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23,25-Dihydroxy-24-oxovitamin D_3: A Metabolite of Vitamin D_3 Made in the Kidney†

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ABSTRACT: Kidney homogenates of rats produced a new metabolite of 25-hydroxyvitamin D_3 which has been isolated in pure form after five column chromatographic steps. It was identified as 23,25-dihydroxy-24-oxovitamin D_3 by means of ultraviolet and infrared absorption spectrophotometry, mass spectrometry, and proton nuclear magnetic resonance spectrometry. The stereochemistry at the C-23 position is as yet unknown. 25-Hydroxy-24-oxovitamin D_3, which also has been isolated in pure form from this system, was found to be the precursor of the new metabolite in vitro. The production of the new metabolite was induced by two different methods: (a) perfusion of the kidneys with 1,25-dihydroxyvitamin D_3 contained in the perfusate and (b) injection of 1,25-dihydroxyvitamin D_3 in the intact animal. 23,25-Dihydroxy-24-oxovitamin D_3 was not biologically active in an assay for intestinal calcium transport and bone calcium mobilization in the vitamin D deficient chick at a dose level of 5.3 nmol. A metabolic pathway is proposed to describe the results; it leads from 25-hydroxyvitamin D_3 → 24(*R*),25-dihydroxyvitamin D_3 → 25-hydroxy-24-oxovitamin D_3 → 23,25-dihydroxy-24-oxovitamin D_3.

The secosteroid vitamin D_3 is known to undergo metabolic conversion before exerting its biological effects (Norman, 1979). The major circulating form of the vitamin, 25-OH-D_3,[1] is further processed by the kidney to yield 1,25$(OH)_2D_3$ or 24(*R*),25$(OH)_2D_3$. The steroid hormone-like actions of 1,25$(OH)_2D_3$ on the intestine (Tsai et al., 1972; Boyle et al., 1972), skeleton (Wong et al., 1972a,b), and a variety of other target tissues (Walters et al., 1981) are well established. In contrast, the contribution of 24(*R*),25$(OH)_2D_3$ to the spectrum of biological responses attributable to vitamin D_3 is still a controversial issue (Norman et al., 1982a,b). On the basis of experiments with a 24,24-difluorinated analogue of 25-OH-D_3, some workers have concluded that 24-hydroxylation does not play a role in the known actions of vitamin D (Miller et al., 1981; Ameenuddin et al., 1982). On the other hand, it was reported that 24(*R*),25$(OH)_2D_3$ is required for normal bone formation in the chick (Ornoy et al., 1978; Malluche et al., 1980) as well as in man (Kanis et al., 1978; Bordier et al., 1977). Also, it was shown that administration of 24(*R*),25$(OH)_2D_3$, along with 1,25$(OH)_2D_3$, was more effective in healing vitamin D deficient osteomalacia in man than 1,25$(OH)_2D_3$ alone (Bordier et al., 1978). 24(*R*),25$(OH)_2D_3$ increases intestinal absorption of calcium and phosphorus in chicks and rats (Henry et al., 1976) as well as in man (Kanis et al., 1978) and suppresses the secretion of parathyroid hormone (Canterbury et al., 1978). In addition, it was reported that 24(*R*),25$(OH)_2D_3$ is essential for normal hatchability of fertile eggs (Henry & Norman, 1978) and may stimulate sulfate incorporation into proteoglycans in isolated chondrocytes (Corvol et al., 1978).

In the chick, the further metabolic pathway of 24(*R*),25$(OH)_2D_3$ may lead to 25-OH-24-oxo-D_3 (Takasaki et al., 1981, 1982; Wichmann et al., 1981) and/or to 25,26,27-trinorvitamin D-carboxylic acid (DeLuca & Schnoes, 1979). The rate of renal synthesis of 24(*R*),25$(OH)_2D_3$ is known to be regulated by 1,25$(OH)_2D_3$ (Tanaka & DeLuca, 1974; Henry, 1979; Omdahl et al., 1980). Using isolated perfused kidneys from

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[1] Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 24(*R*),25$(OH)_2D_3$, 24(*R*),25-dihydroxyvitamin D_3; 23(*S*),25$(OH)_2D_3$, 23(*S*),25-dihydroxyvitamin D_3; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 25-OH-24-oxo-D_3, 25-hydroxy-24-oxovitamin D_3; 23,25$(OH)_2$-24-oxo-D_3, 23,25-dihydroxy-24-oxovitamin D_3; 25-OH-D_3-26,23-lactone, 25-hydroxyvitamin D_3 26,23-lactone; 23,25,26$(OH)_3D_3$, 23,25,26-trihydroxyvitamin D_3; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; $NaBH_4$, sodium borohydride; ICA, intestinal calcium absorption; BCM, bone calcium mobilization; $(Me_3Si)_3$, tris(trimethylsilyl) ether; $(Me_3Si)_2$, bis(trimethylsilyl) ether; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EtOH, ethanol; Tris, tris(hydroxymethyl)aminomethane.

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vitamin D replete rats, we found recently that exposure of the kidneys to 1,25$(OH)_2D_3$ in vitro results in increased production of 24(*R*),25$(OH)_2D_3$ as well as another vitamin D compound, which we have designated peak X (Reddy et al., 1982a). In this paper we report the isolation, identification, and biological activity of peak X and describe its metabolic pathway from 25-OH-D_3.

Experimental Procedures

Instruments. Ultraviolet absorption spectra were recorded with a Beckman Model 26 spectrophotometer (Beckman Instruments, Fullerton, CA) in 95% ethanol. Mass spectra were obtained with an AEI MS-902B spectrometer (Associated Electrical Industries, Ltd., Manchester, UK) interfaced with a PDP 11/40 computer (Digital Equipment Corp., Maynard, MA). The compound (1 μg) was applied on a direct insertion probe; all spectra were taken at 70 eV, and the source temperature was increased from 150 to 210 °C during each run. The 200-MHz proton nuclear resonance spectra were taken in $CDCl_3$ (internal standard used $CHCl_3$) with a JEOL FX-200 (Jeol Ltd., Tokyo, Japan). The Fourier transform infrared spectrum was recorded with a Nicolet 7199 (Nicolet Instruments Corp., Madison, WI) in CCl_4. High-performance liquid chromatography was carried out with a Waters Model 6000 A equipped with a detector, Model 440, monitoring UV-absorbing material at 254 nm (Waters Associates, Milford, MA). Radioactivity determinations were carried out with a Beckman LS 8000 liquid scintillation counter. Samples were dried under a stream of air and dissolved in 7 mL of toluene counting solution (20 g of butyl-PBD in 4 L of toluene).

