Isolation and Identification of 1,23-Dihydroxy-24,25,26,27-tetranorvitamin D_3, a New Metabolite of 1,25-Dihydroxyvitamin D_3 Produced in Rat Kidney†

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ABSTRACT: A new metabolite of vitamin D_3 was produced in vitro by perfusing rat kidneys with 1,25-dihydroxyvitamin D_3 (4×10^{-6} M). It was isolated and purified from the lipid extract of the kidney perfusate by high-performance liquid chromatography. By means of ultraviolet absorption spectrophotometry, mass spectrometry, chemical derivatization, and chemical synthesis, the new metabolite was identified as 1,23-dihydroxy-24,25,26,27-tetranorvitamin D_3. Along with the new metabolite, three other previously identified metabolites, namely, 1,24,25-trihydroxyvitamin D_3, 1,25-dihydroxy-24-oxovitamin D_3, and 1,23,25-trihydroxy-24-oxovitamin D_3, were also isolated. The new metabolite was also formed when 1,23,25-trihydroxy-24-oxovitamin D_3 was used as the substrate. Thus, the new metabolite fits into the following metabolic pathway: 1,25-dihydroxyvitamin D_3 → 1,24(*R*),25-trihydroxyvitamin D_3 → 1,25-dihydroxy-24-oxovitamin D_3 → 1,23,25-trihydroxy-24-oxovitamin D_3 → 1,23-dihydroxy-24,25,26,27-tetranorvitamin D_3. Further, we used 1α,25-dihydroxy[1β-^{3}H]vitamin D_3 in the kidney perfusion system and demonstrated 1,23-dihydroxy-24,25,26,27-tetranorvitamin D_3 as the major further metabolite of 1,25-dihydroxyvitamin D_3, circulating in the final perfusate when kidneys were perfused with 1,25-dihydroxyvitamin D_3 (6×10^{-10} M) for 4 h. The biological activity of 1,23-dihydroxy-24,25,26,27-tetranorvitamin D_3 (C-23 alcohol) and its metabolic relationship to 1-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D_3 (calcitroic acid or C-23 acid), the other previously identified side-chain cleavage metabolite of 1,25-dihydroxyvitamin D_3, are unknown and are presently undergoing investigation.

Vitamin D_3 undergoes several metabolic alterations in vivo before exerting its various biological effects. The first metabolic alteration occurs in the liver where vitamin D_3 is hydroxylated at C-25 to form 25(OH)D_3,[1] which is further hydroxylated in the kidney at C-1 or C-24 to form 1,25(OH)$_2D_3$ or 24,25(OH)$_2D_3$, respectively. Presently, 1,25(OH)$_2D_3$ is considered to be the most potent metabolite of vitamin D_3, and its steroid hormone-like actions in intestine, bone, and other target tissues are well established (Norman et al., 1982). Recently, several investigations have been directed to define the further metabolic pathways for 1,25(OH)$_2D_3$; these have led to the isolation and identification of several new intermediary metabolites that are formed before 1,25(OH)$_2D_3$ is completely inactivated and excreted. It has been demonstrated that both intestine and kidney convert 1,25(OH)$_2D_3$ into 1,24(*R*),25(OH)$_3D_3$, which is further metabolized into 1,25-(OH)$_2$-24-oxo-D_3 and 1,23,25(OH)$_3$-24-oxo-D_3 (Holick et al., 1973; Kumar et al., 1978; Mayer et al., 1983a; Napoli & Horst, 1983; Napoli & Martin, 1984; Ishizuka et al., 1984). 1,25(OH)$_3D_3$ is also metabolized into 1,23(*S*),25(OH)$_3D_3$, 1,25,26(OH)$_3D_3$ (Horst et al., 1984; Reinhardt et al., 1981; Tanaka et al., 1981), and 1,25(OH)$_2D_3$-26,23-lactone (Horst et al., 1984). Also, it has been demonstrated that 1,25(OH)$_2D_3$ undergoes side-chain cleavage between C-23 and C-24 and forms 1-OH-23-COOH-24,25,26,27-tetranor D_3 (calcitroic acid), which was recently identified as a major in vivo metabolite of 1,25(OH)$_2D_3$ (Esvelt et al., 1979). As the further metabolic pathways of 1,25(OH)$_2D_3$ are being actively investigated, it also became obvious that 25(OH)D_3 undergoes similar metabolic alterations (Reddy et al., 1982b; Mayer et al., 1983c; Jones et al., 1983, 1984; Takasaki et al., 1980, 1981, 1982; Yamada et al., 1983). As a result, the following major metabolic pathway in the kidney for 25(OH)D_3 has emerged: 25(OH)D_3 → 24,25(OH)$_2D_3$ → 25(OH)-24-oxo-D_3 → 23,25(OH)$_2$-24-oxo-D_3 → → 23(OH)-24,25,26,27-tetranor-D_3. Thus, it is now obvious that the kidney has the enzymatic capability to cleave the side chain of 25(OH)D_3 between C-23 and C-24 to form 23(OH)-24,25,26,27-tetranor-D_3. As it is now well established that both 25(OH)D_3 and 1,25(OH)$_2D_3$ follow analogous metabolic pathways, we hypothesized that 1,25(OH)$_2D_3$ may also undergo side-chain cleavage between C-23 and C-24 to form an analogous metabolite, 1,23-(OH)$_2$-24,25,26,27-tetranor-D_3. Also, we hoped that finding

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[1] Abbreviations: 25(OH)D_3, 25-hydroxyvitamin D_3; 1,25(OH)$_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25(OH)$_2D_3$, 24(*R*),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; 23(OH)-24,25,26,27-tetranor-D_3, 23-hydroxy-24,25,26,27-tetranorvitamin D_3; 25,26(OH)$_2D_3$, 25-(*S*),26-dihydroxyvitamin D_3; 1,24,25(OH)$_3D_3$, 1,24(*R*),25-trihydroxyvitamin D_3; 1,25(OH)$_2$-24-oxo-D_3, 1,25-dihydroxy-24-oxovitamin D_3; 1,23,25(OH)$_3$-24-oxo-D_3, 1,23,25-trihydroxy-24-oxovitamin D_3; 1,23-(OH)$_2$-24,25,26,27-tetranor-D_3, 1,23-dihydroxy-24,25,26,27-tetranorvitamin D_3; 1-OH-23-COOH-24,25,26,27-tetranor-D_3, 1-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D_3; 1,23,25(OH)$_3D_3$, 1,23(*S*),25-trihydroxyvitamin D_3; 1,25,26(OH)$_3D_3$, 1,25(*S*),26-trihydroxyvitamin D_3; 1,25(OH)$_2D_3$-26,23-lactone, 1,25(*R*)-dihydroxyvitamin D_3 26,23(*S*)-lactone; HPLC, high-performance liquid chromatography.

