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Isolation and Identification of 1,24,25-Trihydroxyvitamin D_2, 1,24,25,28-Tetrahydroxyvitamin D_2, and 1,24,25,26-Tetrahydroxyvitamin D_2: New Metabolites of 1,25-Dihydroxyvitamin D_2 Produced in Rat Kidney†

G. Satyanarayana Reddy*
Department of Pediatrics, Case Western Reserve University at Cleveland Metropolitan General Hospital, Cleveland, Ohio 44109
Kou-Yi Tserng
Department of Pharmacology, Case Western Reserve University and Veterans Administration Medical Center, Cleveland, Ohio 44106
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ABSTRACT: Three new metabolites of vitamin D_2 were produced in vitro by perfusing isolated rat kidneys with 1,25-dihydroxyvitamin D_2. They were isolated and purified from the kidney perfusate by the techniques of methanol–methylene chloride lipid extraction and high-performance liquid chromatography. By means of ultraviolet absorption spectrophotometry, mass spectrometry, and specific chemical reactions, the metabolites were identified as 1,24,25-trihydroxyvitamin D_2, 1,24,25,28-tetrahydroxyvitamin D_2, and 1,24,25,26-tetrahydroxyvitamin D_2. Both 1,24,25,28-tetrahydroxyvitamin D_2 and 1,24,25,26-tetrahydroxyvitamin D_2 were also produced when a kidney was perfused with 1,24,25-trihydroxyvitamin D_2. Thus, it becomes clear that 1,25-dihydroxyvitamin D_2 is first hydroxylated at C-24 to form 1,24,25-trihydroxyvitamin D_2, which is then further hydroxylated at C-28 and C-26 to form 1,24,25,28-tetrahydroxyvitamin D_2 and 1,24,25,26-tetrahydroxyvitamin D_2, respectively. From several recent studies, it has been well established that 1,25-dihydroxyvitamin D_3 is converted into various further metabolites in the kidney as a result of chemical reactions such as C-23, C-24, and C-26 hydroxylations, C-24 ketonization, and C-23:C-26 lactonization. From our study it is obvious that 1,25-dihydroxyvitamin D_2 does not undergo all of the aforementioned chemical reactions except C-24 and C-26 hydroxylations. Also, our study indicates that C-28 hydroxylation plays a significant role in the further metabolism of 1,25-dihydroxyvitamin D_2. Thus, for the first time, we describe a novel further metabolic pathway for 1,25-dihydroxyvitamin D_2 in a mammalian kidney.

It has been known that there are species differences in the biological activity of ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). For example, vitamin D_2 is about 10 times less biologically active in avian species when compared to vitamin D_3 (Chen & Bosmann, 1964). On the other hand, it is being assumed that both vitamins have similar biological activity in mammals. Recent studies, however, demonstrated that mammals also show differences in the biological activity of the two vitamins (Hunt et al., 1972; Sjoden et al., 1985; Tjellesen et al., 1985a,b). There may be several reasons for the above observations. One of the reasons may be the differences in the further metabolism of these vitamins (Horst et al., 1982; Napoli & Horst, 1985). It is already well established that both vitamins undergo hydroxylations at C-25 (in liver) and C-1 (in kidney) to form 1,25-dihydroxyvitamin D_2 [1,25$(OH)_2D_2$][1] and 1,25-dihydroxyvitamin D_2 [1,25$(OH)_2D_3$], respectively (Jones et al., 1975; Norman et al., 1982). Also, it became obvious that the further metabolism of 1,25-$(OH)_2D_3$ in both kidney and intestine is mainly due to the chemical alterations of its side chain (Napoli et al., 1983; Napoli & Horst, 1983; Mayer et al., 1983; Horst et al., 1984; Ishizuka et al., 1984). Because of the structural differences between the side chains of the two vitamins (the side chain of vitamin D_2 has an extra methyl group at C-24 and a double bond between C-22 and C-23 when compared to the side chain of vitamin D_3), we hypothesized that the side-chain metabolism of 1,25$(OH)_2D_2$ differs from that of 1,25$(OH)_2D_3$. In this investigation, we studied the further metabolism of 1,25-

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* Address correspondence to this author at the Department of Pediatrics, Cleveland Metropolitan General Hospital.

[1] Abbreviations: 24(OH)D_2, 24-hydroxyvitamin D_2; 25(OH)D_2, 25-hydroxyvitamin D_2; 24,25$(OH)_2D_2$, 24(*R*),25-dihydroxyvitamin D_2; 1,24,25$(OH)_3D_2$, 1,24,25-trihydroxyvitamin D_2; 1,24,25,28$(OH)_4D_2$, 1,24,25,28-tetrahydroxyvitamin D_2; 1,24,25,26$(OH)_4D_2$, 1,24,25,26-tetrahydroxyvitamin D_2; 1(OH)-24-keto-25,26,27-trinor-D_2, 1-hydroxy-24-keto-25,26,27-trinorvitamin D_2; 1,25$(OH)_2$-24-keto-28-nor-D_2, 1,25-dihydroxy-24-keto-28-norvitamin D_2; 1,24$(OH)_2$-25-keto-26-nor-D_2, 1,24-dihydroxy-25-keto-26-norvitamin D_2; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; HPLC, high-performance liquid chromatography; Me_3Si, trimethylsilyl; $NaIO_4$, sodium metaperiodate.

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$(OH)_2D_2$ using the technique of isolated perfused rat kidney and described a novel further metabolic pathway for 1,25-$(OH)_2D_2$ in a mammalian kidney. The results of our study, for the first time, indicate that there are definite differences in the side-chain metabolism of 1,25$(OH)_2D_2$ and 1,25-$(OH)_2D_3$.

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 5985 B 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$(OH)_2D_3$ and 1,25$(OH)_2D_2$ were a generous gift from Dr. Milan Uskokovic (Hoffmann-La-Roche, Inc., Nutley, NJ).

Solvents. All solvents were purchased from Burdick & Jackson Laboratories, Muskega, 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 previous studies, we demonstrated that the increase in the activity of the enzymes involved in the further metabolism of 1,25$(OH)_2D_3$ can be induced in the kidneys by pretreating the rats with 1,25$(OH)_2D_3$ (Reddy et al., 1982b, 1986; Mayer et al., 1983). Therefore, in an analogous fashion in this study the rats were given an intracardiac injection of 1 μg of 1,25$(OH)_2D_2$ in 50 μL of ethanol 6 h prior to isolation of the kidney from the animal to increase the activity of enzymes responsible for the further metabolism of 1,25$(OH)_2D_2$.