Chemicals. 25-OH-D_3, 23(*S*),25$(OH)_2D_3$, 24(*R*),25$(OH)_2D_3$, and 24(*S*),25$(OH)_2D_3$ were kindly donated by Dr. Milan Uskokovič (Hoffmann-La Roche Co., Nutley, NJ). 25-OH-[26,27-^{3}H]-D_3 (specific radioactivity 19.2 Ci/mmol) was purchased from Amersham/Searle. 24(*R*),25$(OH)_2$-[26,27-$^3H_2$]-D_3 and 25-OH-24-oxo-[26,27-$^3H_2$]-D_3 were prepared enzymatically. The incubation procedure and the purification steps were carried out as detailed below. Kidney homogenate (1 mL) and buffer (2 mL; 50 mM Tris-HCl, pH 7.4, 3.3 mM $MgCl_2$, and 16.7 mM succinate, pH 7.4) were incubated with 25-OH-[26,27-$^3H_2$]-D_3 (0.5 μCi; 8.5 nM). The incubation was carried out for 30 min at 37 °C. Isolated and purified 24(*R*),25$(OH)_2$-[26,27-$^3H_2$]-D_3 was reincubated with the kidney homogenate by using the same incubation procedure to yield 25-OH-24-oxo-[26,27-$^3H_2$]-D_3.

Isopropyl alcohol and dichloromethane (both AR grade) were purchased from Mallinckrodt (Los Angeles, CA). Isopropyl alcohol was redistilled in glass. Hexane (UV grade, distilled in glass) was purchased from Burdick & Jackson (Muskegon, MI).

Animals. Male, albino Wistar rats weighing 250–300 g were obtained from the Hilltop Co. (Chatsworth, CA). The animals were kept in hanging wire cages and fed a normal rat chow.

Isolation of the Metabolite. First Trial. The enzymes which convert 25-OH-D_3 to peak X were induced by 1,25$(OH)_2D_3$ in vitro by the isolated kidney perfusion technique as described by Reddy et al. (1982a,b). The right kidneys of three rats were isolated and perfused for 15 min with a Krebs–Henseleit bicarbonate buffer (pH 7.4, 37 °C) containing bovine serum albumin (6 g/dL), glucose (5 mM), a mixture of L-amino acids (Rosenthal et al., 1980), and 1,25$(OH)_2D_3$ (5×10^{-8} M). Then, the perfusion of the kidneys was continued for 4 h with the same buffer without 1,25$(OH)_2D_3$ included. Subsequently, a 20% homogenate (w/v) was prepared in 10 mM Hepes buffer (pH 7.4) containing 0.2 M sucrose and 10 mM KCl. A crude mitochondrial fraction was prepared by centrifugation of the homogenate at 3000 *g* for 10 min. The pellet was transferred to a 500-mL Erlenmeyer flask containing 50 mL of incubation buffer (20 mM Hepes, pH 7.4, 100 mM KCl, 2 mM $MgSO_4$, and 20 mM succinate) and 25 mL of the 20% (w/v) homogenate. The flask was gassed for 30 s with 100% oxygen, and subsequently, the substrate 25-OH-D_3 (20 μg) dissolved in 100 μL of ethanol was added. After incubation at 37 °C for 30 min in a shaking water bath, the reaction was terminated by the addition of CH_2Cl_2–MeOH (1:2 v/v). The extraction was carried out as described by Lambert et al. (1977). The initial purification step was carried out on Sephadex LH 20 columns (0.6 × 12 cm) which were eluted with *n*-hexane–chloroform–methanol (9:1:1). The fractions containing putative 25-OH-24-oxo-D_3 (4–10 mL) and peak X (11–20 mL) were pooled separately and evaporated to dryness. Further purification of these two pooled regions was achieved by using HPLC. A μPorasil column (0.39 × 30 cm; Waters) was eluted with hexane–isopropyl alcohol (98:2 v/v) at a flow rate of 2 mL/min. Putative 25-OH-24-oxo-D_3 (eluted at 22–26 mL), peak X (46–53 mL), and 24(*R*),25$(OH)_2D_3$ (72–84 mL) were collected separately and further purified on HPLC with a reverse-phase column (μBondapak C_{18}, 0.4 × 30 cm; Waters). Putative 25-OH-24-oxo-D_3 was eluted from 20 to 25 mL and 24(*R*),25$(OH)_2D_3$ from 17 to 21 mL with water–methanol (25:75 v/v) as solvent. With water–methanol (35:65 v/v) as solvent, peak X was eluted from 28 to 46 mL. Finally, putative 25-OH-24-oxo-D_3 and peak X were rechromatographed on HPLC with the straight phase system described above. The yield of products from the incubations utilizing the homogenates of 24 kidneys was peak X (4.8 μg), 25-OH-24-oxo-D_3 (2.2 μg), and 24(*R*),25$(OH)_2D_3$ (6 μg).

Second Trial. Twenty-four rats were given 1,25$(OH)_2D_3$ (42 nmol) in 0.2 mL of 1,2-propanediol–ethanol (1:1 v/v) subcutaneously. Six hours later the animals were killed by decapitation and the kidneys removed. A crude mitochondrial fraction of a 10% homogenate (w/v) was prepared in 0.25 M sucrose. The incubation mixture contained 36 mL of 50 mM Tris-HCl (pH 7.4), 3.3 mM $MgCl_2$, and 16.7 mM succinate (pH 7.4) and 14 mL of the 10% homogenate. The substrate, 24(*R*),25$(OH)_2D_3$ (20 μg), was added in 100 μL of EtOH before the start of the incubation. 24(*R*),25$(OH)_2D_3$ was used as substrate, because, as described below, it was found to be an intermediate in the metabolic pathway leading from 25-OH-D_3 to peak X. Each 50-mL aliquot was incubated in a 125-mL Erlenmeyer flask at 37 °C for 30 min in a shaking water bath. For extraction, 4-L bottles containing 2 L of chloroform–methanol (1:1 v/v) were used. The reaction was terminated by pouring the reaction mixtures into the organic solvent (13 flasks per bottle). The chloroform extracts were dried under vacuum by rotary evaporation. The concentrated extracts were divided into three aliquots, and each was applied separately to a Sephadex LH 20 column (1.5 × 22 cm) which was equilibrated and eluted with chloroform–hexane (65:35 v/v). The fractions eluted from 25 to 150 mL were pooled and evaporated to dryness in vacuo. Subsequently, the residue was reapplied onto a single Sephadex LH 20 column (1.5 × 22 cm) which was again eluted with chloroform–hexane (65:35 v/v). The fractions eluted from 30 to 150 mL were collected. The Sephadex LH 20 columns used were calibrated before use with [^{3}H]-25-OH-D_3 (eluting from 30 to 55 mL) and [^{3}H]-24,25-$(OH)_2D_3$ (eluting from 80 to 120 mL). Further purification was achieved by high-performance liquid chromatography. A μPorasil column was equilibrated and eluted with

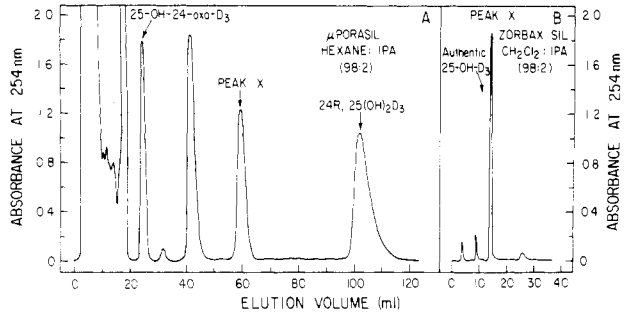

FIGURE 1: Purification of 23,25$(OH)_2$-24-oxo-D_3 (peak X) by high-performance liquid chromatography. A μPorasil column was eluted with hexane–isopropyl alcohol (98:2 v/v) at a flow rate of 2 mL/min (panel A). Subsequently peak X was rechromatographed on a Zorbax SIL column which was eluted with dichloromethane–isopropyl alcohol (98:2 v/v) at a flow rate of 1 mL/min (panel B).