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a metabolite such as 1,23$(OH)_2$-24,25,26,27-tetranor-D_3 may help to fill the gaps in our understanding of metabolic relationship between the various recently identified further metabolites of 1,25$(OH)_2D_3$ and calcitroic acid. Therefore, the following study was undertaken.

Materials and Methods

General. Ultraviolet absorbance spectra were taken in 2-propanol with a Beckman DU 8 recording spectrophotometer. High-performance liquid chromatography (HPLC) was performed with a Waters Model 600 equipped with a detector (Model 440) to monitor UV-absorbing material at 254 nm (Waters Associates, Milford, MA). Mass spectra (70 eV) were obtained on a Hewlett-Packard 5985B mass spectrometer. Samples of metabolites (0.5–1 μg) were introduced into the ion source maintained at 200 °C via a direct-insertion probe.

Vitamin D Compounds. Crystalline 25(*S*),26$(OH)_2D_3$, 1,25$(OH)_2D_3$, 1,24(*R*),25$(OH)_3D_3$, and 1,25$(OH)_2D_3$-26,23-lactone were a generous gift from Dr. M. R. Uskokovic, Hoffmann-La Roche, Nutley, NJ. 1α,25$(OH)_2$[1β-^{3}H]D_3 (sp act. 16 Ci/mmol) was a kind gift from Dr. S. Ishizuka, Teijin Institute for Biomedical Research, Tokyo, Japan). 1α,25-$(OH)_2$[1β-^{3}H]D_3 was synthesized by the method described by Holick et al. (1980). 1α,25$(OH)_2$[26,27-^{3}H]D_3 was purchased from Amersham Corp., Arlington Heights, IL.

Solvents. All solvents were from Burdick & Jackson Laboratories, Muskegan, MI.

Animals. Male Sprague-Dawley rats (about 350 g) purchased from Zivic-Miller Laboratories, Inc., Allison Park, PA, were fed a regular rodent diet, sufficient in calcium, phosphorus, and vitamin D. In our initial perfusions with pharmacological concentration of 1,25$(OH)_2D_3$, we used the kidneys, isolated from rats that were given an intracardiac injection of 1 μg of 1,25$(OH)_2D_3$ in 50 μL of ethanol, 6 h prior to isolation of the kidney from the animal. Pretreatment of the rats with 1,25$(OH)_2D_3$ is known to increase the enzyme activity needed for the further metabolism of 1,25$(OH)_2D_3$ as indicated in our previous studies (Reddy et al., 1982b; Mayer et al., 1983a). However, in our later perfusion experiments, designed to study the metabolism of 1,25$(OH)_2D_3$ under physiological conditions, we used the kidneys straight from the rats and did not pretreat the rats with 1,25$(OH)_2D_3$.

Study of 1,25$(OH)_2D_3$ Metabolism Using the Technique of Kidney Perfusion. Kidney perfusions were performed exactly as described before in detail (Reddy et al., 1982a, 1983; Reddy & Tserng, 1986). Metabolism of 1,25$(OH)_2D_3$ was studied by introducing cold 1,25$(OH)_2D_3$ (400 nmol in 100 μL of ethanol) into 100 mL of perfusate after 5 min of stabilization, following the isolation of the kidney. Aliquots of perfusate (4 mL) were taken every 2 h, and the kidney perfusion was continued for 8 h. Out of each 4-mL perfusate sample, only 2 mL of perfusate was used for lipid extraction and HPLC analysis. The various further metabolites of 1,25$(OH)_2D_3$ and the remaining unmetabolized 1,25$(OH)_2D_3$ in the lipid extract of each 2-mL perfusate sample were analyzed and quantified by the technique of HPLC described later.

Lipid Extraction. Lipid extraction of the kidney perfusate was performed according to the procedure of Bligh and Dyer (1959) except that methylene chloride was substituted for chloroform.

High-Performance Liquid Chromatography. (1) Analysis of Lipid Extracts of Perfusate Samples for Various Further Metabolites of 1,25$(OH)_2D_3$. Lipid extracts of perfusate samples (2 mL each) obtained at 2-h intervals during the kidney perfusion experiment were analyzed by using the chromatographic conditions described in the legend for Figure 1. The elution position of each metabolite was determined via monitoring of the UV absorbance at 254 nm. A clear separation of all metabolites was obtained by a single HPLC run as shown in Figure 1. Also, at this stage, the amounts of various vitamin D metabolites present in the lipid extract of each 2 mL of perfusate were calculated by using the method described in the legend for Figure 2.

(2) Isolation and Purification of 1,23$(OH)_2$-24,25,26,27-tetranor-D_3 and the Various Previously Identified Further Metabolites of 1,25$(OH)_2D_3$ from the Final Perfusate. Bulk lipid extract obtained from the final perfusate (80 mL) was divided into four portions. Each portion of the lipid obtained from 20 mL of perfusate was subjected directly to HPLC with the same analytical Zorbax-SIL column and solvent system as described in the legend for Figure 1. Fractions of each individual metabolite from all the first HPLC runs were pooled and subjected to a second HPLC run with the same Zorbax-SIL column (4.6 mm × 25 cm) eluted with methylene chloride–2-propanol (96:4) at a flow rate of 2 mL/min. The elution volume of each metabolite is as follows: 1,25-$(OH)_2$-24-oxo-D_3 (13–15 mL); 1,23,25$(OH)_3$-24-oxo-D_3 (22–25 mL); 1,23$(OH)_2$-24,25,26,27-tetranor-D_3 (23–25 mL); 1,24,25$(OH)_3D_3$ (34–40 mL). During the second HPLC run, we noticed that each metabolite peak obtained from the first HPLC run is homogeneous and did not contain any other metabolite. Finally, each metabolite obtained from the second HPLC run was further purified with the first HPLC run twice. At this point, each metabolite was sufficiently pure for its structure identification process.

Derivatization of 1,23$(OH)_2$-24,25,26,27-tetranor-D_3. Preparation of the trimethylsilyl ether derivative of 1,23-$(OH)_2$-24,25,26,27-tetranor-D_3 was performed by treating 1 μg of the compound in 15 μL of pyridine with 10 μL of *N,O-bis*(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylsilyl chloride. After 45 min at 55 °C, reagents were removed under a stream of nitrogen, and the samples were rechromatographed on a Zorbax-SIL column eluted with hexane–ethyl acetate (99:1) at a flow rate of 2 mL/min. The purified product, eluting at 7–8 mL, was collected and subjected to mass spectrometry.

Chemical Synthesis of 1,23$(OH)_2$-24,25,26,27-tetranor-D_3. To definitely confirm the assigned structure to the biologically produced 1,23$(OH)_2$-24,25,26,27-tetranor-D_3, chemical synthesis of 1,23$(OH)_2$-24,25,26,27-tetranor-D_3 was carried out by the scheme outlined in Figure 4. The individual steps of the reaction are described in detail below.