Study of 1,25$(OH)_2D_2$ Metabolism Using the Technique of Kidney Perfusion. Kidney perfusions were performed as described before in detail (reddy et al., 1982a, 1983). Metabolism of 1,25$(OH)_2D_2$ was studied by introducing cold 1,25$(OH)_2D_2$ (400 mmol in 100 μL of ethanol) into 100 mL of perfusate after a 5-min stabilization period, 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 new metabolites of 1,25$(OH)_2D_2$ and the remaining unmetabolized 1,25$(OH)_2D_2$ in the lipid extract of each 2-mL perfusate sample were analyzed and quantified by the technique of HPLC described later. A control perfusion was performed with cold 1,25$(OH)_2D_2$ (400 nmol in 100 μL of ethanol) in 100 mL of perfusate. Aliquots of perfusate (2 mL) were taken every 2 h while the perfusate was pumped through the perfusion apparatus for 8 h in the absence of a kidney. The lipid extract of each 2-mL perfusate sample was analyzed under the same HPLC conditions used for the above experiment.

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

High-Performance Liquid Chromatography. (A) Analysis of Lipid Extracts of Perfusate Samples for Various New Metabolites of 1,25$(OH)_2D_2$. Lipid extracts of perfusate samples (2 mL each) obtained at 2-h intervals from the above kidney perfusion experiments (with or without kidney) were analyzed by the technique of HPLC. A clear separation of all the metabolites of 1,25$(OH)_2D_2$ was obtained by a single HPLC run under the chromatographic conditions described in detail in the legend for Figure 1. The amounts of unmetabolized 1,25$(OH)_2D_2$ and each new metabolite present in the lipid extract of each 2-mL perfusate sample were quantified by comparing the peak area of each metabolite with the corresponding peak area in a standard curve, produced with 1 nmol of each new metabolite. Nonradioactive 25(*S*),26-$(OH)_2D_3$ (0.5 μg) was added as an internal standard to each 2-mL perfusate sample before lipid extraction to monitor recovery of vitamin D_2 metabolites, and thus, appropriate corrections were made for the metabolite losses that might have occurred during the procedures of lipid extraction and HPLC.

(B) Isolation and Purification of New Metabolites of 1,25$(OH)_2D_2$ from the Final Perfusate. Bulk lipid extract obtained from the final perfusate of 300 mL, belonging to three kidney perfusions, was divided into 10 portions. Each lipid portion was then subjected directly to HPLC under the same chromatographic conditions described in the legend for Figure 1 without overloading the column with lipid. Fractions of each individual metabolite from all the first 10 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 (94:6) at a flow rate of 2 mL/min. The elution volume of each metabolite was as follows: 1,24,25-$(OH)_3D_2$, 12–14 mL; 1,24,25,28$(OH)_4D_2$, 30–36 mL; 1,24,25,26$(OH)_4D_2$, 40–48 mL. Each metabolite obtained from the second HPLC run was rechromatographed twice with the first HPLC run [6% 2-propanol in hexane for 1,24,25-$(OH)_3D_3$ and 10% 2-propanol in hexane for the tetrahydroxy metabolites]. At this time, the purity of each metabolite was adequate for its structure identification process.

Chemical Modification of New Metabolites of 1,25-$(OH)_2D_2$. (A) Periodate Oxidation. The susceptibility of each metabolite to periodate oxidation was tested in order to locate the exact position of the hydroxyl groups in each new metabolite. It is well-known that $NaIO_4$ cleaves the carbon bond between two carbons when either both carbons bear two hydroxyl groups or one carbon bears a hydroxyl group and the other bears a keto group. Each new metabolite (1.0 μg each) was dissolved in 15 μL of methanol and was allowed to react with 10 μL of 5% aqueous sodium metaperiodate ($NaIO_4$). All the reactions were carried out at room temperature (25 °C). The appropriate incubation period and the HPLC system used to produce and isolate the periodate cleavage products of the three new metabolites are described in detail in the legend for Figure 3.

(B) Trimethylsilylation. Preparation of trimethylsilyl ether derivatives of all three new metabolites of 1,25$(OH)_2D_2$ were performed by treating 1 μg of each metabolite 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 elution volume of the trimethylsilyl ether derivative of each metabolite was as follows: 1,24,25$(OH)_3D_2$, 17–20 mL; 1,24,25,28$(OH)_4D_2$, 10–12 mL; 1,24,25,26$(OH)_4D_2$, 13–15 mL. Mass spectra of all the trimethylsilyl ether derivatives indicated that the C-24 hydroxyl group of all the three new metabolites did not undergo derivatization with the mild incubation conditions used in our study. This phenomenon seems to be unique to all the C-24 hydroxylated vitamin D_2 metabolites as the C-24 hydroxyl

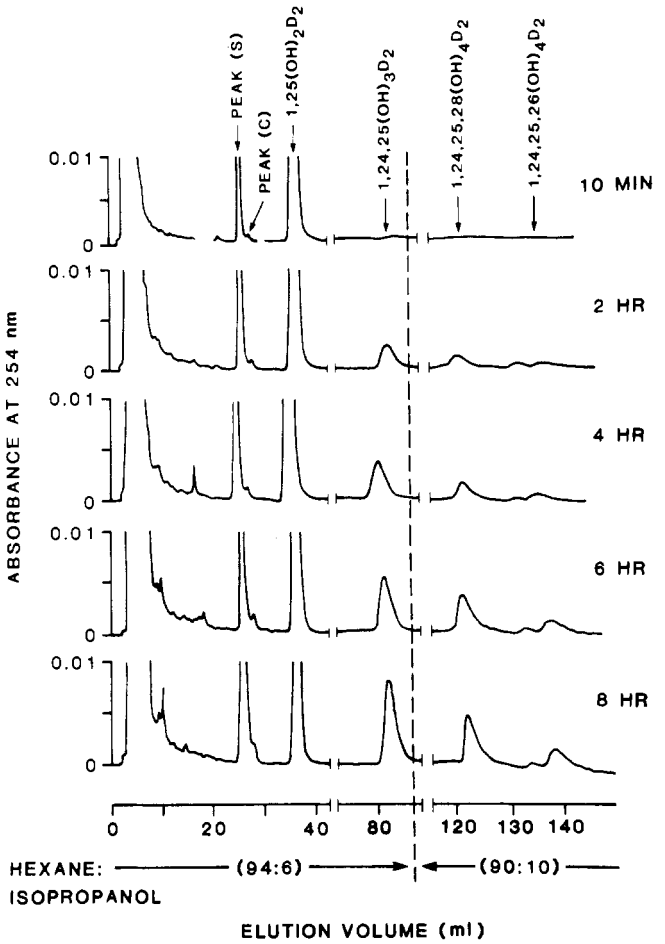

FIGURE 1: HPLC analysis of lipid extracts of perfusate samples, obtained by perfusing a kidney for 8 h with 400 nmol of $1,25(OH)_2D_2$ in 100 mL of perfusate (4×10^{-6} M): Lipid extracts of perfusate samples (2 mL each) obtained at different time points were analyzed by HPLC on a Zorbax-SIL column (25 cm × 4.6 mm). The column was first eluted with hexane–2-propanol (94:6) until $1,24,25(OH)_3D_2$ was eluted out of the column. Then, the first solvent system was changed to a second solvent system, hexane–2-propanol (90:10), to elute the tetrahydroxy metabolites of vitamin D_2 out of the column. The various metabolites of $1,25(OH)_2D_2$ were identified by monitoring their UV absorbance at 254 nm. 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 in general. 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.

group is sterically hindered due to the presence of the C-28 methyl group at C-24.