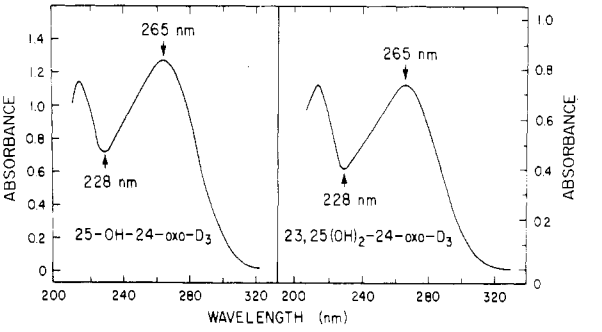

FIGURE 2: Ultraviolet spectra of isolated vitamin D metabolites 25-OH-24-oxo-D_3 (left panel) and 23,25$(OH)_2$-24-oxo-D_3 (right panel).

hexane–isopropyl alcohol (98:2 v/v). Authentic 25-OH-D_3 eluted at 22–27 mL, 23(*S*),25$(OH)_2D_3$ at 56–64 mL, and 24(*R*),25$(OH)_2D_3$ at 97–115 mL. A UV-absorbing peak, which appeared exactly in the position of 25-OH-D_3, was later determined to be 25-OH-24-oxo-D_3. Peak X was coeluted with authentic 23(*S*),25$(OH)_2D_3$ (see Figure 1A). The compounds were separately rechromatographed on a Zorbax SIL column (0.46 × 25 cm; Du Pont, Wilmington, DE) which was equilibrated and eluted with 2% isopropyl alcohol in dichloromethane (v/v). 25-OH-24-oxo-D_3 was eluted at 8–9.5 mL and peak X at 13–15 mL (see Figure 1B). The column was calibrated before use with authentic 25-OH-D_3, 23(*S*),25$(OH)_2D_3$, and 24(*R*),25$(OH)_2D_3$, which was eluted at 10–11, 35–38, and 27–30 mL, respectively. The compounds were finally purified by HPLC on a μPorasil column which was eluted with 2.5% isopropyl alcohol in hexane (v/v) as described above. The yields were 69 μg of peak X and 113 μg of 25-OH-24-oxo-D_3.

Ultraviolet and mass spectra of the metabolites produced in vitro were carried out with the compounds obtained from both the first and the second trials. NMR spectra were obtained on the material from the second trial. The respective ultraviolet and mass spectra of putative 25-OH-24-oxo-D_3 and peak X isolated during both trials were virtually identical.

Chemical Modification of the Metabolites 25-OH-24-oxo-D_3 and Peak X. (*A*) *$NaBH_4$ Reduction.* 25-OH-24-oxo-D_3 (4 μg) was incubated in 100 μL of ethanol containing 0.1% potassium hydroxide with an excess of $NaBH_4$ at room temperature for 5 h. Then 500 μL of water was added, and the compound was extracted 3 times with 500 μL of dichloromethane. The sample was dried under a stream of nitrogen and redissolved in 50 μL of 2% isopropyl alcohol in hexane. Thereafter, the compound was purified by HPLC on a μPorasil column (0.39 × 30 cm) which was eluted with the same solvents.

(*B*) *Trimethylsilylation.* The new metabolite, peak X (2 μg), the material derived from the reduction of 25-OH-24-oxo-D_3 (3 μg), and isolated 24(*R*),25$(OH)_2D_3$ (2 μg; obtained from first trial) each were dissolved in 50 μL of pyridine and reacted for 45 min at 55 °C with 50 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylsilyl chloride (Me_3SiCl). After evaporation of the solvent under nitrogen, the samples were subjected to HPLC on a 0.39 × 30 cm μPorasil column which was eluted with 2% ethyl acetate in hexane (v/v) as described by Takasaki et al. (1981). The chromatographic profile of reduced 25-OH-24-oxo-D_3 and of isolated 24(*R*),25$(OH)_2D_3$ revealed a single UV-absorbing peak eluted at 3.0–3.7 mL. In contrast, the derivatization of the new metabolite peak X resulted in the appearance of two UV-absorbing peaks on HPLC, the minor peak eluted at 4.7–5.2 mL and the major peak eluted at 15–16.5 mL. These two compounds, as well as the Me_3Si derivatives of the reduced 25-OH-24-oxo-D_3 and the isolated 24(*R*),25$(OH)_2D_3$, were collected and analyzed by mass spectrometry.

Metabolism of 25-OH-[^3H]-D_3 to ^3H-Labeled Peak X. Two rats were primed in vivo with 1,25$(OH)_2D_3$ (3.25 nmol) 6 h before sacrifice. A 10% homogenate (w/v) of the kidneys was prepared in 0.25 M sucrose. The incubation mixture consisted of 1 mL of kidney homogenate, 2 mL of the buffer described above (second isolation trial), and 36 000 dpm of either [^{3}H]-25-OH-D_3, [^{3}H]-24,25$(OH)_2D_3$, or [^{3}H]-25-OH-24-oxo-D_3. The reactions were carried out for 30 min and were then stopped by the addition of 3 volumes of chloroform–methanol (1:1 v/v). All incubations were carried out in duplicate. The pooled extracts were subjected to HPLC by using a Zorbax SIL column (0.46 × 25 cm), which was eluted with dichloromethane–isopropyl alcohol (98:2 v/v). The retention of authentic 25-OH-D_3, 24(*R*),25$(OH)_2D_3$, and 23(*S*),25$(OH)_2D_3$ was monitored by UV absorbance at 254 nm. Fractions (0.5 mL) were collected, and the radioactivity contained was determined by liquid scintillation counting of 250 μL of each fraction (see Figure 7). The radioactive peaks were rechromatographed on a μPorasil column with 2.5% isopropyl alcohol in hexane as the solvent system.

