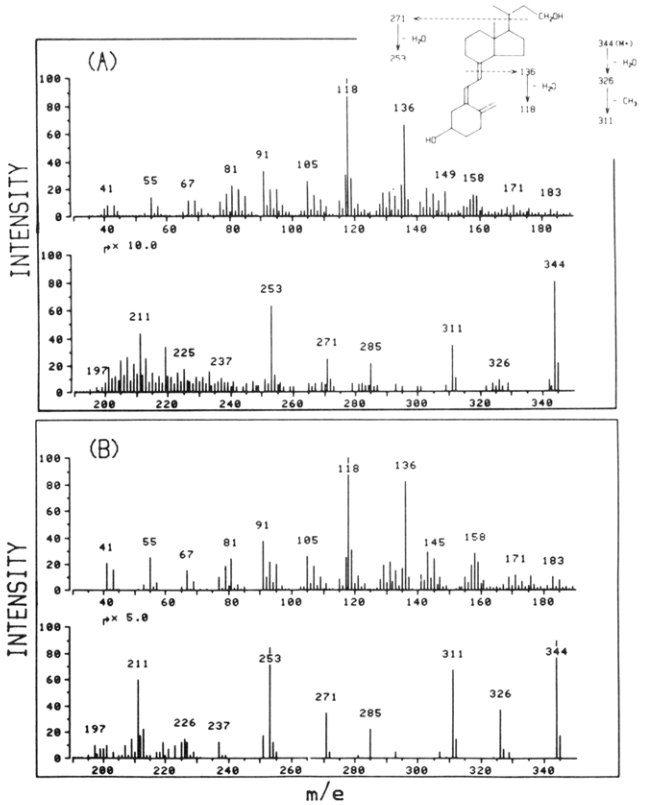

FIGURE 4: Mass spectra of (A) putative 24,25,26,27-tetranor-23-$(OH)D_3$ from kidney perfusate and (B) synthetic 24,25,26,27-tetranor-23-$(OH)D_3$.

the following characteristics: M^+ at *m/z* 344 and fragments at *m/z* 311 (loss of 33), 285, 271 (loss of side chain), 253 (271 – H_2O), 136 (*cis*-triene cleavage), and 118 (136 – H_2O). Synthetic 24,25,26,27-tetranor-23-$(OH)D_3$ had a virtually identical mass spectrum (Figure 4B). Cochromatography of the renal and synthetic metabolites showed that retention times were identical in at least two chromatographic systems. On Zorbax-SIL (4.6 mm × 25 cm) with the solvent hexane–2-propanol–methanol (94:5:1) at a flow rate of 1.5 mL/min, retention times of renal and synthetic 24,25,26,27-tetranor-23-$(OH)D_3$ metabolites were 13.85 and 13.81 min, respectively. [For comparison: 25-$(OH)D_3$, 10.37 min; 24(*R*),25-$(OH)_2D_3$, 17.09 min; 1,25-$(OH)_2D_3$, 35.19 min.] On Zorbax-CN (4.6 mm × 25 cm) with the solvent hexane–2-