(1) Periodate Cleavage of 1,23,25$(OH)_3$-24-oxo-D_3. 1,23,25$(OH)_3$-24-oxo-D_3 was produced biologically by perfusing kidneys with 1,24(*R*),25$(OH)_3D_3$, and its structure was confirmed by mass spectrometry and derivatization (data not shown). A total of 5 μg of 1,23,25$(OH)_3$-24-oxo-D_3 was dissolved in 15 μL of methanol and was allowed to react with 10 μL of 5% aqueous sodium metaperiodate. After 30 min at 25 °C, the reaction product was dried under nitrogen and was subjected to HPLC with Zorbax-SIL column eluted with methylene chloride–2-propanol (96:4) at a flow rate of 2 mL/min. About 2 μg of periodate cleavage product emerged at 11–13 mL. The purified periodate cleavage product (0.5 μg) was subjected to mass spectrometry and was identified as 1-OH-23-oxo-24,25,26,27-tetranor-D_3 (C-23 aldehyde) (Figure 5A). Thus, as expected periodate cleavage of 1,23,25-$(OH)_3$-24-oxo-D_3 resulted in the formation of C-23 aldehyde.

(2) Sodium Borohydride ($NaBH_4$) Reduction of 1-OH-23-oxo-24,25,26,27-tetranor-D_3. 1-OH-23-oxo-24,25,26,27-

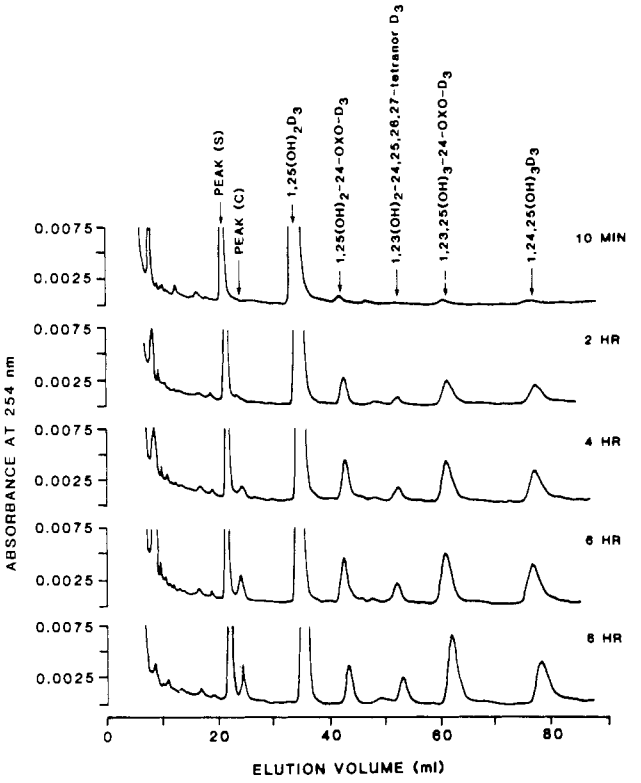

FIGURE 1: HPLC analysis of the lipid extracts of perfusate samples, obtained by perfusing a kidney for 8 h with 400 nmol of $1,25(OH)_2D_3$ in 100 mL of perfusate (4×10^{-6} M): Lipid extracts of perfusate samples (2 mL each) obtained at different times points were analyzed by HPLC under the following chromatographic conditions: Zorbax-SIL column, 25 cm × 4.6 mm; solvent, hexane–2-propanol (93:7 v/v); flow, 2 mL/min. The various metabolites of $1,25(OH)_2D_3$ were identified by monitoring their UV absorbance at 254 nm. On this HPLC system, synthetic $1,25(OH)_2D_3$-26,23-lactone elutes at 102–110 mL, and there was no significant amount of UV-absorbing material to produce a peak at the elution volume of 102–110 mL (not shown in the figure). Peak S represents cold $25(S),26(OH)_2D_3$ (0.5 μg), added to each 2-mL perfusate sample at the time of lipid extraction to monitor recovery of vitamin D metabolites. Peak C represents a non vitamin D lipid contaminant produced by the kidney. The UV absorption spectrum of the lipid material obtained from peak C exhibited a UV maximum at 243 nm and did not exhibit a spectrum characteristic for D vitamins.

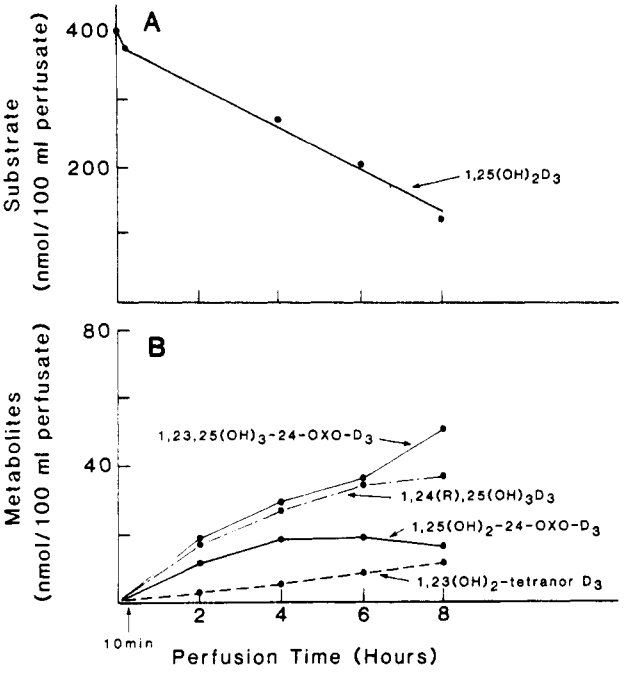

FIGURE 2: Graphic representation of results, recalculated from Figure 1. The concentration of each metabolite was quantified by measuring its peak area and comparing it with the corresponding peak area in a standard curve produced by 1 nmol of each metabolite. Panel A indicates rate of disappearance of $1,25(OH)_2D_3$ from the perfusate, and panel B indicates rate of appearance of various further metabolites of $1,25(OH)_2D_3$ into the perfusate.

tetranor-D_3 (1.5 μg) was incubated in 50 μL of ethanol containing 1 mg of $NaBH_4$. After 30 min at 25 °C, the reaction product was dried under nitrogen and was dissolved in 2 mL of 4% 2-propanol in methylene chloride. The sample was filtered through a syringe fitted with a swinney filter holder containing a 0.45-μm Teflon filter (Millipore, Milford, MA) and was concentrated under nitrogen up to a volume of 100 μL, which was then chromatographed on a Zorbax-SIL column eluted with methylene chloride–2-propanol (96:4) at a flow rate of 2 mL/min. About 1 μg of $NaBH_4$ reduction product emerged at 23–25 mL, and it comigrated with the biologically produced putative $1,23(OH)_2$-24,25,26,27-tetranor-D_3 (data not shown). The purified $NaBH_4$ reduction product was then subjected to mass spectrometry. As expected, $NaBH_4$ reduction of 1-OH-23-oxo-24,25,26,27-tetranor-D_3 (C-23 aldehyde) resulted in the formation of $1,23(OH)_2$-24,25,26,27-tetranor-D_3 (C-23 alcohol) (Figure 5B).