Biological Assays. Intestinal calcium absorption (ICA) and bone calcium mobilization (BCM) were measured as described by Hibberd & Norman (1969). One-day-old chicks (Pace Setter Hatcheries, Alta Loma, CA) were raised for 3 weeks on a diet devoid of vitamin D containing 0.6% calcium and 0.4% phosphorus. The animals were placed on a zero Ca^{2+} diet 72 h before assay; 24 h before assay, the birds were injected intramuscularly either with 5.3 nmol of the test compound in 0.2 mL of 1,2-propanediol–ethanol (1:1 v/v) or with vehicle. The chicks were lightly anesthetized with ether, and 4 mg of Ca^{2+} along with 1.6 μCi of $^{45}Ca^{2+}$ (New England Nuclear, Boston, MA) was placed into the duodenal loop. The animals were decapitated 30 min later, and blood was collected. The radioactivity content of the serum (0.2 mL) was measured by liquid scintillation counting to determine the amount of $^{45}Ca^{2+}$ absorbed. BCM activity was estimated from the increase of total serum calcium concentration as determined by atomic absorption spectrophotometry (Model 4000; Perkin-Elmer Corp., Norwalk, CT).

Results

The ultraviolet absorption spectra of both metabolites of vitamin D, namely, peak X and 25-OH-24-oxo-D_3, showed a λ_{max} at 265 nm and a λ_{min} at 228 nm (Figure 2), demonstrating the presence of the 5,6-*cis*-triene chromophore characteristic

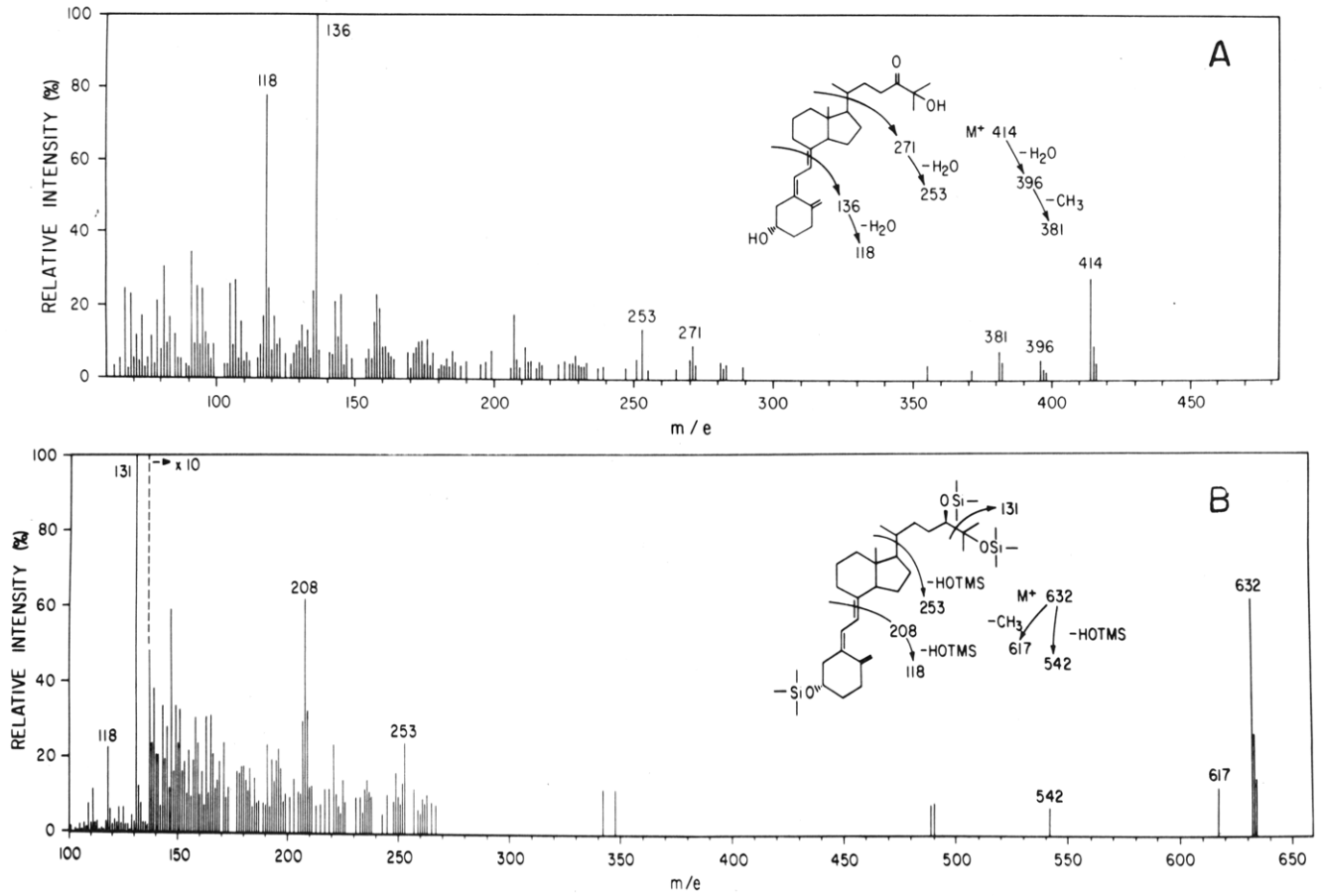

FIGURE 3: Mass spectra of isolated 25-OH-24-oxo-D_3 (panel A) and the tris(trimethylsilyl) ether derivative of 25-OH-24-oxo-D_3 which was reduced to 24,25$(OH)_2D_3$ with $NaBH_4$ before silylation (panel B).

of the D vitamins. The mass spectrum of the putative 25-OH-24-oxo-D_3 is shown in Figure 3A. Major ions, relative intensities, and structural assignments are as follows: *m/e* 414, 28, M^+; 396, 5, $M^+ - H_2O$; 381, 8, $M^+ - H_2O - CH_3$; 271, 9, M^+ – side chain; 253, 14, 271 – H_2O; 136, 100 (A ring + $C_6 + C_7)^+$; 118, 79, 136 – H_2O. The apparent molecular ion at *m/e* 414 suggests the incorporation of an oxygen atom and one degree of unsaturation in the molecule as compared with 25-OH-D_3. Characteristic ions at *m/e* 271, 253, 136, and 118 also support the presence of an unaltered vitamin D nucleus with modifications having occurred only at the side chain (Okamura et al., 1976). Treatment of the metabolite with borohydride and subsequent HPLC analysis on a μPorasil column (solvent system hexane–isopropyl alcohol, 98:2 v/v) showed that two compounds had been produced. Two overlapping peaks of equal size appeared, which could be cochromatographed with authentic 24(*R*),25$(OH)_2D_3$ and 24(*S*),25$(OH)_2D_3$, respectively. This finding suggested that the two compounds produced were the two possible epimers of 24,25$(OH)_2D_3$. Subsequent silylation of the reduced putative 25-OH-24-oxo-D_3 resulted in a product whose mass spectrum is shown in Figure 3B. Major ions, relative intensities, and structural assignments are as follows: *m/e* 632, 6, M^+; 617, 1, $M^+ - CH_3$; 542, 1, $M^+ - HOMe_3Si$; 253, 2, M^+ – side chain – $HOMe_3Si$; 208, 6, (A ring + C_6 + $C_7)^+$; 131, 100, $C_3H_6OMe_3Si^+$; 118, 22, 208 – $HOMe_3Si$. This mass spectrum was identical with the spectra of the tris(trimethylsilyl) ether derivatives of authentic 24(*R*),25$(OH)_2D_3$ as well as of 24(*R*),25$(OH)_2D_3$, isolated in the first trial (data not shown).

