24,26-Dihydroxyvitamin D2: a unique physiological metabolite of vitamin D2

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detailed understanding of the effects of Zn^{2+} on the molecular structure of tRNA.

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24,26-Dihydroxyvitamin D_2: A Unique Physiological Metabolite of Vitamin D_2†

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ABSTRACT: A new vitamin D_2 metabolite, 24,26-dihydroxyvitamin D_2, has been detected in the plasma of rats fed physiologic amounts of vitamin D_2. The identity of the new metabolite (isolated from cow plasma) was established by ultraviolet absorbance, mass spectroscopy, chemical reactivity, and NMR spectroscopy. Among these, the mass spectrum was unique for the presence of a peak at M − 48 that was attributed to an intramolecular rearrangement involving both the C-24 and C-26 hydroxyl groups. A 300-MHz ^1H NMR spectrum of 40 μg of metabolite indicated a downfield shift of the C-28 methyl group signal to δ 1.30 and a multiplet at δ 3.66 corresponding to the hydroxylated C-26 methyl group. We determined that the formation of 24,26-dihydroxyvitamin D_2 represented a major pathway for further metabolism of 24-hydroxyvitamin D_2 in rats, exceeding the formation of 24,25-dihydroxyvitamin D_2. Standard bioassays revealed that 24,26-dihydroxyvitamin D_2 possessed very little biological activity and most likely represents a deactivation pathway for 24-hydroxyvitamin D_2.

The metabolism of vitamin D_3 has been extensively characterized and largely consists of oxidative modifications occurring in the side chain (Horst et al., 1983; Jones et al., 1983, 1984; Napoli & Horst, 1982; Napoli et al., 1982; Norman, 1984; Tanaka et al., 1981; Wichmann et al., 1981). The metabolism and biological activity of vitamin D_2 are generally thought to parallel those of vitamin D_3, and as such, it is freely administered to both humans and commercially important mammals (DeLuca, 1978; Norman, 1979). As a result, vitamin D_2 is prescribed for patients with a wide variety of

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vitamin D and calcium-related diseases.

Inherent differences in vitamin D_2 metabolism from that of vitamin D_3 would appear to be inevitable on the basis of its altered side-chain structure. The presence of the C-22 alkene, coupled with the methylation of C-24, can be viewed as a barrier to normal oxidative processes known to occur in vitamin D_3 (Horst et al., 1982; Reddy & Tserng, 1986). In human patients treated with phenobarbitone/phenytoin, the actions of vitamin D_2 and vitamin D_3 were seen to be quite different with respect to bone metabolism (Tjellesen et al., 1985). In addition, the 25-hydroxylase activity in human liver exhibited different rates of mitochondrial hydroxylation for vitamin D_2 and vitamin D_3 (Holmberg, 1984; Holmberg et al., 1986). Consistent with these findings was the recent observation that other mammals such as the pig and rat discriminated between vitamin D_2 and vitamin D_3 (Horst et al., 1982).

In conjunction with an ongoing study concerning the toxicity of vitamin D compounds, we now report the isolation and unequivocal identification of a unique vitamin D_2 metabolite, 24,26-$(OH)_2D_2$.[1] This represents the first significant deviation of vitamin D_2 metabolism from the more extensively characterized metabolism of vitamin D_3.