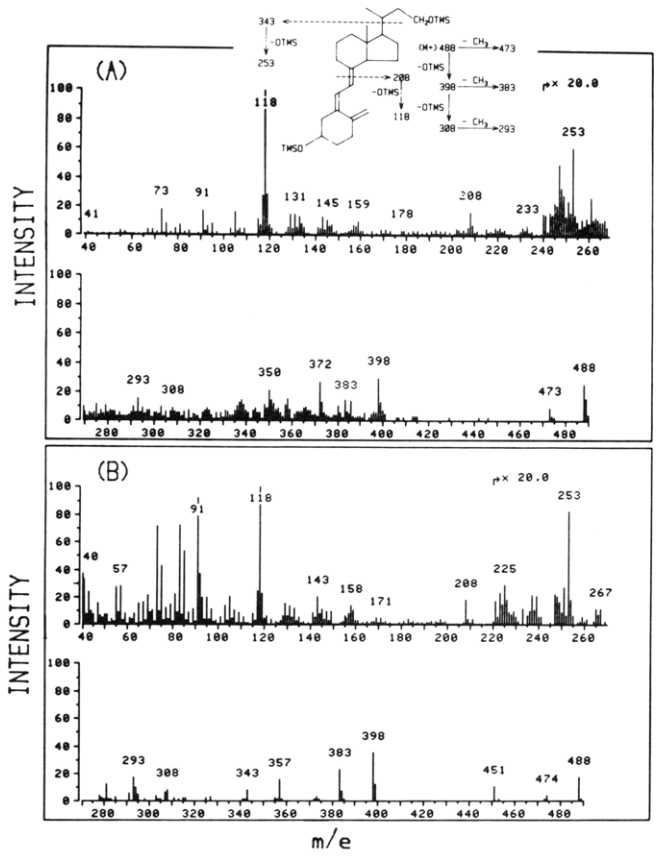

FIGURE 5: Mass spectra of (A) di-TMS derivative of putative 24,25,26,27-tetranor-23-(OH)D_3 and (B) di-TMS derivative of synthetic 24,25,26,27-tetranor-23-(OH)D_3.

propanol–methanol (94:5:1) at a flow rate of 1.3 mL/min, retention times of renal and synthetic 24,25,26,27-tetranor-23-(OH)D_3 metabolites were 9.12 and 9.13 min, respectively. [For comparison: 25-(OH)D_3, 6.69 min; 24(*R*),25-$(OH)_2D_3$, 10.87 min; 1,25-$(OH)_2D_3$, 17.66 min.]

Trimethylsilylation of synthetic 24,25,26,27-tetranor-23-(OH)D_3 resulted in a di-TMS derivative with a retention time of 4.45 min on Zorbax-SIL with 0.25% 2-propanol in hexane. The di-TMS derivative of perfusate 24,25,26,27-tetranor-23-(OH)D_3 had a retention time of 4.49 min under similar conditions.

The mass spectrum of the di-TMS derivative of the natural metabolite is shown in Figure 5A and was similar to that of the di-TMS derivative of the synthetic compound (Figure 5B). Each featured a molecular ion M^+ at m/z 488, with major fragments at m/z 473 ($M^+ - CH_3$), 398 (M^+ – TMSOH), 383 (398 – CH_3), 308 (398 – TMSOH), 293 (308 – CH_3), 253 (398 – side chain), 208 (*cis*-triene cleavage), and 118 (208 – TMSOH). In summary, the two forms of 24,25,26,27-tetranor-23-(OH)D_3, either synthetic or natural, appeared to be identical as defined by the chromatographic and mass spectrometric methods that were available to us.

Rate of Metabolism of 24(R),25(OH)$_2$D$_3$ in the Kidneys from Vitamin D Intoxicated Rats. Figure 6 illustrates the time course of the appearance of metabolites of 24(*R*),25-$(OH)_2D_3$ in the kidneys from rats given 50 000 IU of D_3 per day for 5 days. The results are significantly different from control studies where 24(*R*),25-$(OH)_2D_3$ was used in perfusions of kidneys from rats fed a normal D-replete diet with no supplements (Jones et al., 1983). Initial synthesis of 24-oxo-25-(OH)D_3 and 24-oxo-23,25-$(OH)_2D_3$ over the 0–2-h period was more rapid in D-treated kidneys than in controls [24-oxo-25-(OH)D_3, 6.07 ± 0.31 nmol/2 h in D treated significantly different from 4.33 ± 0.23 nmol/2 h in control, p less than 0.005; 24-oxo-23,25-$(OH)_2D_3$, 10.80 ± 1.30 nmol/2 h in D treated significantly different from 3.08 ± 0.23 nmol/2 h in control, p less than 0.005]. However, maximal concentrations of each metabolite in the perfusate were reached at around 4 h, and rates of synthesis over the 0–4-h period appeared similar between the groups. [24-Oxo-25-(OH)D_3, 5.75 ± 0.71 nmol/4 h in D treated vs. 7.40 ± 0.11 nmol/4 h in control, not significant; 24-oxo-23,25-$(OH)_2D_3$, 16.66 ● 1.31 nmol/4 h in D treated vs. 17.2 ± 1.20 nmol/4 h in control, not significant]. Accumulation of 24,25,26,27-tetranor-23-(OH)D_3 continued for the whole 6-h perfusion period.