The 200-MHz NMR spectrum of putative 25-OH-24-oxo-D_3 (Figure 4) indicated the intact vitamin D-5,6-*cis*-triene system including the signals at δ 6.24 (H_6, d, J = 11 Hz), 6.05

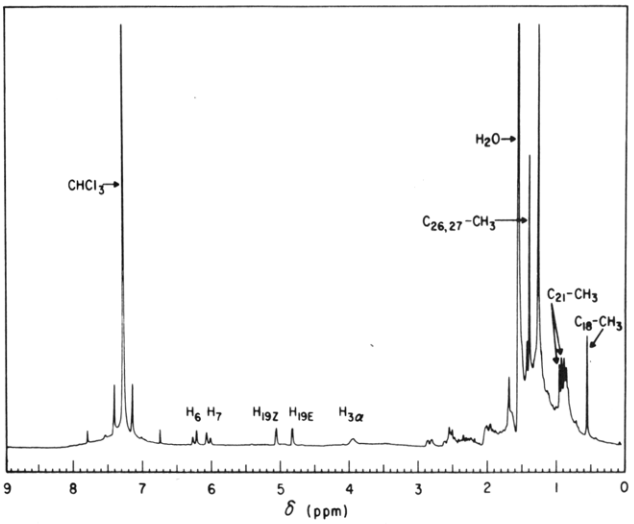

FIGURE 4: Proton nuclear magnetic resonance spectrum of 25-OH-24-oxo-D_3.

(H_7, d, J = 11 Hz), 5.06 (H_{19Z}, br s), and 4.82 (H_{19E}, br s). Further signals present are δ 3.95 ($H_{3\alpha}$, m), 0.56 (C_{18}–CH_3, s), 0.94 (C_{21}-CH_3, d, J = 6.4 Hz), and 1.39 ($C_{26,27}$-CH_3, s). In agreement with NMR data of synthetic 25-OH-24-oxo-D_3 as described by Takasaki et al. (1982), these data support the assigned structure for this vitamin D metabolite as 25-OH-24-oxo-D_3.

The mass spectrum of peak X is shown in Figure 5A. Major ions, relative intensities, and structural assignments are as follows: *m/e* 430, 22, M^+; 412, 5, $M^+ - H_2O$; 397, 7, 412 – CH_3; 372, 9, $M^+ - C_3H_6O$; 271, 7, M^+ – side chain; 253,

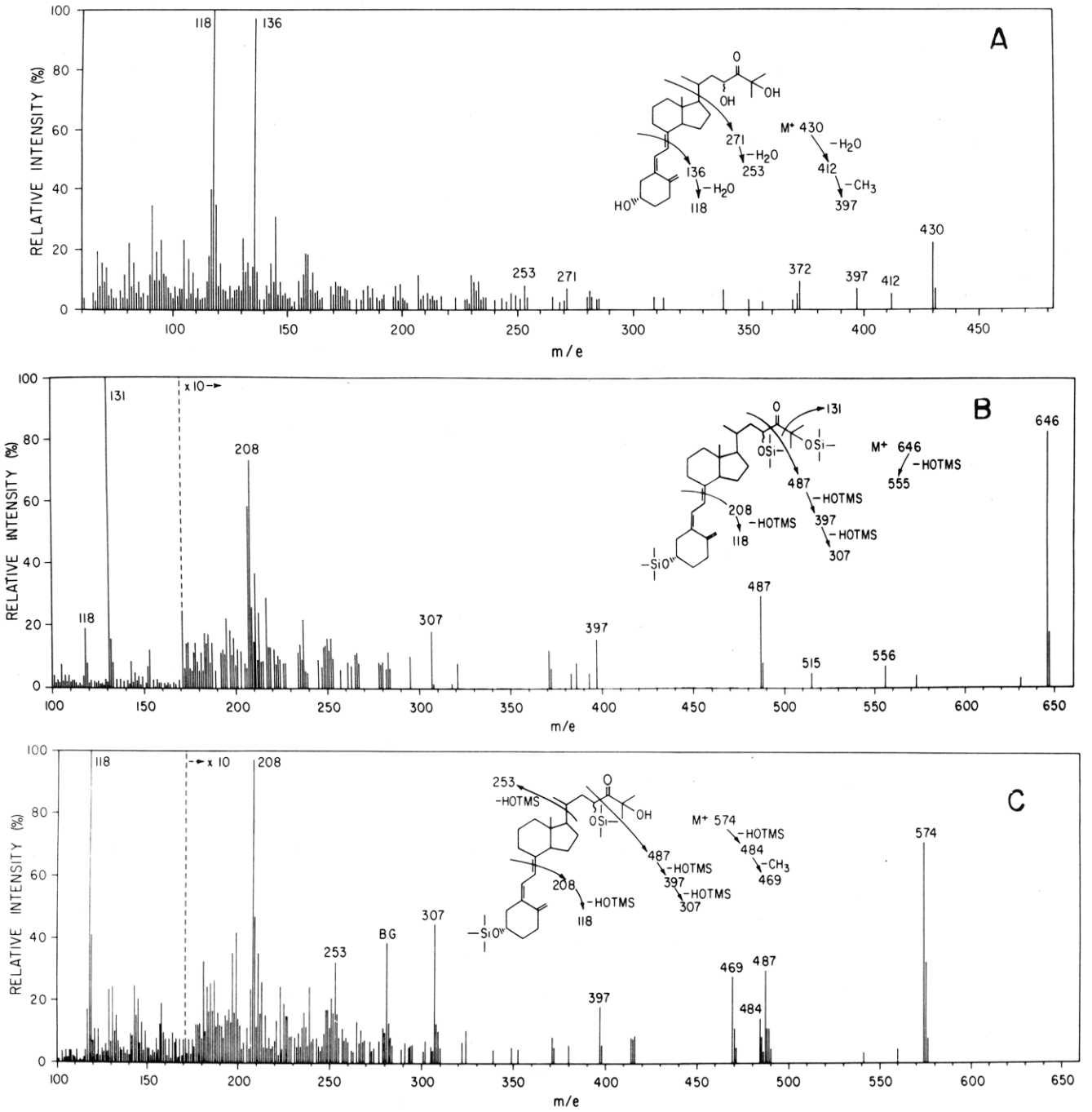

FIGURE 5: Mass spectra of isolated 23,25(OH)$_2$-24-oxo-D$_3$ and its (Me$_3$Si)$_3$ and (Me$_3$Si)$_2$ ether derivatives.