Materials and Methods

General. High-performance liquid chromatography (HPLC) was performed with Waters Associates ALC/GPC 204 liquid chromatographic equipment. HPLC columns were purchased from Du Pont Instruments (Wilmington, DE).[2] Zorbax-Sil refers to microparticulate silica gel columns. Zorbax-ODS refers to the octadecasilane derivative of the microparticulate silica gel columns. Zorbax-NH_2 refers to the 3-aminopropyl triethoxy derivative of the microparticulate silica gel columns. HPLC solvents were purchased from Burdick and Jackson Laboratories (Muskegon, MI) or Fisher Scientific Co. (Fair Lawn, NJ). Florisil was purchased from Fisher Scientific Co. UV spectra were obtained in ethanol with a Beckman Model 25 recording spectrometer. Low-resolution mass spectra were obtained with solid probes on a Finnigan Model 4000 quadrupole GC–MS. The analyses were carried out at 70 eV with an ionizer temperature of 150 °C while the probe was heated from ambient temperature to 350 °C. High-resolution mass spectra were obtained with solid probes on a Kratos MS 50 GC–MS mass spectrometer. The 300-MHz proton NMR spectra were taken in deuteriated chloroform on a Nicolet NT-300 FT NMR spectrometer. Gas–liquid chromatographic analysis with flame ionization detection was carried out on a Hewlett-Packard Model 5840A gas–liquid chromatograph.

Compounds. Vitamin D_2 was purchased from Sigma Chemical Co. (St. Louis, MO). Inhoffen-Lythgoe diol was a gift from Dr. M. Uskokovic (Hoffmann-La Roche, Nutley, NJ). Standard 25(*R*),26-$(OH)_2D_2$ and 25(*S*),26-$(OH)_2D_2$ were gifts from Dr. D. Williams (University Chemical Laboratory, Cambridge, England). Deuteriated chloroform (99.8 atom % D) and pyridinium chlorochromate were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Isolation of Vitamin D_2 Metabolites from Cow Plasma. A Jersey cow was given weekly intramuscular injections of 1 g (40×10^6 units) of vitamin D_2. After 4 weeks of treatment, blood was collected in heparizined containers. Plasma was prepared by centrifugation and stored at −20 °C.

Vitamin D metabolites were extracted by the following procedure. To 7 L of plasma was added 1400 g of ammonium sulfate. After this was shaken for 1 h, 8 L of acetonitrile was added, and the mixture was shaken until an emulsion formed. The phases were separated by the addition of 2 L of methanol. The resulting mixture was filtered to remove the precipitated proteins, and the remaining pellet was reextracted with 5.4 L of acetonitrile. Water (3 volumes) was added to the combined organic phases, and the mixture was applied to an 18 cm × 18 cm ODS column (Waters Associates, Milford, MA) equilibrated in 25% water in acetonitrile. After the sample was applied to the column bed, 5 L of 40% water in methanol was applied to elute the more polar lipids. The vitamin D metabolites were then eluted by the addition of 8 L of acetonitrile. The acetonitrile was removed by vacuum, and the resultant lipids were dissolved in 2 mL of 2-propanol/methylene chloride/hexane (1:10:89). The dissolved lipids were applied to a Zorbax-Sil HPLC prep column (2.2 cm × 25 cm), and the vitamin D metabolites were eluted by use of a gradient of 2-propanol/methylene chloride/hexane (1:10:89 to 30:10:60).

Metabolism and Biological Evaluation Studies. Metabolites, including 24-OHD_2 and 25-OHD_2 for use in evaluation studies, were purified further according to methods previously described (Napoli et al., 1986). Initial experiments to study in vivo metabolism of various monohydroxylated vitamin D_2 substrates were conducted with male weanling rats (Holtzman, Madison, WI) divided into four groups. Three of the groups were maintained on a vitamin D deficient diet for 4 weeks and then received daily oral supplements of 100 IU of either vitamin D_2 (group I) or vitamin D_3 (other two groups) for 3 weeks. During the last week of supplementation with vitamin D_3 one group of rats received daily intraperitoneal injections of 5 μg of 24-OHD_2 (group II) in 100 μL of propylene glycol carrier for 5 days, while the other group was similarly injected with 25-OHD_2 (group III). The fourth group of rats (group IV) was maintained on a low-calcium (0.005%), vitamin D deficient diet for 4 weeks, and then given intraperitoneal injections with 24-OHD_2 as described. At the end of the treatment periods, blood was collected by cardiac puncture in heparinized syringes, and the plasma was frozen at −20 °C.

Extraction of the plasma and isolation of the vitamin D_2 metabolites were carried out as described previously (Horst et al., 1981a; Napoli et al., 1986).