Table I: Metabolism of 24-Oxo-25-(OH)D_3 in the Vitamin D Replete Rat Kidney

time of perfusion (h)	concn in perfusate (nmol/100 mL)		
	24-oxo-25-(OH)D_3	24-oxo-23,25-$(OH)_2D_3$	24,25,26,27-tetranor-23-(OH)D_3
1	44.53	3.41	0.06
2	27.80	8.37	0.33
3	16.53	12.26	1.01
4	12.96	14.97	2.29
5	8.46	14.21	2.99
6	8.11	15.77	3.96

Table II: Metabolism of 24,25-$(OH)_2D_3$ in the Vitamin D Deplete Rat Kidney[a]

time of perfusate (h)	concn in perfusate (nmol/100 mL)			
	24,25-$(OH)_2D_3$	24-oxo-25-(OH)D_3	24-oxo-23,25-$(OH)_2D_3$	1,24,25-$(OH)_3D_3$
1	47.71	0	0	2.52
2	46.20	0	0	4.20
3	37.66	0.22	0	4.51
4	38.13	0.66	0.27	6.64
5	32.26	0.92	0.50	6.70
6	33.01	1.23	2.02	8.95

[a] Vitamin D deplete rats were fed a diet deficient in vitamin D for 6 weeks prior to the study as described in the text

Rate of Metabolism of 24-Oxo-25-(OH)D$_3$ in D-Replete Kidney. 24-Oxo-25-(OH)D_3, at a concentration of 40 μg/100 mL perfusate, was rapidly metabolized by the D-replete rat kidney into two peaks: 24-oxo-23,25-(OH)D_3 and 24,25,26,27-tetranor-23-(OH)D_3. Mass spectral analysis of the purified 24,25,26,27-tetranor-23-(OH)D_3 peak again gave a spectrum with a molecular ion of m/z 344 and a fragmentation pattern similar to that in Figure 4. Table I shows the rate of appearance of these two compounds as well as indicating the dramatic disappearance of the substrate. A rapid initial rise in 24-oxo-23,25-$(OH)_2D_3$ is accompanied by a slower but steady rise in the concentration of the 24,25,26,27-tetranor-23-(OH)D_3.

Metabolism of 23(S),25-(OH)$_2$D$_3$ in the D-Replete Kidney. When 23(*S*),25-$(OH)_2D_3$ was used as a substrate at a concentration of 50 μg/100 mL of perfusate, there was no production of 24-oxo-23,25-$(OH)_2D_3$ or 24,25,26,27-tetranor-23-(OH)D_3, but instead, we observed the formation of a peak cochromatographing with authentic 25-(OH)D_3-26,23-lactone. [At 1, 4, and 6 h, 23(*S*),25-$(OH)_2D_3$ concentrations in perfusate were 38.75, 18.73, and 12.89 nmol/100 mL. The corresponding concentrations of 25-(OH)D_3-26,23-lactone were 0.59, 4.52, and 7.96 nmol/100 mL.]

Induction of Enzymes of C-23- and C-24-Oxidation Pathway. 24(*R*),25-$(OH)_2D_3$ (50 μg/100 mL of perfusate) was used as the substrate in a 1-hydroxylating vitamin D deplete kidney (Table II). Initial metabolism was to a peak with a retention time of 27.5 min on Zorbax-CN and with the probable structure 1,24(*R*),25-$(OH)_3D_3$. Over the first 3 h