Study of 1,24,25(OH)$_3$D$_2$ Metabolism by the Perfused Kidney. In order to study the further metabolism of $1,24,25(OH)_3D_2$, a kidney perfusion was performed with 100 nmol of $1,24,25(OH)_3D_2$ in 50 mL of perfusate for a period of 4 h. The $1,24,25(OH)_3D_2$ used in this experiment was produced by perfusing rat kidneys with $1,25(OH)_2D_2$ and was purified from the perfusate as described above. A control kidney perfusion was also performed at the same time by perfusing a kidney with 100 nmol of $1,25(OH)_2D_2$ in 50 mL of perfusate for a period of 4 h. Lipid extracts obtained from 4 mL of final perfusate of both kidney perfusions were analyzed under the HPLC conditions described in the legend for Figure 7.

RESULTS

Metabolism of 1,25(OH)$_2$D$_2$ by the Perfused Rat Kidney. From Figure 1, it becomes obvious that $1,25(OH)_2D_2$ is metabolized by the isolated perfused rat kidney into three major vitamin D_2 metabolites as represented by the UV-absorbing peaks. Figure 2 summarizes the rate of disappearance of $1,25(OH)_2D_2$ from the perfusate and the rate of appearance of the three new metabolites into the perfusate. The amounts of $1,24,25(OH)_3D_2$, $1,24,25,28(OH)_4D_2$, and 1,24,25,26-

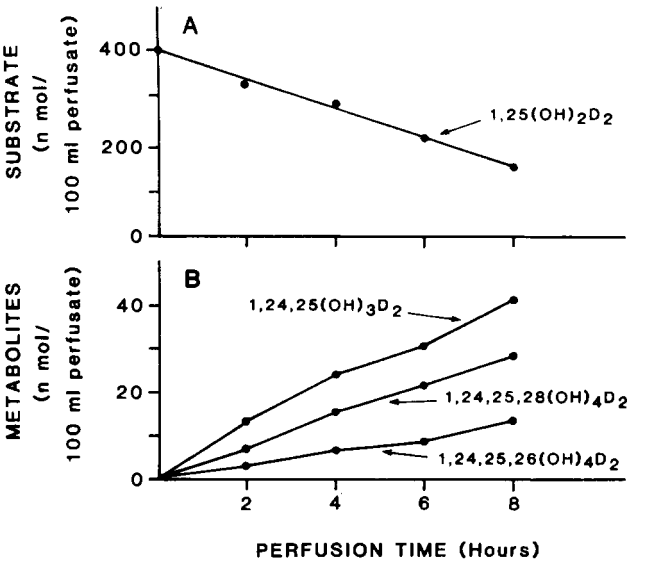

FIGURE 2: Graphic representation of results, recalculated from Figure 1. The concentration of each metabolite was quantified by measuring its peak area and comparing it with the corresponding peak area in a standard curve produced by 1 nmol of each metabolite. Panel A indicates rate of disappearance of $1,25(OH)_2D_2$ from the perfusate, and panel B indicates rate of appearance of various metabolites of $1,25(OH)_2D_2$ into the perfusate.

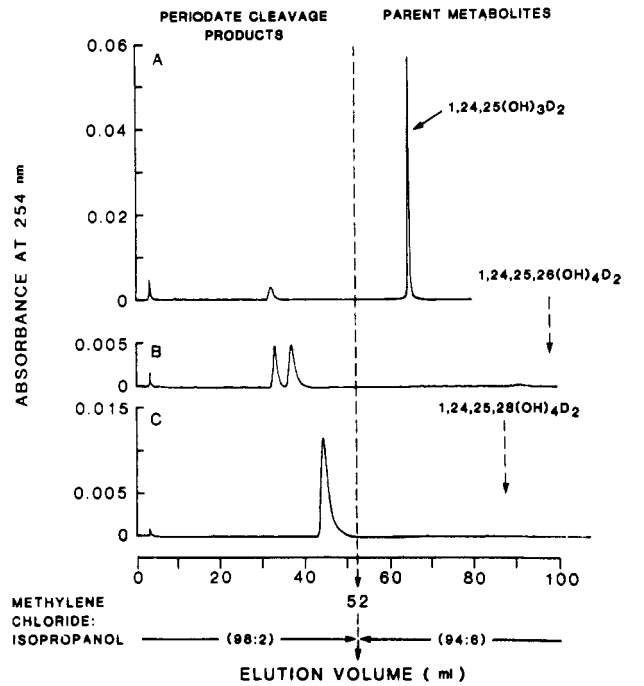

FIGURE 3: HPLC analysis of reaction product, obtained by treating 1 μg of each new metabolite with sodium metaperiodate for 5 min: $1,24,25(OH)_3D_2$ (panel A); $1,24,25,26(OH)_4D_2$ (panel B); $1,24,25,28(OH)_4D_2$ (panel C). HPLC was performed on a Zorbax-SIL column (25 cm × 4.6 mm). The column was first eluted with methyolene chloride–2-propanol (98:2) at a flow rate of 2 mL/min until the periodate cleavage product(s) of each metabolite eluted out of the column. Then, the solvent was switched to methylene chloride–2-propanol (94:6) at the same flow rate to elute the unreacted parent metabolites. Arrows indicate the elution position of the parent metabolites.