Biological activity of vitamin D_2 metabolites was determined with male weanling rats (Holtzman, Madison, WI) as described previously (Horst et al., 1986b).

Silylation. 24,26-Dihydroxyvitamin D_2 (500 ng) was allowed to react with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (50 μL) at 90 °C for 2 h to effect persilylation. The mixture was dried under a stream of N_2 and purified by reverse-phase HPLC. The silylated product was eluted with 0.1% methylene chloride in methanol and analyzed by low-resolution mass spectroscopy.

Ozonolysis. A solution of methylene chloride at −90 °C was saturated with ozone, and the resultant blue solution was stored on dry ice. To the metabolite (2 μg) in 100 μL of pyridine

[1] Abbreviations: 24,26-$(OH)_2D_2$, 24,26-dihydroxyvitamin D_2; 24-OHD_2, 24-hydroxyvitamin D_2; 25OHD_2, 25-hydroxyvitamin D_2; 24,25-$(OH)_2D_2$, 24,25-dihydroxyvitamin D_2; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; 25,26-$(OH)_2D_2$, 25,26-dihydroxyvitamin D_2; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; GC–MS, gas chromatography–mass spectroscopy; NMR, nuclear magnetic resonance; PCC, pyridinium chlorochromate; ICT, intestinal calcium transport; BCR, bone calcium resorption.

[2] Mention of a trade name, proprietary product, or vendor does not constitute a guarantee or warranty by the U.S. Department of Agriculture or cooperators and does not imply its approval to the exclusion of other products or vendors that may be suitable.

at −90 °C was added 100 μL of the methylene chloride/ozone solution. The mixture was kept at −90 °C for 30 s and then dried at room temperature under a stream of N_2. The residue was dissolved in 10 μL of ethanol and analyzed by GLC using a 2 mm × 10 ft 3% SP 2100 column employing N_2 as carrier gas at a flow rate of 25 mL/min. Temperature programming was as follows: 100 °C/1 min; 10 °C/min; 240 °C/30 min. The major product peak eluted at 12.75 min at a temperature of 217 °C. The low-resolution mass spectrum was obtained by GC–MS.

Oxidation of Inhoffen-Lythgoe Diol. The diol (100 μg) was dissolved in 200 μL of methylene chloride to which 2 mg of PCC was added. The suspension was kept at room temperature for 20 min with intermittent vortexing, and then 100 μL of 2-propanol was added. The mixture was dried under a stream of N_2. The brown residue was dissolved in methylene chloride and then filtered through a short column of Florisil, and the column was washed with ether. The eluent was dried under a stream of N_2 to ca. 50 μL and analyzed by low-resolution GC–MS as described.

Radioligand Binding Assay. The comparison of the relative binding affinity of 24,26-$(OH)_2D_2$ to other vitamin D metabolites for the rat plasma vitamin D binding protein was carried out as previously described (Horst et al., 1981b).

Plasma Assays. Vitamin D_2 and Vitamin D_3 metabolite concentrations were assayed by HPLC or competitive protein binding as described by Horst et al. (1981a) or by radioimmunoassay as described by Hollis and Napoli (1985).

Results

Because of the lack of commercially available vitamin D_2 metabolites, plasma from a cow intoxicated with vitamin D_2 was used in anticipation of isolating large quantities of 25-OHD_2, 24,25-$(OH)_2D_2$, and 25,26-$(OH)_2D_2$ for use as laboratory standards. During the isolation and identification procedures, we observed that the metabolite tentatively identified (because of its HPLC migration character; Napoli et al., 1986) as 25,26-$(OH)_2D_2$ reacted unpredictably in several independent identification procedures. Most notable were the insensitivity of the metabolite to periodate (data not shown) and an uncharacteristic mass spectrum. Subsequently, more extensive identification techniques were employed to positively identify the new metabolite.