Study of 1,23,25(OH)$_3$-24-oxo-D$_3$ Metabolism in Perfused Rat Kidney. In order to demonstrate the conversion of $1,23,25(OH)_3$-24-oxo-D_3 into $1,23(OH)_2$-24,25,26,27-tetranor-D_3, the following experiment was performed. First, $1,23,25(OH)_3$-24-oxo-D_3 was produced biologically by perfusing rat kidneys with $1,24(R),25(OH)_3D_3$, and its structure was verified by both mass spectrometry and derivatization before it was used in this experiment. A kidney perfusion was performed with 50 mL of perfusate containing 50 nmol of $1,23,25(OH)_3$-24-oxo-D_3 for a period of 30 min. Lipid extract obtained from 10 mL of final perfusate was analyzed under the chromatographic conditions described in the legend for Figure 1. The HPLC chromatograph exhibited only two major UV-absorbing peaks (see Figure 7). The material in the less polar UV-absorbing peak was subjected to mass spectrometry for its structure identification.

Study of 1,25(OH)$_2$D$_3$ Metabolism in Perfused Rat Kidney under Physiological Conditions. To demonstrate the formation of $1,23(OH)_2$-24,25,26,27-tetranor-D_3 under physiological conditions, kidney perfusions (n = 3) were performed for 4 h with 100 mL of perfusate containing 1 μCi of $1,25(OH)_2$-[1-^{3}H]D_3 (sp act. 16 Ci/mmol). To demonstrate the unsuitability of side-chain-labeled $1,25(OH)_2D_3$ to trace $1,23(OH)_2$-24,25,26,27-tetranor-D_3, a kidney perfusion was also performed for 4 h with 100 mL of perfusate containing 1 μCi of $1,25(OH)_2$-[26,27-^{3}H]D_3 (sp act. 16 Ci/mmol). Unlike the perfusions with pharmacological concentration of $1,25(OH)_2D_3$, in all the above perfusions the rats were not pretreated with $1,25(OH)_2D_3$. Also, in order to demonstrate the lack of further metabolism and the stability of $1,25(OH)_2D_3$ in the perfusion apparatus, a control perfusion was performed by passing 1 μCi of $1,25(OH)_2$[1-^{3}H]D_3 in 100 mL of perfusate through the perfusion apparatus for 4 h without a kidney. At the end each perfusion, the lipid extract from 5 mL of final perfusate was analyzed for the various tritiated vitamin D metabolites by HPLC, using the conditions described in the legend for Figure 8.

RESULTS

Structure Identification of 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$. From Figure 1, it becomes obvious that $1,25(OH)_2D_3$ is metabolized by the isolated perfused rat kidney into four

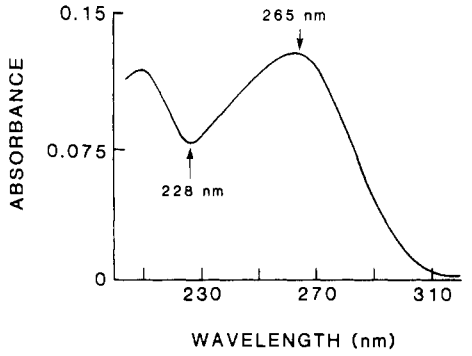

FIGURE 3: Ultraviolet absorption spectrum of $1,23(OH)_2$-24,25,26,27-tetranor-D_3.

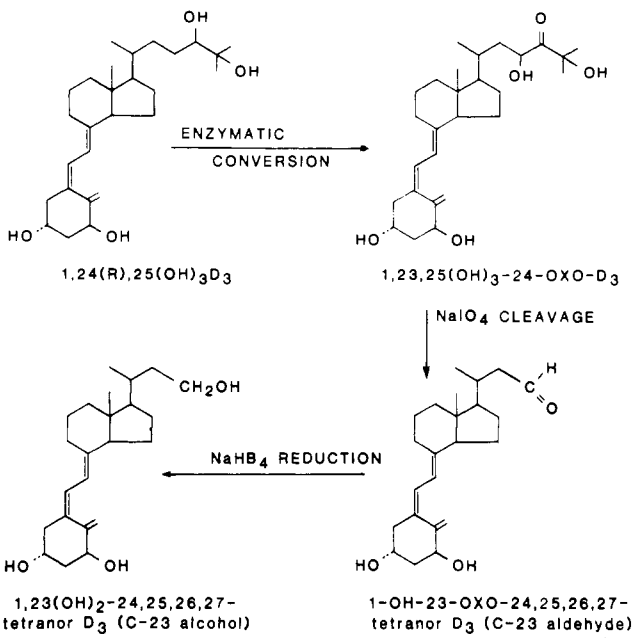

FIGURE 4: Chemical synthesis of $1,23(OH)_2$-24,25,26,27-tetranor-D_3: $1,23,25(OH)_3$-24-oxo-D_3, produced by perfusing kidneys with 1,24-(*R*),$25(OH)_3D_3$, was reacted with sodium metaperiodate ($NaIO_4$) to produce C-23 aldehyde. Authentic C-23 alcohol was finally obtained by subjecting C-23 aldehyde to sodium borohydride ($NaBH_4$) reduction.

major vitamin D metabolites as represented by the UV-absorbing peaks. Figure 2 summarizes the rate of disappearance of $1,25(OH)_2D_3$ from the perfusate and the rate of appearance of the various metabolites into the perfusate. By study of the mass spectra of all four vitamin D metabolites obtained in pure form, it became obvious that three out of the four metabolites demonstrated mass spectra identical with those of 1,24,25-$(OH)_3D_3$, $1,25(OH)_2$-24-oxo-D_3, and $1,23,25(OH)_3$-24-oxo-D_3, which have been described previously (Mayer et al., 1983a) (data not shown). The fourth metabolite is a new metabolite and is identified in this paper as $1,23(OH)_2$-24,25,26,27-tetranor-D_3, and its structure assignment is based on the following data. (a) The purified metabolite demonstrated a UV spectrum with an absorbance maximum at 265 nm and an absorbance minimum at 228 nm, indicating the presence of an intact 5,6-*cis*-triene chromophore, which is characteristic for the D vitamins (Figure 3). (b) Its mass spectrum (Figure 5C) shows the following characteristics: *m/e* 360 (M^+), 342 ($M^+ - H_2O$), 324 ($M^+ - 2H_2O$), 327 ($M^+ - H_2O - CH_3$), 309 ($M^+ - 2H_2O - CH_3$), 287 (M^+ - side chain), 269 (M^+ - side chain - H_2O), 251 (M^+ - side chain - 2 H_2O), 152 [(A-ring fragment)$^+$], and 134 [(A-ring fragment)$^+ - H_2O$]. The fragment ions at *m/e* 287, 269, 251, 152, and 134 are also