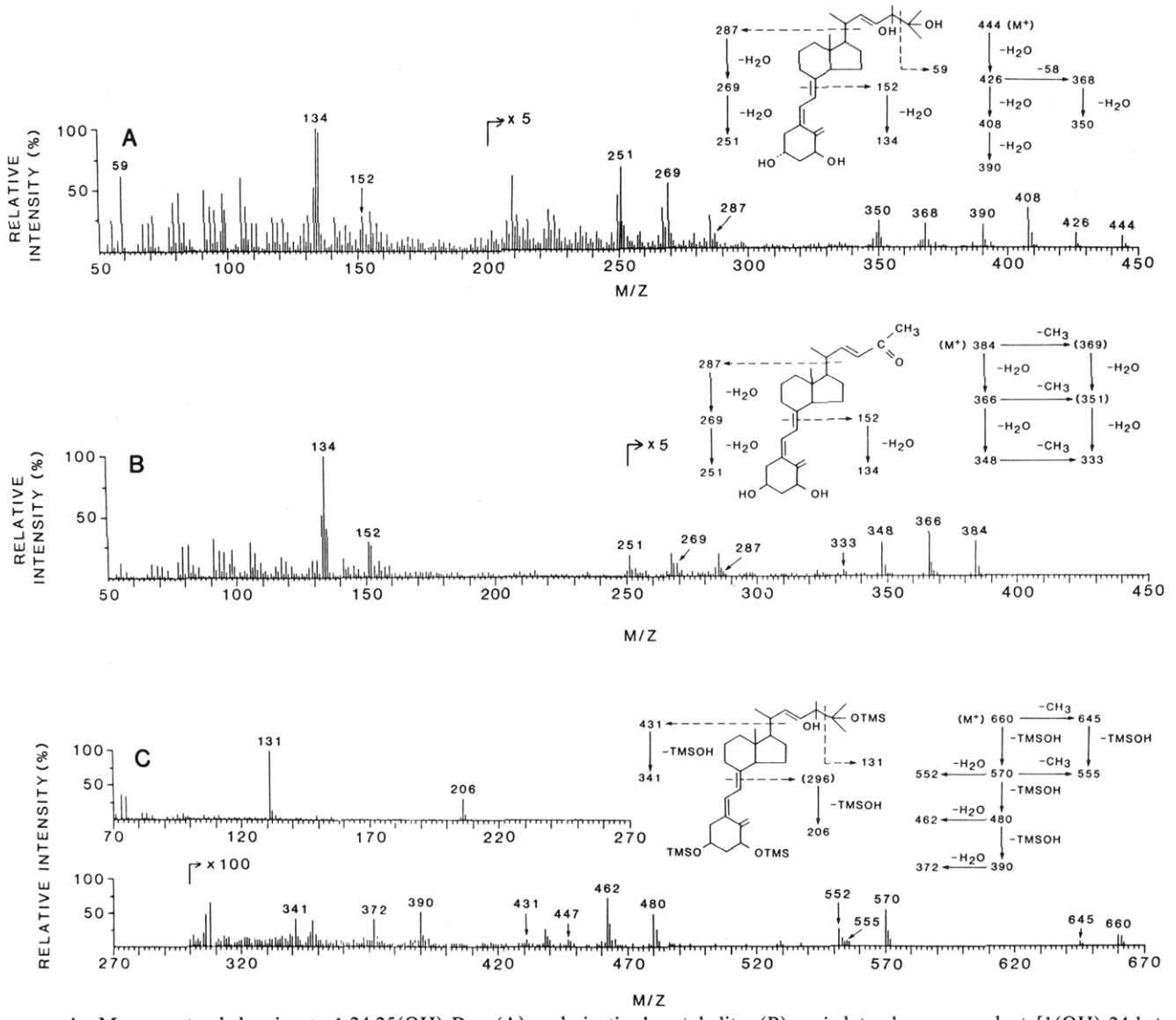

FIGURE 4: Mass spectra belonging to $1,24,25(OH)_3D_2$: (A) underivatized metabolite; (B) periodate cleavage product [1(OH)-24-keto-25,26,27-trinor-D_2]; (C) $(Me_3Si)_3$ derivative.

$(OH)_4D_2$ appearing into the kidney perfusate are in the ratio of about 3:2:1 (Figure 2B). There was no further metabolism or loss of $1,25(OH)_2D_2$ from the perfusate when a perfusion was performed without a kidney for a period of 8 h (data not shown).

Differences in Susceptibility of New Metabolites to Periodate Oxidation. All three new metabolites were susceptible to periodate oxidation. However, during an incubation period of 5 min, only 5% of $1,24,25(OH)_3D_2$ was converted into its corresponding periodate cleavage product whereas both $1,24,25,28(OH)_4D_2$ and $1,24,25,26(OH)_4D_2$ were completely converted into their corresponding periodate cleavage product(s) (Figure 3). Therefore, in order to produce 1-(OH)-24-keto-25,26,27-trinor-D_2 in a sufficient quantity, we had to increase the incubation period to 1 h (data not shown). The differences in the susceptibility of the metabolites to periodate oxidation can be explained on the basis of differences in the exposure of the vicinal diol in each metabolite to $NaIO_4$. The vicinal diol at C-24 and C-25 in $1,24,25(OH)_3D_2$ is not readily susceptible to periodate oxidation as it is sterically hindered by C-28, C-27, and C-26 methyl groups. The vicinal diol at C-24 and C-28 in $1,24,25,28(OH)_4D_2$ and the vicinal diol at C-25 and C-26 in $1,24,25,26(OH)_4D_2$ are not sterically hindred and, hence, are highly susceptible to periodate oxidation.

Structure Identification of New Metabolites of 1,25-$(OH)_2D_2$. A correct structure assignment to each further metabolite of $1,25(OH)_2D_2$ was achieved by the techniques of ultraviolet absorption spectrophotometry, mass spectrometry, and specific chemical reactions.

(A) UV Spectral Findings. All the new metabolites purified from the kidney perfusate exhibited UV spectra (data not shown) with an absorbance maximum at 265 nm and an absorbance minimum at 228 nm. This finding indicated that all the metabolites contained an intact 5,6-*cis*-triene chromophore. *cis*-triene chromophore.

(B) Mass Spectral Findings, Indicating That All New Metabolites Are Formed as a Result of Changes Occurring on the Side Chain of Their Parent 1,25$(OH)_2D_2$. The mass spectra of all the metabolites (Figures 4A, 5A, and 6A) exhibit a peak at m/z 287, which is due to the side-chain cleavage from the main steroid molecule (C-17/C-20 cleavage). The peaks at m/z 269 and 251 are a result of two sequential losses of water from the peak at m/z 287. The cleavage between carbons 7 and 8 results in the peak at m/z 152, which represents the A ring plus the carbon 6 and 7 fragment. Loss of water from the peak at m/z 152 results in the base peak at m/z 134. Collectively, the peaks at m/z 287, 269, 251, 152, and 134 in all the new metabolites indicate that the secosteroid