8, HOMe$_3$Si; – H$_2$O; 136, 97, (A ring + C$_6$ + C$_7$)$^+$; 118, 100, 136 – H$_2$O. Characteristic vitamin D cleavage ions at *m/e* 271, 253, 136, and 118 illustrate that the secosteroid nucleus of the molecule has remained unchanged and that, therefore, the metabolic alterations have been introduced in the side chain. The apparent molecular ion at *m/e* 430 suggests that three oxygen atoms and one degree of unsaturation are present in the side chain of the 25-hydroxylated vitamin D molecule. For localization of the hydroxyl functionalities in the metabolite, peak X was converted to a tris(trimethylsilyl) ether derivative. HPLC analysis revealed that two compounds were produced. The mass spectrum of the minor product is shown in Figure 5B. Major ions, relative intensities, and structural assignments are as follows: *m/e* 646, 8, M$^+$; 556, 1, M$^+$ – HOMe$_3$Si; 515, 1, M$^+$ – C$_3$H$_6$ – OMe$_3$Si; 487, 3, M$^+$ – C$_4$O$_2$H$_6$Me$_3$Si; 397, 2, 487 – HOMe$_3$Si; 307, 2, 397 – HOMe$_3$Si; 208, 7, (A ring + C$_6$ + C$_7$)$^+$; 131, 100, C$_3$H$_6$OMe$_3$Si$^+$; 118, 19, 208 – HOMe$_3$Si. The apparent molecular ion at *m/e* 646 demonstrates that three hydroxyl functionalities are present as trimethylsilyl ether derivatives in the molecule. The ion at *m/e* 487 strongly indicates the presence of a hydroxyl group at C-23 (Tanaka et al., 1981a; Wichmann et al., 1981) as well as that there are no further alterations below C-23. The cleavage peak at *m/e* 131 (base peak) establishes the presence of a hydroxyl group at carbon 25 and illustrates that no further modifications at carbon 26 or carbon 27 have occurred. Therefore, the structure of the new vitamin D metabolite can be proposed as 23,25(OH)$_2$-24-oxo-D$_3$.

The mass spectrum of the major product obtained after silylation of peak X is presented in Figure 5C. Major ions, relative intensities, and structural assignments are as follows: 574, 7, M$^+$; 487, 3, M$^+$ – C$_4$O$_2$H$_6$Me$_3$Si; 484, 1, 574 – HOMe$_3$Si; 469, 3, 484 – CH$_3$; 397, 2, 487 – HOMe$_3$Si; 307,

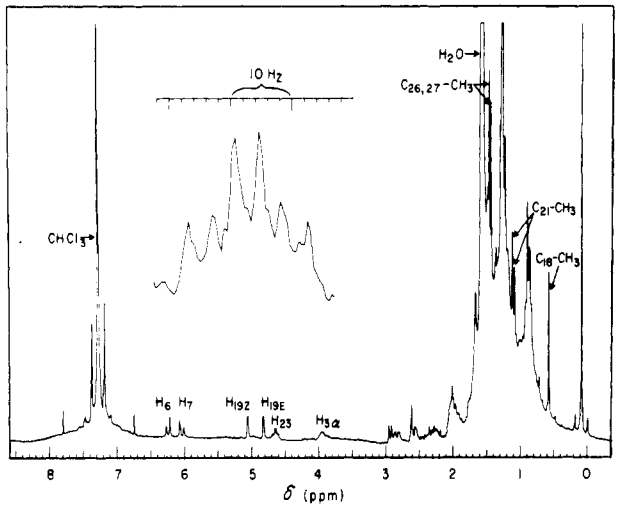

FIGURE 6: Proton nuclear magnetic resonance spectrum of 23,25-$(OH)_2$-24-oxo-D_3 (peak X). Insert shows the 4.57–4.72 ppm region representing the resonance of the C-23 proton.

4, 397 – $HOMe_3Si$; 281, 4, background; 253, 3, M^+ – side chain – $HOMe_3Si$; 208, 10, (A ring + C_6 + C_7)$^+$; 118, 100, 208 – $HOMe_3Si$. The apparent molecular ion at *m/e* 574 illustrates that two hydroxyl groups in the molecule are present in silylated form. The sequence of ions at *m/e* 487, 397, and 307 shows conclusively the presence of a hydroxyl group at C-23 and indicates that no further alterations are present below C-23. The fragmentation ion at *m/e* 208 proves the presence of the second hydroxyl group at C-3. The mass spectrum of this product suggests that 23,25$(OH)_2$-24-oxo-D_3 is present here as bis(trimethylsilyl) ether derivative, in which the hydroxyl at C-25 is not derivatized.

The 200-MHz proton NMR spectrum of peak X is presented in Figure 6. The spectrum shows signals typical of the 5,6-*cis*-triene system of vitamin D (Wing et al., 1975) including the signals at δ 6.24 (H_6, d, J = 11.3 Hz), 6.04 (H_7, d, J = 11.3 Hz), 5.06 (H_{19Z}, br s), and 4.83 (H_{19E}, br s). Other signals characteristic of a vitamin D structure were δ 3.95 ($H_{3\alpha}$, m) and 0.57 (C_{18} methyl, s). The assignment of the side chain structure as being 23,25$(OH)_2$-24-oxo-D_3 is based on three factors: (*a*) *One is the chemical shifts of the C-26 and C-27 methyl groups.* A comparison of the chemical shifts of the C-26,27 methyl groups in 25-OH-24-oxo-D_3 (δ 1.39) and 25-OH-D_3 (δ 1.22) reveals a deshielding effect of 0.17 ppm on the methyl signals upon β-carbonyl substitution at C-24. Also, by substitution of 25-OH-D_3 with a C-23 hydroxyl group [as in 23(*S*),25$(OH)_2D_3$] a deshielding by 0.08 ppm (δ 1.22 vs. δ 1.32 and 1.28) was found [NMR data of 23(*S*),25-$(OH)_2D_3$ described by Tanaka et al. (1981a) and Ikekawa et al. (1981)]. Therefore, the observed chemical shift of the C-26,27 methyl groups in the spectrum of peak X at δ 1.45 and 1.43 is consistent with a carbonyl functionality at C-24 and a hydroxyl group at C-23 in the molecule. (*b*) *Another is the presence of a signal integrating for one proton (H_{23}) at δ 4.65.* Since the C-23 proton of 23(*S*),25$(OH)_2D_3$ appears at 4.1 ppm (Ikekawa et al., 1981; Tanaka et al., 1981b) and since the effect of α-carbonyl substitution of a hydroxyl-substituted methine is deshielding by 0.5 ppm (calculated from model compounds), we conclude that the signal at δ 4.65 in the spectrum of peak X can reasonably be assigned to the C-23 proton of 23,25$(OH)_2$-24-oxo-D_3. (*c*) *The third is the multiplicity of the H_{23} signal at δ 4.65* (t of d, J = 7.5 Hz, J = 4.1 Hz). The signal can be analyzed as a triplet of doublets with the larger coupling (7.5 Hz) due to the adjacent C-22 protons and the smaller coupling (4.1 Hz) due to the hydroxyl