The metabolite, purified from cow plasma, displayed a UV absorbance spectrum characteristic of a 5(*E*),7,10(19)-triene chromophore (not shown). The absorbance maximum was at 265 nm, and the absorbance minimum was at 229 nm. On the basis of the absorbance spectrum, approximately 80 μg of the metabolite was isolated from the bovine source.

An insufficient amount of metabolite was isolated from rat plasma for identification. However, the metabolite isolated from the rat plasma comigrated with the bovine compound on two different HPLC systems. Comigration was observed on a Zorbax-Sil column developed in methanol/2-propanol/methylene chloride/hexane (0.75:2.5:25:72, peak elutes at 24–28 mL) and a Zorbax-NH_2 column developed in methanol/2-propanol/hexane (1:3:96, peak elutes at 40–44 mL). The comigration on two chemically distinct HPLC systems verified that the material isolated from the bovine and rat sources was identical (Napoli et al., 1986). The mobility of this new metabolite relative to several known vitamin D compounds on a Zorbax-Sil column developed in methanol/acetonitrile/methylene chloride (1:20:180) is shown in Figure 1.

The high-resolution mass spectrum of the metabolite (Figure 2) had a molecular ion at m/z 428.3286 indicating an elemental composition of $C_{28}H_{44}O_3$ (calculated value of

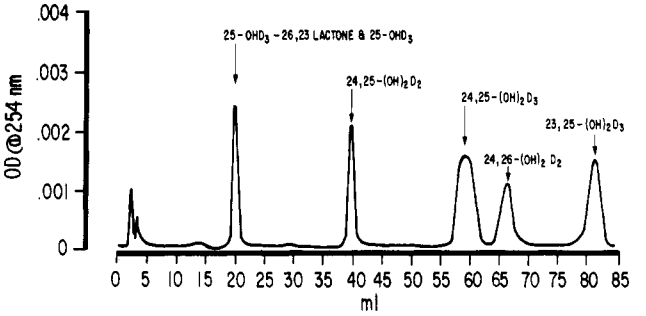


FIGURE 1: Comparison of the relative mobility of 24,26-$(OH)_2D_2$ with various vitamin D metabolites on a Zorbax-Sil column developed in methanol/acetonitrile/methylene chloride (1:20:180).

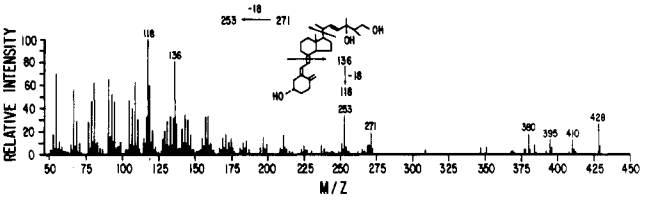
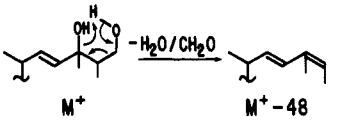


FIGURE 2: High-resolution mass spectrum of 24,26-$(OH)_2D_2$ isolated from bovine plasma.

428.3290). This molecular formula was consistent with a dihydroxylated vitamin D_2 metabolite. The peaks at m/z 410 (M − 18) and 395 (M − 33) can be attributed to sequential losses of water and a methyl group. The characteristically large peaks at m/z 136 and 118 demonstrated that the triene system remained intact and ruled out A-ring substitutions. The peaks at m/z 271 and 253 arise from the cleavage of the steroid side chain and subsequent loss of water. This indicated that the two remaining hydroxyl groups were a part of the side chain. Absence of either a significant M − 58 peak or a peak at 59 amu, often seen in 25-hydroxylated vitamin D_2 compounds, discounted the presence of a 25-hydroxyl group in the metabolite (Napoli et al., 1986). The peak at m/z 380.3075 (M − 48) had an elemental composition of $C_{27}H_{40}O$ (calculated value of 380.3079) representing an unusual loss of CH_4O_2 from the molecular ion. An even-numbered loss of this type strongly suggests a rearrangement. The elimination of 48 amu from the parent ion may arise from an intramolecular proton transfer involving both the C-24 and C-26 hydroxyl functions. This would result in the elimination of water and formaldehyde molecules (C-26) from the molecular ion:



Persilylation of the metabolite produced a compound with a molecular ion at m/z 644, which established that three alcohol groups were present (Figure 3). The peaks at m/z 554 and 464 represented the successive losses of $(CH_3)_3SiOH$ from the parent ion. More significant was the peak at m/z 513. This corresponded to a loss of $CH_3CHCH_2OSi(CH_3)_3$ from the terminus of the side chain as a result of β-cleavage of the silyl ether present at C-24. This also accounted for the peak seen at m/z 131. Cleavage of the side chain resulted in the peak observed at m/z 343. The peak at m/z 333 represented the combined losses of two $(CH_3)_3SiOH$ functions in addition to the previously discussed loss of 131 amu from the molecular ion.

The major product obtained from ozonolysis of the metabolite was characterized by GC–MS (Figure 4). The

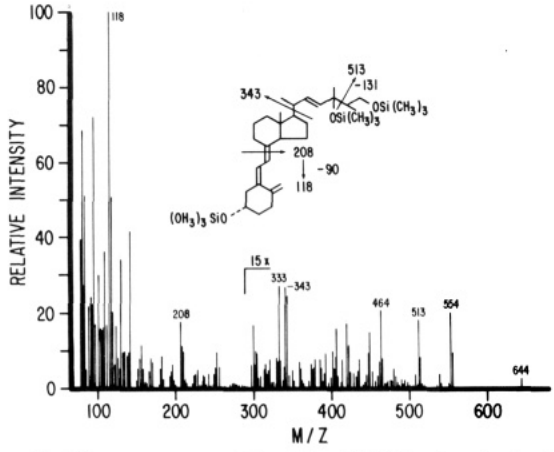


FIGURE 3: Mass spectrum of the trimethylsilyl ether derivative of 24,26-$(OH)_2D_2$.

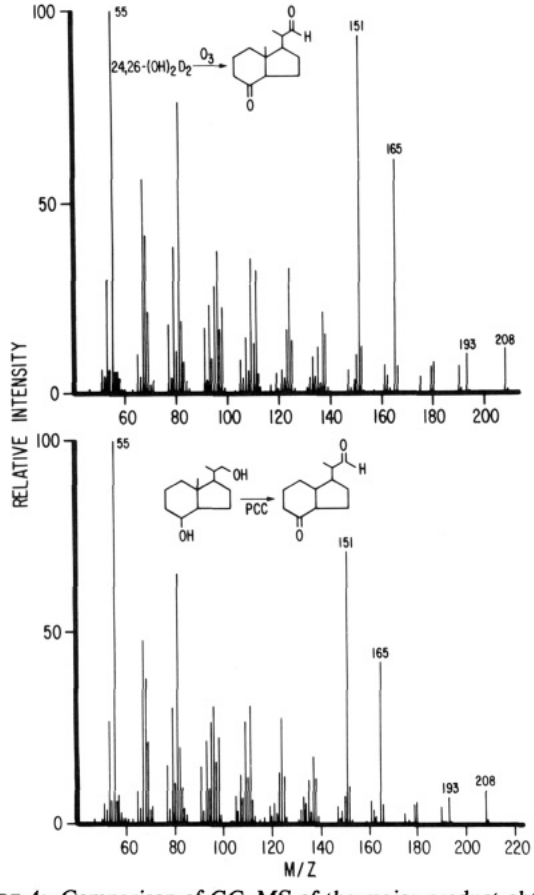


FIGURE 4: Comparison of GC–MS of the major product obtained from the ozonolysis of 24,26-$(OH)_2D_2$ (upper spectrum) and the product obtained from the oxidation of Lythgoe-Inhoffen diol (lower spectrum).

low-resolution mass spectrum indicated a parent ion at m/z 208. This corresponded to cleavage of the C-7/C-8 and C-22/C-23 olefin bonds. Losses of water (M − 18) and a methyl group (M − 15) were readily evident. In addition, cleavage of the side chain accounts for the peak at m/z 151. The mass spectrum of the ozonolysis product was in excellent agreement with the product obtained from oxidation of Inhoffen-Lythgoe diol. This placed the alcohol groups in the terminal portion of the side chain.