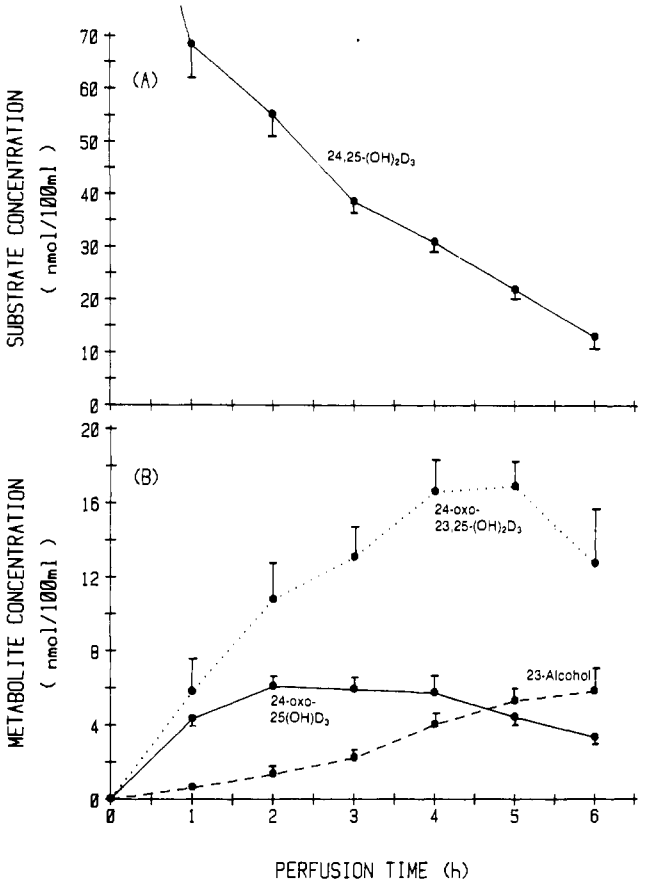

FIGURE 6: (A) Rate of disappearance of 24(*R*),25-$(OH)_2D_3$ and (B) rate of appearance of metabolites of 24(*R*),25-$(OH)_2D_3$ in perfusate of kidneys from rats treated with large doses of vitamin D_3. Substrate concentration at time zero was 1250 nmol/100 mL of perfusate. Datapoints represent mean ■ SEM. In (B): (—) 24-oxo-25-$(OH)D_3$; (···) 24-oxo-23,25-$(OH)_2D_3$; (– –) 24,25,26,27-tetranor-23-$(OH)D_3$. Animals used in (B) received 50 000 IU/day for 5 days prior to sacrifice.

of perfusion, there was no evidence of the formation of 24-oxo-25-$(OH)D_3$ or its derivatives. Subsequently, between 3 and 6 h of perfusion, the appearance of 24-oxo-25-$(OH)D_3$, 24-oxo-23,25-$(OH)_2D_3$, and 24,25,26,27-tetranor-23-$(OH)D_3$ was noted. This pattern is similar to the appearance of the same metabolites from 25-$(OH)D_3$ in a vitamin D deplete kidney (Kano & Jones, 1984).

Discussion

We have demonstrated in this paper that a metabolite derived from 24(*R*),25-$(OH)_2D_3$ in the vitamin D replete rat kidney is indistinguishable from chemically synthesized 24,25,26,27-tetranor-23-$(OH)D_3$. The two vitamin D compounds, renally and synthetically produced, are identical chromatographically, have similar mass spectra, and can be chemically converted to the same di-TMS derivative. Previous work (Jones et al., 1983) has shown that a renal metabolite with a likely structure of 24,25,26,27-tetranor-23-$(OH)D_3$ is formed from 25-$(OH)D_3$ or 24(*R*),25-$(OH)_2D_3$. The additional evidence presented here provides unequivocal support for the interpretation that the metabolite is 24,25,26,27-tetranor-23-$(OH)D_3$.