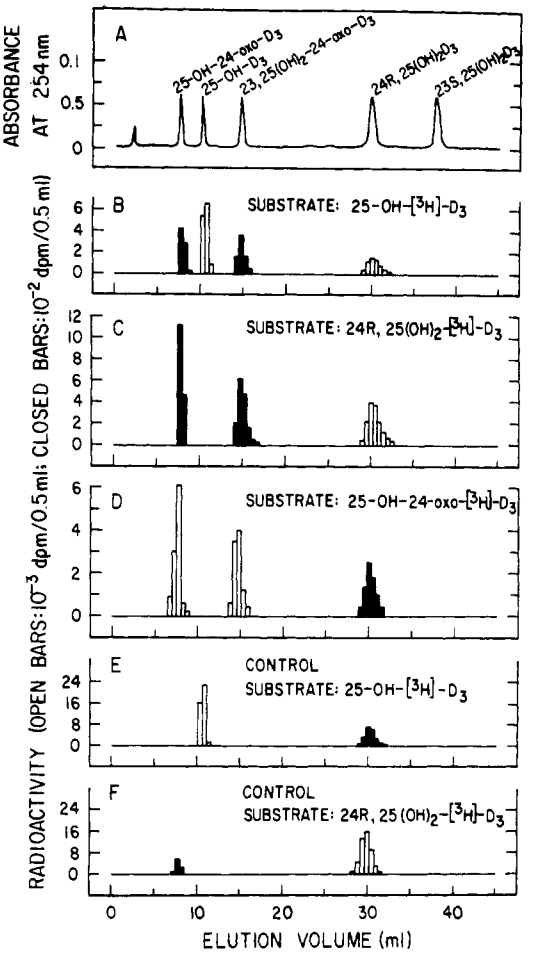

FIGURE 7: High-performance liquid chromatographic profile of metabolites of 25-OH-[26,27-$^3H_2$]-D_3 produced by rat kidney in vitro. A Zorbax SIL column was eluted withdichloromethane–isopropyl alcohol (98:2 v/v) at a flow rate of 1 mL/min. Panel A shows the retention volume of unlabeled, vitamin D metabolites. The profiles obtained in panels B, C, and D are obtained from lipid extracts of incubations with the kidneys of rats which were injected with 0.65 nmol of 1,25$(OH)_2D_3$/100 g of body weight 6 h before sacrifice. In control experiments, rats were injected with vehicle [0.2 mL of 1,2-propanediol–ethanol (1:1 v/v)]. HPLC profiles of lipid extracts of incubations with the kidneys of these not primed rats are depicted in panels E and F. The substrates used are indicated on the corresponding panels. Open bars represent 10^{-3} dpm/0.5 mL of solvent eluting from HPLC and closed bars 10^{-2} dpm/0.5 mL.

proton at C-23 under nonexchange conditions (Silverstein et al., 1981). Alternatively, it is possible that the complexity of the signal at δ 4.65 may be due to virtual coupling between H_{23} and the more remote protons at C-21 (Musher & Corey, 1962). In summary, the proton NMR spectrum of the new metabolite peak X presents strong evidence for the assigned structure as 23,25$(OH)_2$-24-oxo-D_3.

The FT-IR spectrum of peak X (in CCl_4) showed a strong absorbance at 1712 cm^{-1}, indicative of a carbonyl functionality. Therefore, the structure of the new metabolite must be (3*S*,23ξ)-(5*Z*,7*E*)-3,23,25-trihydroxy-9,10-secocholesta-5,7,10(19)-trien-24-one [23,25$(OH)_2$-24-oxo-D_3].

Metabolic Pathway from 25-OH-D_3 to 23,25$(OH)_2$-24-oxo-D_3. The results of experiments carried out to elucidate the metabolic pathway leading from 25-OH-[26,27-$^3H_2$]-D_3 to 23,25$(OH)_2$-24-oxo-[26,27-$^3H_2$]-D_3 are depicted in Figure 7. Using kidney homogenates of rats which were primed with 1,25$(OH)_2D_3$, we found that 24(*R*),25$(OH)_2$-[26,27-$^3H_2$]-D_3 was a 2-fold better substrate than 25-OH-[26,27-$^3H_2$]-D_3 at identical substrate concentrations for the production of 25-OH-24-oxo-[26,27-$^3H_2$]-D_3 and 23,25$(OH)_2$-24-oxo-[26,27-

Table I: Biological Activity Assessment of 23,25$(OH)_2$-24-oxo-D_3 in Comparison to Several Related Vitamin D Metabolites in the Chick[a]

compound	ICA (cpm of $^{45}Ca^{2+}$/0.2 mL of serum)	BCM (serum calcium, mg/100 mL)
vehicle (control)	780 ± 60	5.3 ± 0.1
23,25$(OH)_2$-24-oxo-D_3	650 ± 50[b]	5.1 ± 0.1[b]
25-OH-24-oxo-D_3	830 ± 40[b]	5.6 ± 0.3[b]
24(*R*),25$(OH)_2D_3$	1200 ± 120[c]	6.4 ± 0.5[c]
25-OH-D_3	2500 ± 120[d]	7.4 ± 0.5[d]
23(*S*),25$(OH)_2D_3$	820 ± 60[b]	5.1 ± 0.2[b]

[a] All values represent the mean ± SE; 5.3 nmol of each compound was injected intramuscularly 24 h before assay. [b] Not different from control. [c] Significantly different from control, $p < 0.01$. [d] Significantly different from control, $p < 0.001$.

$^3H_2$]-D_3 (Figure 7, panels B and C). The major metabolite of 25-OH-24-oxo-[26,27-$^3H_2$]-D_3 produced was 23,25-$(OH)_2$-24-oxo-[26,27-$^3H_2$]-D_3, and a minor compound obtained was 24(*R*),25$(OH)_2$-[26,27-$^3H_2$]-D_3 (panel D). In control experiments, where rats were injected with vehicle, only a trace amount of 25-OH-24-oxo-[26,27-$^3H_2$]-D_3 was produced from 24(*R*),25$(OH)_2$-[26,27-$^3H_2$]-D_3 by the rat kidney in vitro (Figure 7, panel F). No further metabolites besides 24(*R*),-25$(OH)_2$-[26,27-$^3H_2$]-D_3 were obtained from the incubations with 25-OH-[26,27-$^3H_2$]-D_3 as substrate (Figure 7, panel E). The radioactive peaks shown in Figure 7 were rechromatographed on HPLC by using a μPorasil column (0.39 × 30 cm) which was eluted with hexane–isopropyl alcohol (98:2 v/v). All radioactive compounds were found homogeneous and coeluted with their corresponding unlabeled compounds.