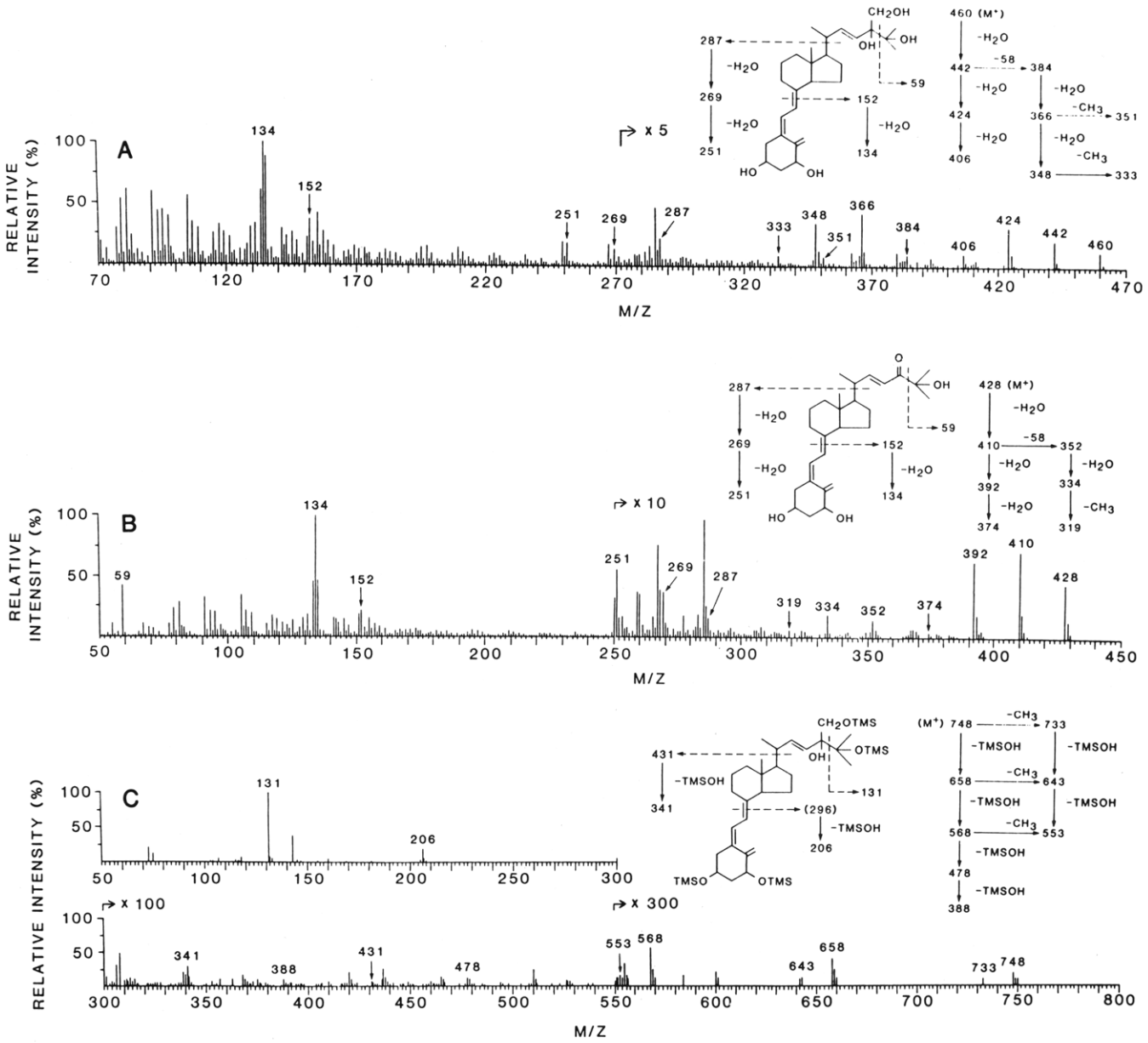

FIGURE 5: Mass spectra belonging to 1,24,25,28(OH)$_4$D$_2$: (A) underivatized metabolite; (B) periodate cleavage product [1,25(OH)$_2$-24-keto-28-nor-D$_2$]; (C) (Me$_3$Si)$_4$ derivative.

nucleus of their parent 1,25(OH)$_2$D$_2$ has remained unchanged. This finding indicates that all the new metabolites are formed as a result of changes occurring only on the side chain of their parent 1,25(OH)$_2$D$_2$. A definite structure assignment of each new metabolite is as follows.

(*a*) *Structure Identification of 1,24,25(OH)$_3$D$_2$.* The molecular ion at *m/z* 444 (M^+) in the mass spectrum (Figure 4A) suggests that this new metabolite is a trihydroxy metabolite of vitamin D$_2$. Therefore, it can be concluded that there is an additional hydroxyl group on the side chain of this new metabolite when compared to its parent 1,25(OH)$_2$D$_2$. The position of the extra hydroxyl group is located as follows: The mass spectrum exhibits a peak at *m/z* 59 and a peak at *m/z* 368 formed as a result of elimination of 58 mass units from the peak at *m/z* 426. This phenomenon is a McLafferty-type rearrangement, which is common in unsaturated alcohols (Budzikiewicz et al., 1967a). This finding and the base peak at *m/z* 131 (corresponding to $C_3H_6OSiMe_3^+$) in the mass spectrum of the trimethylsilyl ether derivative (Figure 4C) indicate that the new metabolite contains an intact C-25 hydroxyl group with no hydroxylations occurring on C-26 or C-27. Also, this metabolite is susceptible to periodate oxidation, and the structure of the periodate cleavage product is identified as 1(OH)-24-keto-25,26,27-trinor-D$_2$ (Figure 4B). This finding suggests that the metabolite contains a vicinal diol. Therefore, the extra hydroxyl group on the side chain of this new metabolite has to be on one of the carbons adjacent to the C-25 hydroxyl group. As we have already demonstrated that no hydroxylations occurred at carbons C-26 and C-27, it can be concluded that the extra hydroxyl group is at C-24. Collectively, from the above data, this new metabolite can be identified as 1,24,25(OH)$_3$D$_2$.

(*b*) *Structure Identification of 1,24,25,28(OH)$_4$D$_2$.* The molecular ion at *m/z* 460 (M^+) in the mass spectrum (Figure 5A) suggests that this new metabolite is a tetrahydroxy metabolite of vitamin D$_2$. Therefore, it can be concluded that there are two additional hydroxyl groups on the side chain of this new metabolite when compared to its parent 1,25(OH)$_2$D$_2$. The exact positions of the two extra hydroxyl groups are located as follows: The mass spectrum exhibits a peak at *m/z* 59 (87% of the base peak; present in the original mass spectrum but not shown in Figure 5A), and the peak at *m/z* 384