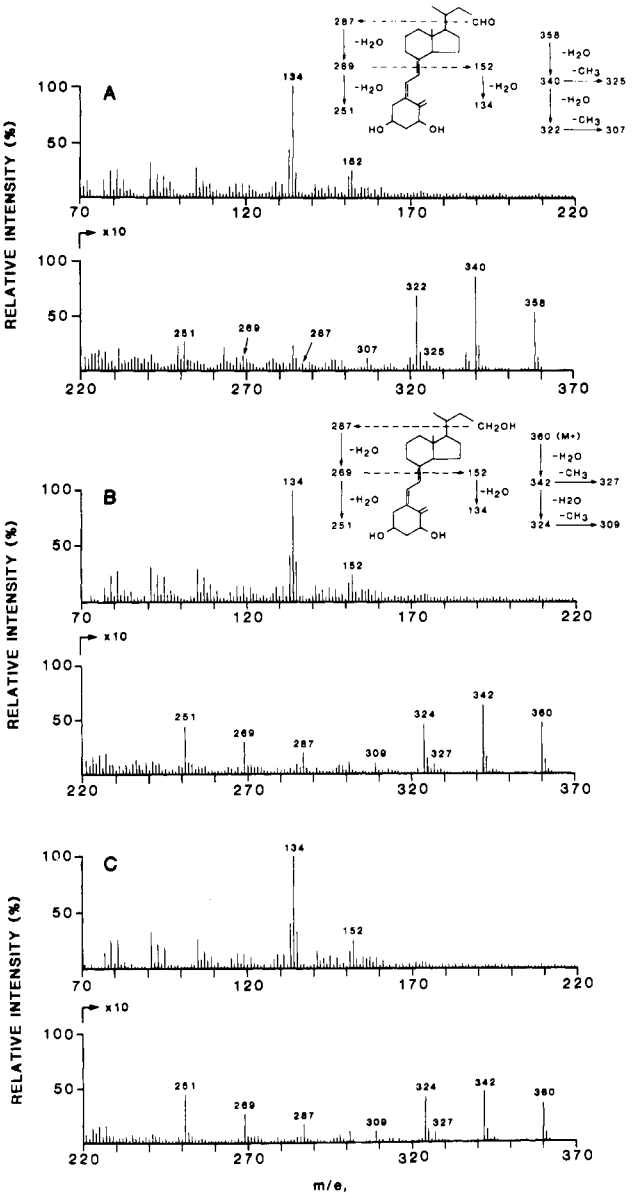

FIGURE 5: Mass spectra of (A) periodate cleavage product of $1,23,25(OH)_3$-24-oxo-D_3 (1-OH-23-oxo-24,25,26,27-tetranor-D_3 or C-23 aldehyde), (B) chemically derived authentic $1,23(OH)_2$-24,25,26,27-tetranor-D_3 produced as a result of $NaBH_4$ reduction of C-23 aldehyde, and (C) putative $1,23(OH)_2$-24,25,26,27-tetranor-D_3 produced in the kidney from $1,25(OH)_2D_3$.

present in the mass spectrum of $1,25(OH)_2D_3$ and indicate that the secosteroid nucleus of its parent $1,25(OH)_2D_3$ has remained unchanged and that all the metabolic alterations took place only on the side chain. The mass spectrum of the trimethylsilyl ether derivative of the new metabolite (Figure 6) shows the following characteristics: *m/e* 576 (M^+), 486 (M^+ - HOTMS), 396 (M^+ - 2HOTMS), 296 [(A-ring fragment)$^+$], and 206 [(A-ring fragment)$^+$ - HOTMS]. The molecular ion at *m/e* 576 indicates that the metabolite contains three hydroxyl groups. Thus, collectively, the above mass spectral data suggest that the new metabolite is formed as a result of loss of four carbons from the side chain of its parent $1,25(OH)_2D_3$ and that the shortened side chain of the new metabolite carries one hydroxyl group. (c) A definite structure identification of the new metabolite was provided by comparing the mass spectrum of the biologically produced metabolite with the mass spectrum of authentic chemically derived 1,23-$(OH)_2$-24,25,26,27-tetranor-D_3. Both the metabolites displayed identical mass spectra (Figure 5) and chromatographic

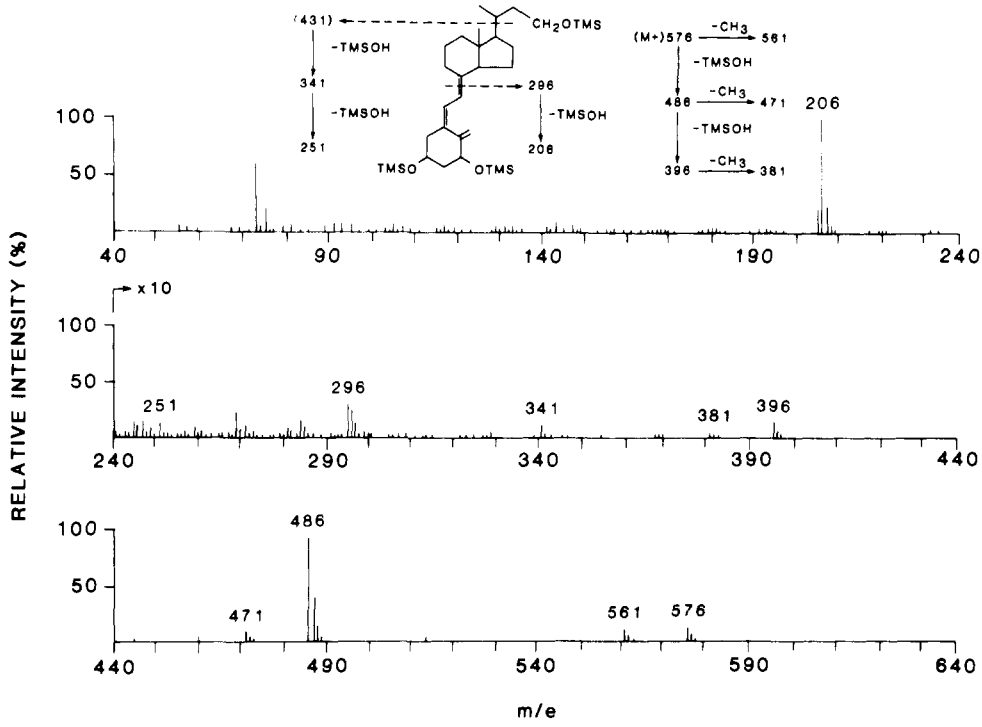

FIGURE 6: Mass spectrum of trimethylsilyl ether derivative of 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$.

mobility (data not shown). (d) Further, indirect evidence for the identification of this new metabolite also comes by comparing the mass spectral data obtained by Jones et al. (1984) for 23(OH)-24,25,26,27-tetranor-D$_3$ with the mass spectral data obtained by us for 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$. Jones et al. used 25(OH)D$_3$ as the substrate to obtain 23-(OH)-24,25,26,27-tetranor-D$_3$, which exhibited a molecular ion at *m/e* 344. We used 1,25(OH)$_2$D$_3$ as the substrate to obtain 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$, which exhibited a molecular ion at *m/e* 360 due to the presence of an extra hydroxyl group at C-1 position. Thus, the new metabolite is unequivocally identified as 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$.