Biological Activity Assessment in the Chick. Both intestinal calcium transport and bone calcium mobilization activity were not enhanced over the control level by 23,25$(OH)_2$-24-oxo-D_3, a result which was also obtained for 25-OH-24-oxo-D_3 and 23(*S*),25$(OH)_2D_3$. 25-OH-D_3 was 5 times more active than 24(*R*),25$(OH)_2D_3$ in the assay for intestinal calcium transport activity and 2 times more effective in mobilizing calcium from bone (Table I).

Discussion

A metabolite of 25-OH-D_3, designated as peak X and previously described by Reddy et al. (1982b), has been biosynthesized in vitro from rat kidney homogenates. The enzymes which catalyze the conversion of 25-OH-D_3 to peak X were induced by exposing the kidneys to 1,25$(OH)_2D_3$ either in vitro by using the isolated kidney perfusion technique or in vivo. The structure of peak X was identified as 23,25-$(OH)_2$-24-oxo-D_3; however, the stereochemistry at C-23 is unknown. Also isolated from these incubations was 25-OH-24-oxo-D_3, a metabolite of vitamin D which had been previously isolated from incubations with chick kidney homogenates (Takasaki et al., 1981) and from the serum of chicks given high doses of vitamin D_3 (Wichmann et al., 1981). Also, Takasaki et al. (1981) reported that 25-OH-24-oxo-D_3 was produced from 25-OH-D_3 via 24(*R*),25$(OH)_2D_3$ in chick kidney homogenates. We prepared 24(*R*),25$(OH)_2$-[^{3}H]-D_3 and 25-OH-24-oxo-[^{3}H]-D_3 from incubations using kidney homogenates of rats which were primed with 1,25$(OH)_2D_3$. Subsequent reincubation of these tritiated compounds clearly demonstrated that 23,25$(OH)_2$-24-oxo-D_3 is produced from 25-OH-D_3 via 24(*R*),25$(OH)_2D_3$ and 25-OH-24-oxo-D_3. The enzyme which hydroxylates 25-OH-24-oxo-D_3 at C-23 appears to be highly specific for this substrate, because no evidence for the production of 23,25$(OH)_2D_3$ or 23,24,25$(OH)_3D_3$ was found in this system when 25-OH-D_3 and 24(*R*),25$(OH)_2D_3$, respectively, were employed as a substrate.

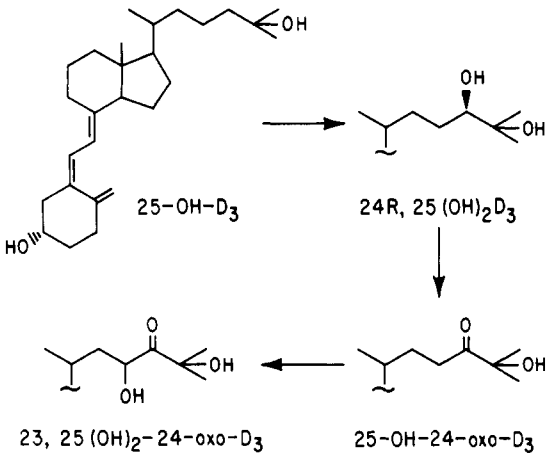

FIGURE 8: Proposed metabolic pathway from 25-OH-D_3 to 23,25-$(OH)_2$-24-oxo-D_3 in rat kidney.

The further metabolic fate of 23,25$(OH)_2$-24-oxo-D_3 is unknown. However, we have reincubated 23,25$(OH)_2$-24-oxo-[26,27-$^3H_2$]-D_3 with kidney homogenates of rats primed with 1,25$(OH)_2D_3$. Under these conditions, the amount of radioactivity in the chloroform layer decreased with time, while no further tritiated metabolite could be detected (data not shown). Therefore, we speculate that the side chain of 23,25$(OH)_2$-24-oxo-[26,27-$^3H_2$]-D_3 is cleaved to yield the C-23 acid of 25-OH-D_3 and the labeled side chain fragment. Studies are currently in progress to assess this possibility.

1,25$(OH)_2D_3$ is well-known as the most potent stimulator of the renal 25-OH-D_3-24-hydroxylase (Tanaka & DeLuca, 1974; Henry, 1980). In this report we demonstrate that 1,25$(OH)_2D_3$ also induces enzyme activity for the oxidation of 24(*R*),25$(OH)_2D_3$ at C-24 and for the subsequent hydroxylation of 25-OH-24-oxo-D_3 at C-23 to yield 23,25-$(OH)_2$-24-oxo-D_3. Thus, the administration of 1,25$(OH)_2D_3$ might mediate the coordinated induction of all three enzymatic activities.

Biological activity assessment in the vitamin D deficient chick revealed that at the dose level studied, both 25-OH-24-oxo-D_3 and 23,25$(OH)_2$-24-oxo-D_3 are devoid of activity in enhancing intestinal calcium transport as well as in mobilizing calcium from bone. Therefore, it seems conceivable that oxidation of 24(*R*),25$(OH)_2D_3$ at C-24 to yield 25-OH-24-oxo-D_3 and the subsequent hydroxylation of the molecule at C-23 is a route of inactivation of 24(*R*),25$(OH)_2D_3$. However, it is also conceivable that these further metabolites of 24(*R*),25$(OH)_2D_3$ may play an important role at the local tissue level. By systemic administration to vitamin D deficient chicks, these compounds have possibly not reached their target tissues and therefore have failed to show biological activity.

We can now observe two different patterns for modifications on the side chain of 25-OH-D_3 in kidney tissue. One pathway leads through 23,25$(OH)_2D_3$ to 23,25,26$(OH)_3D_3$ and 25-OH-D_3-26,23-lactone (Tanaka et al., 1981a,b; Ishizuka et al., 1982) and the other through 24(*R*),25$(OH)_2D_3$ to 25-OH-24-oxo-D_3 and 23,25$(OH)_2$-24-oxo-D_3 (this report; Figure 8). To establish the determinants for the hydroxylation step at C-23 vs. C-24 is an important issue for future investigations, because this step directs the subsequent metabolic fate of the principal circulating form of vitamin D, namely, 25-OH-D_3.

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Registry No. 23,25$(OH)_2$-24-oxo-D_3, 84164-55-6; 1,25$(OH)_2D_3$, 32222-06-3; 25-OH-24-oxo-D_3, 74886-61-6; 24(*R*),25$(OH)_2D_3$, 55721-11-4; 25-OH-D_3, 19356-17-3.

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