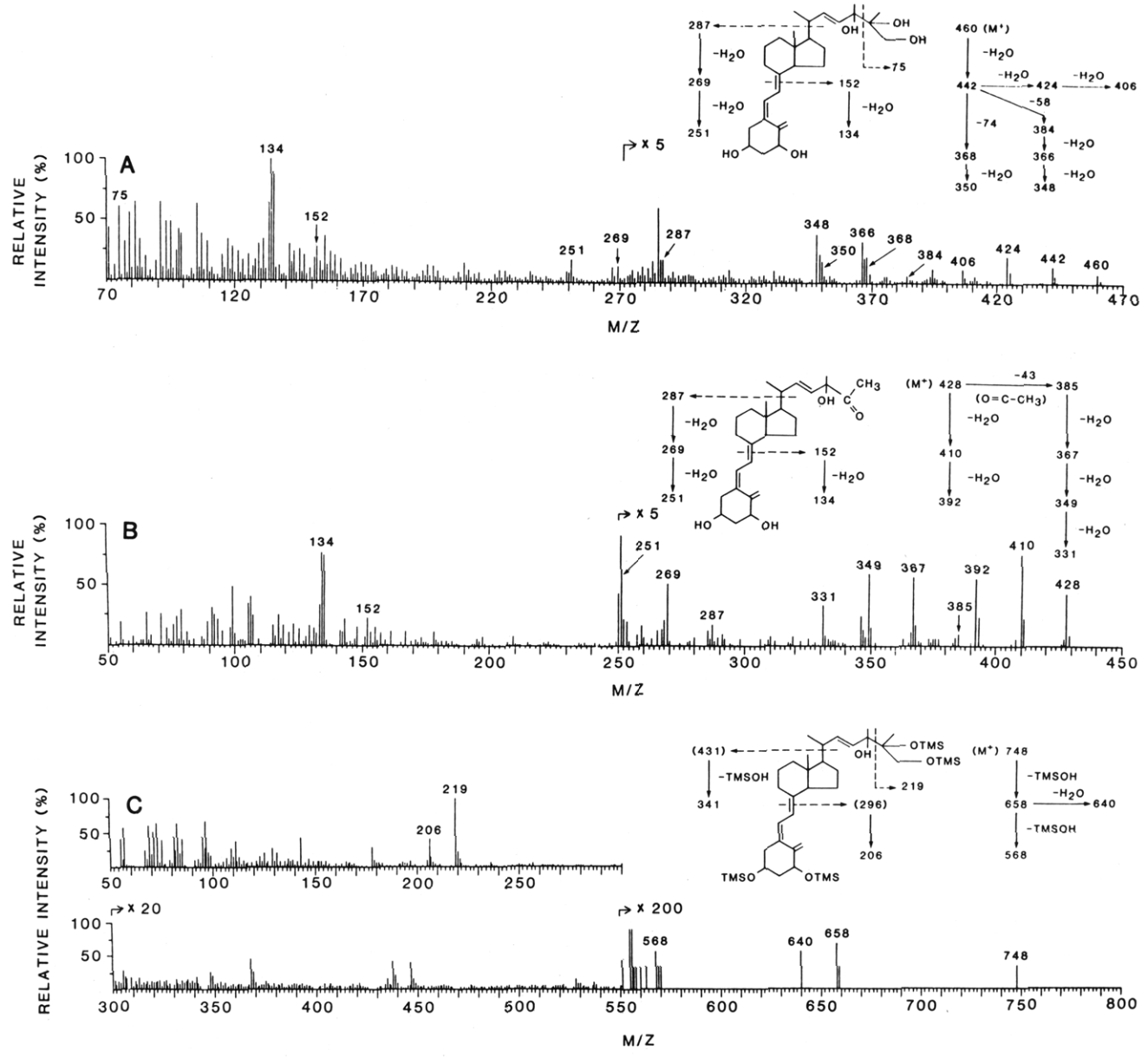

FIGURE 6: Mass spectra belonging to $1,24,25,26(OH)_4D_2$: (A) underivatized metabolite; (B) periodate cleavage product [$1,24(OH)_2$-25-keto-26-nor-D_2]; (C) $(Me_3Si)_4$ derivative.

formed as a result of elimination of 58 mass units from the peak at m/z 442 (McLafferty-type rearrangement). This finding and the base peak at m/z 131 (corresponding to $C_3H_6OSiMe_3^+$) in the mass spectrum of the trimethylsilyl ether derivative (Figure 5C) indicate that the new metabolite contains an intact C-25 hydroxyl group with no hydroxylations occurring on C-26 and C-27. Also, this metabolite is susceptible to periodate oxidation, and the mass spectrum of the periodate cleavage product (Figure 5B) shows a molecular ion at m/z 428, which indicates that the parent compound has lost CH_3OH (32 mass units) during the process of periodate oxidation. The characteristic peak at m/z 352 formed as a result of elimination of 58 mass units from the peak at m/z 410 (McLafferty-type rearrangement) indicates that the periodate cleavage product still contains an intact C-25 hydroxyl group like its parent. With this information, the periodate cleavage product is identified as $1,25(OH)_2$-24-keto-28-nor-D_2, the formation of which is only possible due to the presence of a vicinal diol at C-24 and C-28. This new metabolite, therefore, has to possess hydroxyl groups at C-24 and C-28 and is identified as $1,24,25,28(OH)_4D_2$.

(*C*) *Structure Identification of* $1,24,25,26(OH)_4D_2$. The molecular ion at m/z 460 (M^+) in the mass spectrum (Figure 6A) indicates that this new metabolite is a tetrahydroxy metabolite of vitamin D_2. Therefore, it can be concluded that there are two additional hydroxyl groups on the side chain of this new metabolite when compared to its parent $1,25(OH)_2D_2$. The positions of the two extra hydroxyl groups are located as follows: The mass spectrum exhibits a peak at m/z 75 and another peak at m/z 368 due to the elimination of 74 mass units from the peak at m/z 442 (McLafferty-type rearrangement). This finding and the base peak at m/z 219 [corresponding to $C_3H_5(OSiMe_3)_2^+$] in the mass spectrum of the trimethylsilyl ether derivative (Figure 6C) indicate that the new metabolite contains an intact C-25 hydroxyl group with an extra hydroxyl group at C-26. Other evidence for C-26 hydroxylation also comes due to the presence of characteristic mass fragments at m/z 384, 366, and 348 (Figure 6A). These mass fragments are produced as a result of McLafferty-type rearrangement of an α-substituted β-hydroxy aldehyde resulting from the dehydration of the molecular ion (M^+), followed by a loss of CH_3CH_2CHO (58 mass units). This

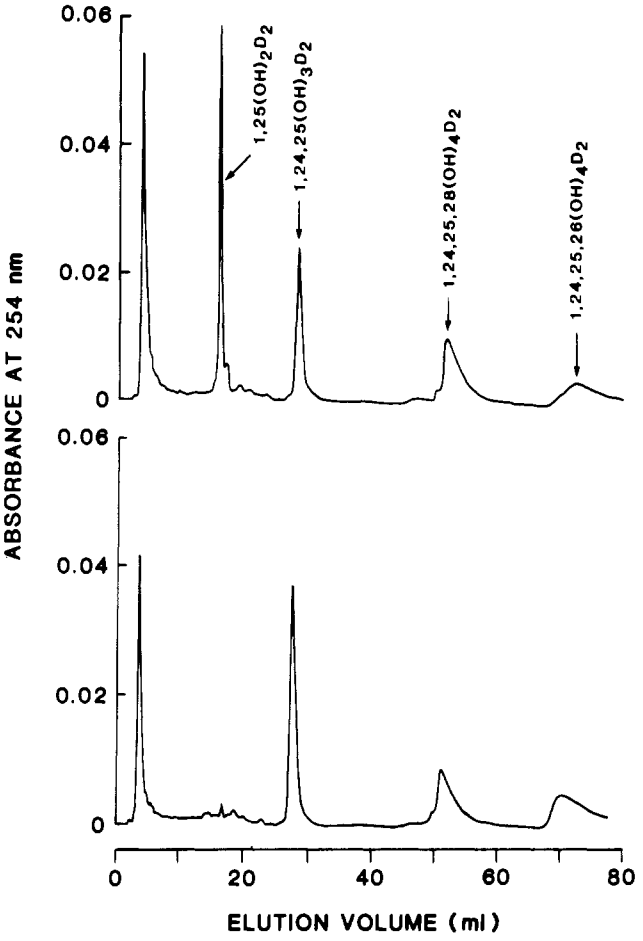

FIGURE 7: HPLC analysis of the lipid extract of 4 mL of final perfusate obtained by perfusing a kidney for 4 h with 100 nmol of either $1,25(OH)_2D_2$ in 50 mL of perfusate (upper panel) or $1,24,25(OH)_3D_2$ in 50 mL of perfusate (lower panel). HPLC chromatographic conditions are as follows: Zorbax-SIL column (25 cm × 4.6 mm) eluted with hexane–2-propanol (90:10 v/v) at a flow rate of 2 mL/min. Elution positions of various metabolites are shown by the arrows.