Demonstration of 1,23,25(OH)$_3$-24-oxo-D$_3$ as Precursor of 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$. Figure 7 illustrates the conversion of 1,23,25(OH)$_3$-24-oxo-D$_3$ into a less polar metabolite whose chromatographic mobility and mass spectrum were similar to that of 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$ produced from 1,25(OH)$_2$D$_3$ (data not shown). Thus, we have demonstrated 1,23,25(OH)$_3$-24-oxo-D$_3$ as the precursor of 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$.

Demonstration of Formation of 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$ under Physiological Conditions. The HPLC analysis of the lipid extract of the final perfusate from one of the kidney perfusions (n = 3) with 1,25(OH)$_2$[1-^{3}H]D$_3$ is shown in Figure 8A. The concentration of each metabolite (pg/mL of perfusate, mean ± SE) is as follows: 1,24,25-(OH)$_3$D$_3$ (4.0 ± 0.4), 1,25(OH)$_2$-24-oxo-D$_3$ (4.0 ± 0.2), 1,23,25(OH)$_3$-24-oxo-D$_3$ (4.5 ± 0.7), 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$ (7.8 ± 0.6), and 1,25(OH)$_2$D$_3$-26,23-lactone (0.5 ± 0.1). Thus, it becomes obvious that 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$ is the major metabolite of 1,25(OH)$_2$D$_3$ formed in the kidneys perfused with 1,25-(OH)$_2$D$_3$ at a concentration as low as 6 × 10^{-10} M. At the same time, we also demonstrated that 1,25(OH)$_2$[26,27-^{3}H]D$_3$ is not suitable to trace 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$ due to the obvious loss of tritium from the side chain of 1,25-(OH)$_2$[26,27-^{3}H]D$_3$ during the formation of 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$ (Figure 8B). Further, we also demonstrated that no metabolism of 1,25(OH)$_2$[1-^{3}H]D$_3$ occurs in the perfusion apparatus in the absence of a kidney (Figure 8C).

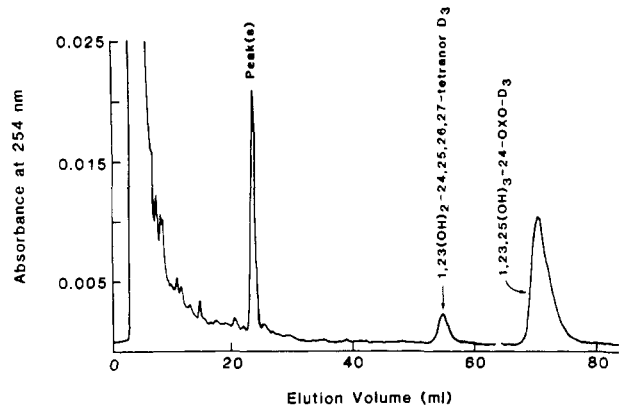

FIGURE 7: HPLC analysis of lipid extract of final perfusate sample obtained by perfusing a kidney for 30 min with 50 nmol of 1,23,25(OH)$_3$-24-oxo-D$_3$ in 50 mL of perfusate. The lipid extract of 10 mL of final perfusate sample was analyzed by HPLC under the chromatographic conditions described in the legend for Figure 1. Also, the description of peak S was the same as in the legend for Figure 1.

Discussion

This paper reports the isolation and identification of 1,23-(OH)$_2$-24,25,26,27-tetranor-D$_3$, a new renal metabolite of 1,25(OH)$_2$D$_3$. The structural assignment of the new metabolite is based on several lines of evidence as described in detail under Results. Recently, it has been established that 1,25-(OH)$_2$D$_3$ is further metabolized in both the kidney and the intestine according to the following pathway: 1,25(OH)$_2$D$_3$ → 1,24(*R*),25(OH)$_3$D$_3$ → 1,25(OH)$_2$-24-oxo-D$_3$ → 1,23,25-(OH)$_3$-24-oxo-D$_3$ (Mayer et al., 1983a; Napoli et al., 1983; Napoli & Horst, 1983). In our study, we have demonstrated that 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$ is produced in the kidney from 1,25(OH)$_2$D$_3$ along with 1,24,25(OH)$_3$D$_3$, 1,25(OH)$_2$-24-oxo-D$_3$, and 1,23,25(OH)$_3$-24-oxo-D$_3$. Also, we have provided direct evidence to suggest that 1,23,25-