24,25,26,27-Tetranor-23-$(OH)D_3$ was formed when 24-oxo-25-$(OH)D_3$ was used as a substrate in the vitamin D replete kidney. Neither 24-oxo-23,25-$(OH)_2D_3$ nor 24,25,26,27-tetranor-23-$(OH)D_3$ was produced when 23,25-$(OH)_2D_3$ was used as a substrate in the vitamin D replete kidney, suggesting that 24-oxo-25-$(OH)D_3$ and not 23(*S*),25-$(OH)_2D_3$ is the precursor of these two compounds in the normal kidney. Thus, taken with the earlier evidence that 24-oxo-25-$(OH)D_3$, 24-oxo-23,25-$(OH)_2D_3$, and 24,25,26,27-tetranor-23-$(OH)D_3$ are produced from 25-$(OH)D_3$ or 24(*R*),25-$(OH)_2D_3$ in the vitamin D replete kidney (Jones et al., 1983) and the timing of the appearance of 24-oxo-23,25-$(OH)_2D_3$ and 24,25,26,27-tetranor-23-$(OH)D_3$ in the perfusate (Figure 6), the following metabolic pathway seems most likely:

$$25\text{-}(OH)D_3 \rightarrow 24,25\text{-}(OH)_2D_3 \rightarrow 24\text{-oxo-}25\text{-}(OH)D_3 \rightarrow 24\text{-oxo-}23,25\text{-}(OH)_2D_3 \rightarrow 24,25,26,27\text{-tetranor-}23\text{-}(OH)D_3$$

Whether the final step in this pathway is a single step or a two-step process is not yet clear since we have been unable to find evidence for the presence of a 23-aldehyde or 23-acid intermediate.

Previously (Jones et al., 1983), we have shown that for all but the final step, 24-oxo-23,25-$(OH)_2D_3$ → 24,25,26,27-tetranor-23-$(OH)D_3$, metabolism along this pathway occurs irrespective of substrate concentrations. Physiological (25 nM) and pharmacological (1250 nM) concentrations of 25-$(OH)D_3$ result in qualitatively similar conversions to 24-oxo-23,25-$(OH)_2D_3$. In most of the present studies, we were forced to use pharmacological concentrations of 24,25-$(OH)_2D_3$ or 24-oxo-25-$(OH)D_3$ to overcome the problem that radioactive [26,27-^{3}H]- or [23,24-^{3}H]-25-$(OH)D_3$ or [26,27-^{3}H]- or [23,24-^{3}H]-24,25-$(OH)_2D_3$ substrate preparations lose part of their side chain, and hence their radioactivity, on conversion to 24,25,26,27-tetranor-23-$(OH)D_3$. By using large quantities of nonradioactive substrate, we were able to generate sufficient amounts of 24,25,26,27-tetranor-23-$(OH)D_3$ for UV detection and metabolite identification. More physiological experiments of 24,25,26,27-tetranor-23-$(OH)D_3$ synthesis must await the availability of [^{3}H]-25-$(OH)D_3$ labeled in the nucleus. Meanwhile, our experiments imply that the final step in the pathway operates at superphysiological concentrations of 25-$(OH)D_3$ and 24(*R*),25-$(OH)_2D_3$, a situation that occurs in patients treated with large doses of vitamin D_3 and in hypervitaminosis D_3 in animals (Littledike & Horst, 1982), and may operate at lower concentrations of substrate also.

The 3–4-h delay before the appearance of 24-oxo-25-$(OH)D_3$, 24-oxo-23,25-$(OH)_2D_3$, and 24,25,26,27-tetranor-23-$(OH)D_3$ in the vitamin D deplete kidney perfused with 24,25-$(OH)_2D_3$ implies that the enzymes necessary for this metabolism are absent from the vitamin D deplete kidney and must be induced by the substrate 24,25-$(OH)_2D_3$ over the first 3–4 h of perfusion. Provision of 24(*R*),25-$(OH)_2D_3$ at zero time rules out the fact that the concentration of substrate [24(*R*),25-$(OH)_2D_3$] is limiting due to an absence of 24-hydroxylase activity in the vitamin D deplete kidney. Thus, it appears that these enzymes must be induced in the vitamin D deplete kidney along with 24-hydroxylase activity. This induction appears to require the presence of a vitamin D molecule since 24(*R*),25-$(OH)_2D_3$ was used here and a 3–4-h delay, presumably reflecting the need for a protein synthetic step. This is entirely analogous to the induction of the 24-hydroxylase by a variety of vitamin D molecules (Reddy et al., 1983), a process that can be abolished by protein synthesis inhibitors (Kung et al., 1983).