A 300-MHz ^{1}H NMR spectrum was taken of 40 μg of the metabolite (Figure 5). The signals at δ 6.31, 6.02, 5.03, and 4.81 indicated that the characteristic triene system was intact. These peak positions also confirmed the lack of 1α-

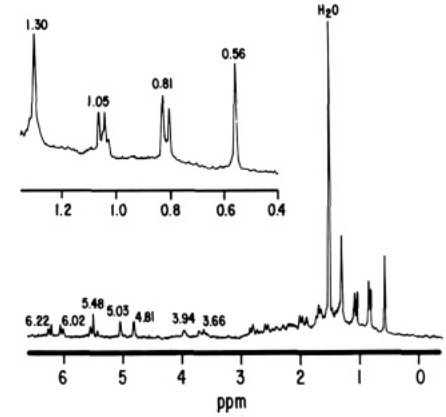


FIGURE 5: The 300-MHz ^{1}H NMR spectrum of 40 μg of 24,26-$(OH)_2D_2$. The insert is an expanded view of the upfield portion of the spectrum.

Table I: Ability of Vitamin D Metabolites To Displace [^{3}H]-25-OHD_3 from the Rat Plasma Vitamin D Binding Protein

metabolite	amount that produces 50% displacement of [^{3}H]-25-OHD_3 (ng)	competitive index relative to 25-OHD_3[a]
25-OHD_2	0.6	100
25-OHD_3	0.6	100
24(R)-25-$(OH)_2D_3$	0.6	100
24(R)-25-$(OH)_2D_2$	1.2	50
24,26-$(OH)_2D_2$	2.0	30

[a] The amount of 25-OHD_3 that displaces 50% of the [^{3}H]-25-OHD_3 divided by the amount of metabolite that will displace 50% of the [^{3}H]-25-OHD_3 times 100.

Table II: Biological Evaluation of 24,26-$(OH)_2D_2$ Using the Rat Bioassay

compounds given	dose (ng/rat)	^{45}Ca(serosal)/ ^{45}Ca(mucosal)[a]	plasma Ca (mg/dL)[b]
24,26-$(OH)_2D_2$	50	1.3 ± 0.1	5.6 ± 0.1
24,26-$(OH)_2D_2$	100	1.5 ± 0.1	5.6 ± 0.2
24,26-$(OH)_2D_2$	200	1.3 ± 0.1	5.3 ± 0.1
25-OHD_2	50	2.4 ± 0.1[c]	6.0 ± 0.2[c]
control		1.4 ± 0.1	5.4 ± 0.2

[a] ICT, intestinal calcium transport. [b] BCR, bone calcium resorption. [c] Differs from control at $p < 0.05$.

hydroxylation in the metabolite. The multiplet corresponding to the C-22/C-23 olefinic protons was located at δ 5.48. The downfield shift of this multiplet, relative to that observed in vitamin D_2, was taken as further evidence of hydroxylation at C-24. The signal at δ 3.94 (1 H) represented the 3α-proton and confirmed the presence of the 3β-hydroxyl group. The multiplet at δ 3.66 (2 H) can be attributed to the presence of the C-26 hydroxyl group. The singlet at δ 0.56 (3 H) and the doublet at δ 1.05 (3 H, J = 6.6 Hz) were consistent for the C-18 and C-21 methyl groups. A lone doublet located at δ 0.81 (3 H, J = 6.9 Hz) corresponded to the C-27 methyl group. The observed splitting pattern further confirmed the absence of substitution at C-25. The C-28 methyl group appeared as a singlet at δ 1.30 and is consistent for C-24 hydroxylation in vitamin D_2 compounds (Koszewski et al., 1987).