phenomenon is similar to the characteristic decomposition pathway described for α-substituted β-hydroxy esters (Budzikiewicz et al., 1967b). Further, this metabolite is also susceptible to periodate oxidation and gives rise to two periodate cleavage products (Figure 3B). The mass spectrum of the more polar periodate cleavage (Figure 6B) product shows a molecular ion at m/z 428, which indicates that the new metabolite had lost CH_3OH (32 mass units) during the process of periodate oxidation. Also, the characteristic loss of $C\text{-}H_3\text{–}C{=}O$ (43 mass units) from the molecular ion gives rise to peak at m/z 385. With this information, the more polar periodate cleavage product is identified as $1,24(OH)_2$-25-keto-26-nor-D_2, the formation of which is only possible due to the presence of a vicinal diol at C-25 and C-26. The less polar periodate cleavage product has exhibited identical mass spectra (data not shown) and chromatographic mobility (see Figure 3) as those of the periodate cleavage product of $1,24,25(OH)_3D_2$ and is identified as 1(OH)-24-keto-25,26,27-trinor-D_2, the formation of which is only possible due to the presence of a vicinal diol at C-24 and C-25. Thus, it becomes obvious that the new metabolite contains two adjacent vicinal diols, one at C-25 and C-26 and the other at C-24 and C-25. Therefore, this new metabolite has to possess hydroxyl groups at C-24, C-25, and C-26 and is identified as $1,24,25,26(OH)_4D_2$.

Metabolism of $1,24,25(OH)_3D_2$ by the Perfused Kidney. Figure 7 compares the HPLC profiles of the lipid extracts of the final perfusate of two kidney perfusion experiments. The lipid extract of the control kidney perfused with $1,25(OH)_2D_2$ gave rise to three UV-absorbing peaks, representing $1,24,25(OH)_3D_2$ and the two tetrahydroxy metabolites. The lipid extract of the experimental kidney perfused with $1,24,25(OH)_3D_2$ gave rise to two UV-absorbing peaks, representing the two tetrahydroxy metabolites that comigrated with the tetrahydroxy metabolites produced from $1,25(OH)_2D_2$. The results of this experiment indicate that both of the tetrahydroxy metabolites are also formed from $1,24,25(OH)_3D_2$. These results further support the mass spectral data, suggesting that the C-24 hydroxyl group is common to both tetrahydroxy metabolites.

Discussion

This paper reports the isolation and identification of three major new metabolites of $1,25(OH)_2D_2$ produced in a mammalian kidney. These were unequivocally identified as $1,24,25(OH)_3D_2$, $1,24,25,28(OH)_4D_2$, and $1,24,25,26(OH)_4D_2$. The structural assignments of all the new metabolites were based on several lines of evidence described in detail under Results. We used the isolated perfused rat kidney system to study the renal metabolism of $1,25(OH)_2D_2$. The various metabolites of $1,25(OH)_2D_2$ in the kidney perfusate were traced by subjecting the perfusate lipid extract directly to a HPLC column and by monitoring the UV absorbance (at 254 nm) of the lipids, eluting out of the HPLC column. In our present study it only became possible to identify the new metabolites by perfusing the kidneys with a pharmacological concentration of $1,25(OH)_2D_2$. However, the demonstration of the formation of the new metabolites under physiological concentrations is not possible until radiolabeled $1,25(OH)_2D_2$ becomes available.

Recently, several investigations were directed to define the further metabolism of $1,25(OH)_2D_3$ in both intestine and kidney, and it became obvious that the side chain of $1,25(OH)_2D_3$ undergoes several metabolic alterations, which include C-23, C-24, and C-26 hydroxylations, C-24 ketonization, lactonization occurring between C-23 and C-26, and side-chain cleavage occurring between C-23 and C-24 (Tanaka et al., 1981; Reinhardt et al., 1981; Napoli et al., 1983; Napoli & Horst, 1983; Mayer et al., 1983; Horst et al., 1984; Ishizuka et al., 1984; Napoli & Martin, 1984; Esvelt et al., 1979; Reddy et al., 1986). Because of the structural differences in the side chains of vitamins D_2 and D_3, some of the aforementioned chemical alterations occurring on the side chain of $1,25(OH)_2D_3$ may not occur on the side chain of $1,25(OH)_2D_2$ especially with respect to C-23 hydroxylation, C-24 ketonization, and C-23:C-26 lactonization. As far as C-24 hydroxylation is concerned, it has been already well established that vitamin D_2 and $25(OH)D_2$ undergo C-24 hydroxylation to form $24(OH)D_2$ and $24,25(OH)_2D_2$, respectively (Jones et al., 1979, 1980). Therefore, $1,25(OH)_2D_2$ may also undergo C-24 hydroxylation to form $1,24,25(OH)_3D_2$. This assumption was recently confirmed in a preliminary report (Napoli & Horst, 1985). In our present study we not only confirmed the above finding but also indicated that $1,24,25(OH)_3D_2$ undergoes further hydroxylations at C-28 and C-26 to form $1,24,25,28(OH)_4D_2$ and $1,24,25,26(OH)_4D_2$, respectively (see Figure 8). Thus, our study for the first time indicates that the differences in the further metabolism of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ become apparent only after the C-24 hydroxylation step.