The finding here that the pathway from 24(*R*),25-$(OH)_2D_3$ to 24-oxo-25-$(OH)D_3$, 24-oxo-23,25-$(OH)_2D_3$, and 24,25,26,27-tetranor-23-$(OH)D_3$ is stimulated by pretreatment of the animal with large doses of vitamin D_3 implies that the enzymes of the side-chain hydroxylation pathway subsequent to the 24-hydroxylase are stimulated also. We and others

(Jones et al., 1980; Littledike et al., 1982) have shown previously that renal 24-hydroxylase of rat is stimulated by prior treatment of the animal with large doses of vitamin D_3, and the studies here avoided the influence of the 24-hydroxylase by utilizing 24(*R*),25-$(OH)_2D_3$ as the substrate.

The functional significance of the pathway culminating in 24,25,26,27-tetranor-23-$(OH)D_3$ is still in question. Though the final product isolated thus far is probably inactive due to its abbreviated side chain, it will be interesting to determine whether its precursors, 24-oxo-25-$(OH)D_3$ or 24-oxo-23,25-$(OH)_2D_3$, have biological activity. Perhaps these intermediates of the pathway represent oxidations of the side chain of the vitamin D molecule in preparation for cleavage between carbons 23 and 24.

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Structural and Conformational Analysis of Sialyloligosaccharides Using Carbon-13 Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: The analysis of the carbon-13 chemical shift data of NeuAcα(2→3)Galβ(1→4)Glc and NeuAcα(2→3)Galβ-(1→4)GlcNAc and their respective NeuAcα(2→6) isomers established distinct and different conformations of the sialic acid residue, depending on the type of anomeric linkage [α-(2→3) vs. α(2→6)]. Interactions between the NeuAc residue and the Glc or GlcNAc residue are particularly strong in the case of the α(2→6) isomers. Similar effects are observed for the larger oligosaccharides [$II^3(NeuAc)_2Lac$ and $IV^6NeuAcLcOse_4$] and even in intact glycoproteins and polysaccharides. It is proposed that the NeuAcα(2→3) isomers assume an extended conformation with the sialic residue at the end (terminal) of the oligosaccharide chain or branch. The NeuAcα(2→6) isomers are assumed to be folded back toward the inner core sugar residues.

Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy has recently emerged as a complementary method to 500-MHz ^1H NMR spectroscopy for structural elucidation of naturally occurring carbohydrate side chains of oligosaccharides (Shashkov et al., 1979; Berman, 1983; Jaques et al., 1980; Nunez et al., 1981; Messer et al., 1982), glycopeptides (Prohaska et al., 1981; Berman & Allerhand, 1981; Daman & Dill, 1983; Dijkstra et al., 1983), glycolipids (Sillerud et al., 1982; Sillerud & Yu, 1983), and glycoproteins (Dill & Allerhand, 1979; Berman et al., 1980; Berman et al., 1981; Barrett-Bee et al., 1982; Goux et al., 1982). It is well documented that glycosidases show a considerable degree of specificity toward the kind of sugar linkages encountered in a particular structure (Kobata, 1979); however, the degree of specificity thus observed is also strongly dependent on the overall carbohydrate structure (Berman & Allerhand, 1981; Kobata, 1979). This may be due to some defined structural features, as in the case of various endo-β-*N*-acetylglucosaminidases (Kobata, 1979), or it may be, to a certain degree, related to a well-defined conformational change. Such conformational effects may be the major reason that most sialidases show substrate specificity toward the $x = 3$ or $x = 6$ linkages in NeuAcα(2→x)Galβ(1→4)Glc[NAc]β1→R^1

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