The affinity of 24,26-$(OH)_2D_2$ for the rat plasma vitamin D binding protein relative to other vitamin D metabolites is presented in Table I. The metabolites were assessed for their ability to displace [^{3}H]-25-OHD_3 from the 4.2S rat plasma vitamin D binding protein. Approximately 3.3 times more 24,26-$(OH)_2D_2$ was needed to achieve 50% displacement than either 25-OHD or 24(R),25-$(OH)_2D_3$. The 24(R),25-

Table III: Plasma Concentrations of Vitamin D Metabolites in Treated Rats

treatment (group)	ng/mL 24,25-$(OH)_2D_3$	24,25-$(OH)_2D_2$	24,26-$(OH)_2D_2$
D_2[a] (I)	ND[b]	4.1 ± 2.3	1.7 ± 1.0
D_3,[a] +24-OHD_2[c] (II)	3.3 ± 0.1	3.3 ± 0.3	14.3 ± 6.9
D_3,[a] +25-OHD_2[c] (III)	2.8 ± 0.8	93 ± 18	ND
−Ca, −D, +24-OHD_2[c] (IV)	ND	4.9 ± 0.8	5.9 ± 0.4
excess vitamin D_2 (cow)	3.5 ± 1.1	106 ± 36	14.7 ± 8.8

[a] Oral supplement of 100 IU/day. [b] Not detected. [c] Intraperitoneal injection of 5 μg/day for 5 days.

$(OH)_2D_2$ exhibited a higher affinity for the binding protein than 24,26-$(OH)_2D_2$ in this assay but was twofold less potent than its vitamin D_3 analogue.

The ICT and BCR responses of rats dosed with various amounts of test compound are presented in Table II. The data indicated that 24,26-$(OH)_2D_2$ has little, if any, effect on ICT and BCR despite increasing the dose up to 200 ng/rat. In contrast, the administration of a minimal dose (50 ng/rat) of 25-OHD_2 was seen to significantly increase both ICT and BCR.

Significant quantities of 24,26-$(OH)_2D_2$ were detected in the plasma of both rats given vitamin D_2 as their sole source of vitamin D and a cow treated with a large amount of vitamin D_2 (Table III). When rats received 24-OHD_2, in addition to a vitamin D_3 supplement, 24,26-$(OH)_2D_2$ was present in a 4-fold higher concentration than 24-25-$(OH)_2D_2$ (group II). The concentration of 24,26-$(OH)_2D_2$ was diminished and only slightly higher than that of 24,25$(OH)_2D_2$ when 24-OHD_2 was administered to rats maintained on a low-calcium, vitamin D deficient diet (group IV). No metabolites comigrating with 24,26-$(OH)_2D_2$ were detected in vitamin D_3 supplemented rats given 25-OHD_2 (group III). Rats supplemented with 25-OHD_2, however, possessed a greatly elevated concentration of 24,25-$(OH)_2D_2$ in their plasma.

Discussion

This paper reports the identification of a new vitamin D_2 metabolite as 24,26-$(OH)_2D_2$. Several independent techniques were employed to characterize the metabolite; however, the mass spectrum was remarkable for the presence of a peak at m/z 380 indicative of a rearrangement of the parent ion. Similar types of rearrangements can be observed in the mass spectra of other 1,3-diols of vitamin D metabolites (Tanaka et al., 1981; Napoli & Horst, 1982). The identification of the metabolite was greatly facilitated by our ability to obtain a high-resolution ^1H NMR spectrum of the material. A conventional interpretation of the NMR data, whereby the C-21 and C-24 methyl group protons have been assigned to the signals at δ 0.90 and 1.01, would have suggested the metabolite's structure as 20,26-$(OH)_2D_2$. However, from the ozonolysis work already discussed, as well as a recent NMR study on vitamin D_2 metabolites by Koszewski et al. (1987), the C-24 and not the C-20 position has been hydroxylated.