It has been well-known that vitamin D_2 is less biologically active when compared to vitamin D_3 in chicks (Chen & Bosman, 1964). The reasons for discrimination between vi-

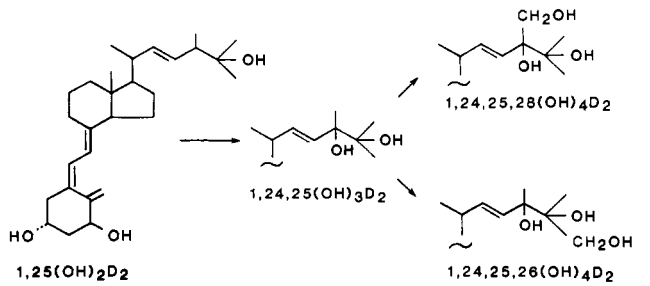

FIGURE 8: Proposed metabolic pathway for $1,25(OH)_2D_2$ in kidney.

tamins D_2 and D_3 in chicks are still not completely understood (Imrie et al., 1967; Drescher et al., 1969). Until recently, vitamin D_2 was considered to be equally bioactive in humans and other mammals. Therefore, vitamin D_2 is being freely substituted for vitamin D_3 as a dietary supplement for humans and most other commercially important mammals. Recent studies (Horst et al., 1982), however, indicated that mammals also show discrimination between vitamins D_2 and D_3. For example, it was demonstrated that there were differences between vitamins D_2 and D_3 in terms of their toxicity. In Rhesus monkeys, hypervitaminosis D caused by vitamin D_2 was found to have less toxic effects when compared to hypervitaminosis D caused by vitamin D_3 (Hunt et al., 1972). Similarly, 1α-hydroxyvitamin D_2 was found to be less toxic when compared to 1α-hydroxyvitamin D_3 in rates (Sjoden et al., 1985). In humans, the clinical condition of anticonvulsant osteomalacia responds to vitamin D_2 supplementation better than to vitamin D_3 or $25(OH)D_3$ supplementation (Tjellesen et al., 1985a,b). The exact mechanism at the molecular level for the above observations is still not clear. Our demonstration of a novel further metabolic pathway for the most active form of vitamin D_2 might form a basis for many future studies that may help to understand the reasons for the observed differences between vitamins D_2 and D_3 in terms of their bioactivity and toxicity both in avian and in mammalian species. Studies to compare the biological activity of the further metabolites of $1,25(OH)_2D_2$ described in this paper with the previously well-studied further metabolites of $1,25(OH)_2D_3$ in terms of (a) calcium absorption by the gut, (b) calcium mobilization from the bone, and (c) their binding affinity to the vitamin D receptor protein are presently in progress in our laboratory.

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Registry No. $1,24,25(OH)_3D_2$, 100496-04-6; $1,24,25,28(OH)_4D_2$, 103305-10-8; $1,24,25,26(OH)_4D_2$, 103305-11-9; $1,25(OH)_2D_2$, 55248-15-2; $1,24,25(OH)_3D_2$ (Me_3Si derivative), 103305-12-0; $1,24,25,28(OH)_4D_2$ (Me_3Si derivative), 103321-13-7; $1,24,25,26(OH)_4D_2$ (Me_3Si derivative), 103321-14-8; 1(OH)-24-keto-25,26,27-trinor-D_2, 103305-13-1; $1,25(OH)_2$-24-keto-28-nor-D_2, 103320-91-8; $1,24(OH)_2$-25-keto-26-nor-D_2, 103321-15-9.

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Interaction and Reconstitution of Carboxyl-Terminal-Shortened B Chains with the Intact A Chain of Insulin†

Chih-Chen Wang and Chen-Lu Tsou*
Institute of Biophysics, Academia Sinica, Beijing 100080, China
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ABSTRACT: With the *S*-(thiomethyl)-A chain and despentapeptide(26–30) and desoctapeptide(23–30) *S*-(thiomethyl)-B chains of insulin at pH 10.8 and a molar ratio of A/B = 1.5, difference spectra of the mixed against the separated chains with negative peaks at 245 and 295 nm and a weak positive peak at 278 nm indicate interaction of the chains leading to Tyr environmental changes as in the case for the intact chains. With the shortened B chains, freshly dissolved from lyophilized powders, it takes some 2 h for the difference spectra to approach completion whereas with the solutions of the shortened B chains left standing overnight at pH 10.8 and 4 °C the difference spectra, similar in shape to that described above, appear almost immediately after mixing. Solvent perturbation with 20% ethylene glycol suggests some ordered structure for the despentapeptide but not for the desoctapeptide B chain. The interactions of the A chain with the shortened B chains appear to be weaker as compared to that with the intact B chain as shown by decreasing reconstitution yields for the intact, despentapeptide, and desoctapeptide B chains respectively with the A chain. The above results indicate that the C-terminal portion of the B chain is important not only for the activity of insulin but also for the correct pairing of the chains.

Although it was suggested some years ago (Du et al., 1961) that the insulin A and B chains pair correctly in solution so that the reoxidation of the reduced chains leads to a fairly good yield of the native hormone, the interaction of the chains has only been recently demonstrated in this laboratory with the *S*-(thiomethyl)-A and -B chains of insulin (Hua et al., 1984, 1985). At pH 10.8 and a molar ratio of the chains of A/B = 1.5, where the resynthesis yield of the hormone by reoxidation of the reduced chains was optimal (Du et al., 1965), both the absorption and the CD[1] spectra of the individual chains show significant changes upon mixing, indicating the interaction of the *S*-(thiomethyl) chains by secondary forces leading to a partial transfer of the Tyr residues from an exposed and hydrophilic to a buried and hydrophobic environment and an increase in ordered secondary structure. Moreover, it is particularly interesting to note that both these changes in absorbance and in ellipticity take about 90 min to approach completion after the mixing of the intact chains. This would seem to suggest that some sort of conformational readjustment of either one or both of the chains is required for their interaction with each other, and this conformational change is a relatively slow process. The presence of a Pro residue at B-28 might be significant in this respect as it is well-known that the cis–trans isomerization of peptide bonds containing the imide nitrogen of prolyl residues is a slow process (Brandts et al., 1975).

A series of insulin derivatives shortened at the C-terminal of the B chain have been prepared, and the role of the C-terminal residues on insulin activity has been studied (Brandenburg, 1981; Lei et al., 1981; Zhang, 1983). It has been found that insulin activity decreases with the increase of the number of residues removed. Despentapeptide(B26–30)-insulin retains about 20% of the activity of native insulin (Gattner, 1975; Zhu et al., 1984) whereas the desoctapeptide(B23–30) derivative is almost completely inactive (Kikuchi et al., 1980). It is therefore considered that the C-terminal sequence of the B chain is important for the binding of the insulin molecule to its receptor and hence to its biological activity.

In this paper, the pairing and the formation of the corresponding insulin derivatives from the C-terminal-shortened despenta- and desoctapeptide B chains with the intact A chain have been studied. The results obtained indicate that the C-terminal sequence of the B chain is important not only for insulin activity but also for the pairing and, hence, the formation of the correct insulin derivatives.

Materials and Methods

Reagents. Porcine insulin was a product of Novo. Pepsin was obtained from E. Merck. DTNB, TPCK-trypsin, collagenase (type II), and albumin (Bovine, fraction V) were Sigma products. Acetyltyrosine ethyl ester was from Dongfong Biochemicals, Shanghai. DTT was from Serva, mercaptoethanol from Fluka, and TFA from Pierce. Urea was a local product twice recrystallized according to Morangos and

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[1] Abbreviations: ATEE, acetyltyrosine ethyl ester; CD, circular dichroism; DP, despentapeptide(B26–30); DO, desoctapeptide(B23–30); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; TPCK, L-1-[(*p*-toluenesulfonyl)amino]-2-phenylmethyl chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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