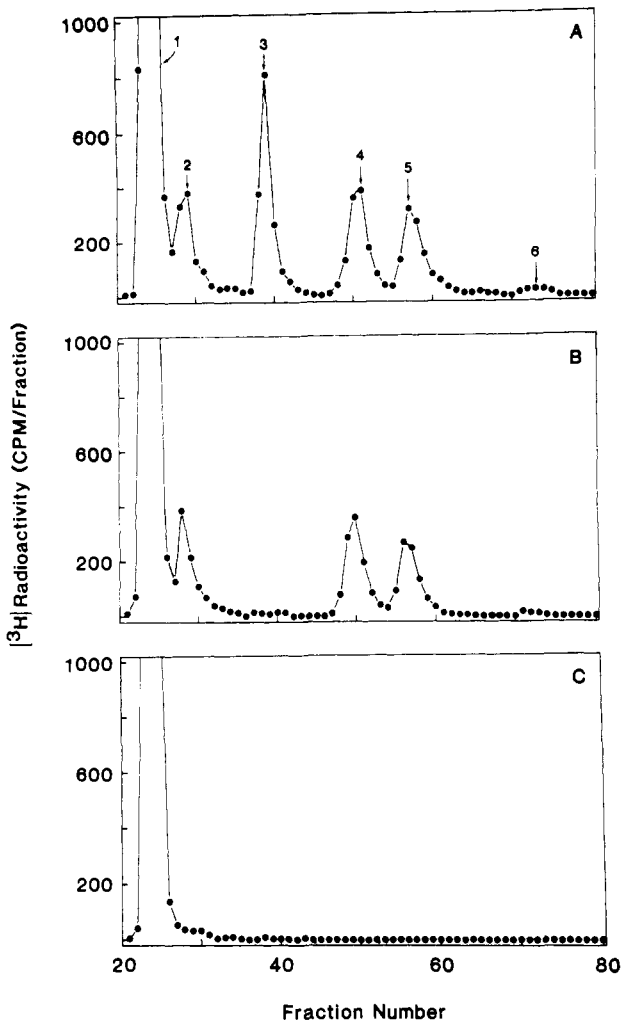

FIGURE 8: HPLC analysis of lipid extracts of final perfusate samples (5 mL each) obtained from three different perfusion experiments: (A) kidney perfusion with $1,25(OH)_2[1\text{-}^3H]D_3$ (B) kidney perfusion with $1,25(OH)_2[26,27\text{-}^3H]D_3$, and (C) control perfusion with $1,25(OH)_2[1\text{-}^3H]D_3$ in the absence of a kidney. All the perfusions were carried on with 1 μCi of the tracer (sp act. 16 Ci/mmol) in 100 mL of perfusate for a period of 4 h. The lipid extract of 5 mL of final perfusate from each perfusion experiment was analyzed by HPLC under the following chromatographic conditions: Zorbax-SIL column, 25 cm × 4.6 mm; solvent, hexane–2-propanol (94:6 v/v); flow, 2 mL/min. Fractions (2 mL) were collected, and the amount of radioactivity was determined. Unlabeled $1,25(OH)_2D_3$ (0.5 μg) was added to each 5-mL perfusate sample at the time of lipid extraction to monitor recovery of vitamin D metabolites. The elution positions of $1,25(OH)_2D_3$ metabolite standards are indicated by arrows: (1) $1,25(OH)_2D_3$; (2) $1,25(OH)_2$-24-oxo-D_3; (3) $1,23(OH)_2$-24,25,26,27-tetranor-D_3; (4) $1,23,25(OH)_3$-24-oxo-D_3; (5) $1,24,25(OH)_3D_3$; (6) $1,25(OH)_2D_3$-26,23-lactone. Also, each metabolite peak from the first HPLC remained as a single peak on a second HPLC system in which methylene chloride–2-propanol (96:4 v/v) was used as the solvent. This finding further indicates that the first HPLC system alone is sufficient to separate all the known major metabolites of $1,25(OH)_2D_3$.

$(OH)_3$-24-oxo-D_3 is the precursor of $1,23(OH)_2$-24,25,26,27-tetranor-D_3. Thus, $1,23(OH)_2$-24,25,26,27-tetranor-D_3 fits into the metabolic pathway shown in Figure 9. However, it is still not clear whether $1,23,25(OH)_3$-24-oxo-D_3 is converted into $1,23(OH)_2$-24,25,26,27-tetranor-D_3 (C-23 alcohol) directly or through the formation of 1-(OH)-23-oxo-24,25,26,27-tetranor-D_3 (C-23 aldehyde) as an intermediate.

In the beginning of our study, when $1,25(OH)_2[1\text{-}^3H]D_3$ was not available, we studied the further metabolism of $1,25(OH)_2D_3$ using pharmacological concentration of $1,25(OH)_2D_3$ in the isolated perfused rat kidney system and traced

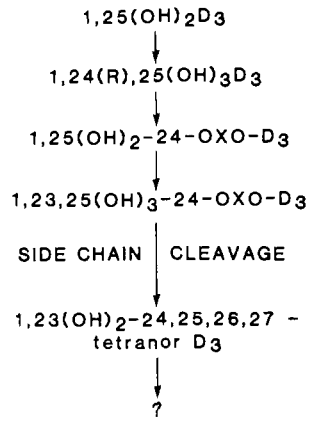

FIGURE 9: Further metabolic pathway for $1,25(OH)_2D_3$ in rat kidney.

the various vitamin D metabolites in the perfusate by subjecting the perfusate lipid extract directly to the HPLC column and by monitoring the UV absorbance (at 254 nm) of the lipids eluting out of the HPLC column. Thus, we were able to trace $1,23(OH)_2$-24,25,26,27-tetranor-D_3 even though it has lost a portion of its side chain. Also, in these perfusion experiments, the rats were pretreated with $1,25(OH)_2D_3$. The phenomenon of $1,25(OH)_2D_3$ enhancing the activity of the enzymes involved in its own further metabolism was well established (Mayer et al., 1983a; Napoli et al., 1983; Napoli & Martin, 1984). Thus, in our initial attempts to isolate $1,23(OH)_2$-24,25,26,27-tetranor-D_3, we not only used pharmacological concentration of $1,25(OH)_2D_3$ in the perfusate but also used the kidneys, isolated from the rats that were pretreated with $1,25(OH)_2D_3$. Later, when $1,25(OH)_2[1\text{-}^3H]D_3$ became available, we demonstrated $1,23(OH)_2$-24,25,26,27-tetranor-D_3 as the major further metabolite of $1,25(OH)_2D_3$, circulating in the perfusate when the kidneys were perfused with $1,25(OH)_2D_3$ at a substrate concentration of 6×10^{-10} M. In these perfusion experiments, the rats were not pretreated with $1,25(OH)_2D_3$. Thus, we demonstrated the formation of $1,23(OH)_2$-24,25,26,27-tetranor-D_3 under physiological conditions.

At present, it is apparent that the metabolism of $1,25(OH)_2D_3$ in both the kidney and the intestine occurs through two major metabolic pathways. One pathway is initiated by C-24 hydroxylation and proceeds further as shown in Figure 9 (Mayer et al., 1983a; Napoli et al., 1983). The other pathway is initiated by C-23 and C-26 hydroxylations and finally results in the formation of $1,25(OH)_2D_3$-26,23-lactone (Ishizuka et al., 1984; Horst et al., 1984). In our kidney perfusion experiments, we did not find significant accumulation of $1,25(OH)_2D_3$-26,23-lactone in the final perfusate when the kidneys were perfused with either pharmacological or physiological concentration of $1,25(OH)_2D_3$. The reasons for the lack of accumulation of $1,25(OH)_2D_3$-26,23-lactone in the kidney perfusate can be due to its reduced production or its rapid further metabolism into a hitherto unidentified polar metabolite(s). Therefore, more studies are needed before we can come to a conclusion about the relative importance of the two aforementioned metabolic pathways.

Earlier studies by Kumar et al. (1976) and Esvelt et al. (1979) resulted in the isolation and identification of 1-OH-23-COOH-24,25,26,27-tetranor-D_3 (calcitroic acid) as a major side-chain cleavage metabolite of $1,25(OH)_2D_3$. The sites for the formation of calcitroic acid were thought to be the intestine and the liver. Kidney was also suspected to be another site for the formation of calcitroic acid but was not definitely established (Esvelt et al., 1981). Our demonstration of the formation of $1,23(OH)_2$-24,25,26,27-tetranor-D_3 in the kidney

definitely indicates that the kidney has the enzymatic capability to produce side-chain cleavage metabolites from $1,25(OH)_2D_3$. Also, our study provided direct evidence to suggest the formation of highly polar, water-soluble metabolite(s) from $1,25(OH)_2D_3$ along with the other less polar, lipid-soluble metabolites. In the perfusion experiments with $1,25(OH)_2$-[1-^{3}H]D_3, it was noted that only about 65% of the total radioactivity in the perfusate was extractable into the methylene chloride layer while the rest of the radioactivity remained in the water/methanol layer. In the control perfusion without the kidney, more than 95% of the total radioactivity in the perfusate was extractable into the methylene chloride layer. This finding definitely indicates that there is formation of hitherto unidentified, highly polar, water-soluble metabolite(s) as the further metabolism of $1,25(OH)_2D_3$ progresses in the kidney. Further studies are needed to find out the chemical nature of the polar metabolite(s) in the water/methanol layer.

While the functional role of the various further metabolites of $1,25(OH)_2D_3$ is not yet known with certainty, it seems likely that they may represent a means of inactivation of the highly biologically active $1,25(OH)_2D_3$. From the studies by Mayer et al. the biological activity of the various further metabolites of $1,25(OH)_2D_3$ seems to be less active in terms of calcium and phosphorus absorption by the gut when compared to their parent hormone (Mayer et al., 1983b). However, recent studies strongly indicate that $1,25(OH)_2D_3$ possesses several unique functions other than maintaining calcium and phosphorus homeostasis (Norman et al., 1982; Bell, 1985). For example, it was found that $1,25(OH)_2D_3$ suppresses the growth and proliferation of both murine and human myeloid leukemic cells (Abe et al., 1981; Miyaura et al., 1981, 1982). $1,25-(OH)_2D_3$ is also known to mediate differentiation of human promyelocytic leukemic cells (HL-60) into monocyte-macrophages (Tanaka et al., 1982, 1983). Thus, $1,25(OH)_2D_3$ seems to have a fundamental role in cell differentiation and growth. Hence, it is highly likely that the further metabolites of $1,25(OH)_2D_3$ may still retain some of the unique functions of $1,25(OH)_2D_3$. Therefore, careful functional studies using the new intermediary metabolites such as $1,23(OH)_2$-24,25,26,27-tetranor-D_3 will help to obtain a better insight into the understanding of the multiple functional roles of $1,25-(OH)_2D_3$. Also, it is important to study the further metabolic fate of $1,23(OH)_2$-24,25,26,27-tetranor-D_3 (C-23 alcohol) especially to understand its metabolic relationship to calcitroic acid (C-23 acid). Such studies are currently under investigation.

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Registry No. BSTFA, 25561-30-2; D_3, 67-97-0; $1,25-(OH)_2D_3$, 32222-06-3; $1,25-(OH)_2$-24-oxo-D_3, 76338-50-6; $1,24,25-(OH)_3D_3$, 50648-94-7; $1,23,25-(OH)_3$-24-oxo-D_3, 87147-48-6; $1,24(R),25-(OH)_3D_3$, 56142-94-0; C-23 aldehyde, 105282-55-1; C-23 alcohol, 97903-37-2; C-23 alcohol (Me_3Si ether derivative), 105282-54-0.

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Free Energy Couplings between Ligand Binding and Subunit Association in Hemoglobin Are of First Order

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ABSTRACT: The calculations presented in a recent paper [Johnson, M. L. (1986) *Biochemistry 25*, 791–797] to the effect that the free energy couplings between oxygen binding and subunit association in hemoglobin A can be of either first or second order are examined. The fitting of the experimental data to a system with second-order couplings carried out by Johnson belongs to a tetramer in which, in contradistinction to hemoglobin A, oxygen binding promotes subunit association.

The analysis of the relations between the binding of ligands to oligomeric proteins and the binding of the monomers to each other within the aggregate may be done by postulating the existence of standard free energy couplings between the two kinds of equilibria involved: protein–protein and protein–ligand associations (Weber, 1972).

The total standard free energy of binding of 4 mol of oxygen to four noninteracting, i.e., independent, subunits is that of the reaction $4M + 4X \rightarrow 4MX$, where M symbolizes the subunit and X the ligand, oxygen in this case. I designate this total free energy as $dG°(sub)$. The standard free energy of binding of 4 mol of oxygen to the tetramer is that of the reaction $T + 4X \rightarrow TX_4$, and I designate this as $dG°(tet)$. In the absence of any coupling between the free energy of subunit interaction and that of oxygen binding, we must have

$$dG°(sub) = dG°(tet) \quad (1)$$

If these two free energies differ from each other, there are two possibilities. The first case is

$$|dG°(sub)| > |dG°(tet)| \quad (2)$$

where the quantities between bars denote absolute values. In this case, the defect in the free energy of binding by the tetramer is the energy employed in overcoming binding constraints that reside at the boundary between subunits. As a consequence, the binding of the ligand promotes oligomer dissociation. The second case is

$$|dG°(sub)| < |dG°(tet)| \quad (3)$$

This case is the opposite of the preceding one. The free energy of ligand binding by the tetramer is augmented by a contribution from increased subunit interaction: Ligand binding promotes association.

Any model proposed for the coupling of oxygen binding to subunit interaction in hemoglobin A should be one that obeys relation 2 as it has been known, for at least 15 years, that oxygen binding promotes subunit dissociation.

I have introduced the distinction between free energy couplings of different orders that I designate as first order, second order, etc. (Weber, 1982). Further, I have concluded (Weber, 1984) that the coupling between the free energy of subunit interaction and that of oxygen binding in hemoglobin A is, unequivocally, of first order.

In a recent paper Johnson (1986) purports to show that second-order couplings, as well as those of first order, can account for the experimental values of the free energies of the four successive additions of oxygen to hemoglobin. A detailed analysis of the orders of coupling is not necessary for our present purpose, and I refer the reader to the original papers (Weber, 1982, 1984) for further information. The calculations that are supposed to demonstrate that both first- and second-order couplings apply to hemoglobin A equally well are shown in Tables III and IV of Johnson's paper. The procedure used in the calculations is essentially that employed by Weber (1982), with the difference that in the calculations by Johnson the free energies of binding of O_2 by the isolated chains are treated as further adjustable parameters while Weber used the experimentally determined values. This difference is unimportant as the free energy conservation relations 1–3 apply in either case. Johnson subjected two sets of experimental data to similar analysis, and as they yield completely concordant results, it is only necessary to examine one set alone. I shall take the one shown in the first two columns of Table III of his paper. The value of $dG°(sub)$ of eq 1–3 is obtained by adding twice the values given for binding by the α and β chains ($\delta_{4\alpha}$ and $\delta_{4\beta}$ of the table), while the value of $dG°(tet)$ results from addition of the four free energies of binding by the tetramer ($\Delta G_{41}'$ to $\Delta G_{44}'$ of the table). The results of these operations, expressed in kcal/mol, are as follows:

	$dG°(sub)$	$dG°(tet)$	difference
first order	−33.48	−27.31	−6.17
second order	−20.80	−27.30	+6.50

The first-order results ($|dG°(sub)| > |dG°(tet)|$) correspond

0006-2960/87/0426-0331$01.50/0