The 24,26-$(OH)_2D_2$ isolated in this study exhibited a lower affinity for the rat plasma vitamin D binding protein than 25-OHD. Hydroxylation at C-26 evidently results in yet a further decrease of a 24-hydroxylated vitamin D_2 metabolite's affinity for the binding protein as seen in the lower competitive index of 24,26-$(OH)_2D_2$ relative to 24(R),25-$(OH)_2D_2$.

The ability of 24,26-$(OH)_2D_2$ to stimulate intestinal calcium transport or bone calcium resorption, as measured in our assays, was insignificant. Horst et al. (1986a) have previously demonstrated that 24-OHD_2 possesses significant biological activity, most likely as a result of its ability to be 1α-hydroxylated. Evidently, the formation of 24,26-$(OH)_2D_2$ represents a pathway for the deactivation of 24-OHD_2. A comparable situation exists with regard to vitamin D_3 metabolism, in that C-26 hydroxylation seemingly represents a means of rendering 25-OHD_3 biologically inactive (Suda et al., 1970).

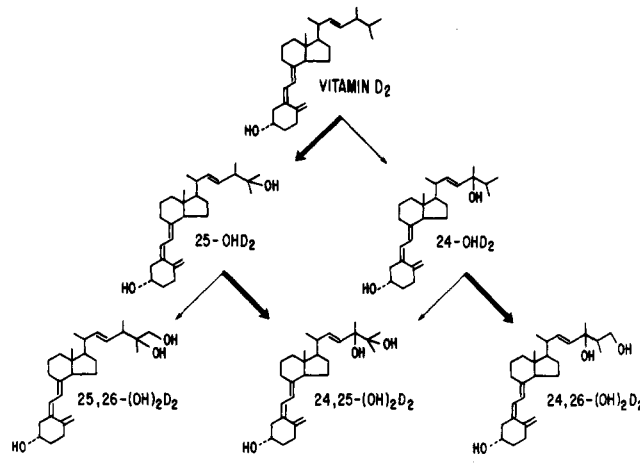


FIGURE 6: Preferred pathways of side-chain oxidations in the metabolism of vitamin D_2 under conditions of normal vitamin supplementation.

Collectively, the data from our controlled rat studies demonstrated that the preferred metabolic pathway for the metabolism of 24-OHD_2 under physiologic conditions is the biosynthesis of 24,26-$(OH)_2D_2$ (Table III). Our data further show that 24-OHD_2 is a poor substrate for the production of 24,25-$(OH)_2D_2$, with 25-OHD_2 being the preferred precursor. The low plasma concentration of 24,26-$(OH)_2D_2$ under conditions of vitamin D deficiency and low calcium probably is a result of the channeling of 24-OHD_2, the sole dietary source of vitamin D in this experiment, to its biologically active 1α-hydroxylated derivative (Horst et al., 1986a). C-26 hydroxylation would be predicted to be attenuated considerably under these dietary conditions.

Also noteworthy is the observed lack of 25,26-$(OH)_2D_2$ production in rats given 25-OHD_2 (data not shown). Consistent with this observation was our demonstration that 25,26-$(OH)_2D_2$ was not detectable in the plasma of a vitamin D_2 intoxicated cow. The only vitamin D_2 metabolite migrating in the 25,26-$(OH)_2D_2$ region of the prep HPLC column used in these experiments was 24,26-$(OH)_2D_2$. 26-Hydroxylation of 25-OHD_2 evidently represents a relatively inefficient process relative to the 26-hydroxylation of 24-OHD_2 or 25-OHD_3.

Figure 6 summarizes the metabolic pathways involved in the side-chain hydroxylations of vitamin D_2 in animals receiving normal dietary calcium and vitamin D_2. Vitamin D_2 is preferentially hydroxylated at the 25-position with C-24 oxidation present as a minor pathway. A major route of 25-OHD_2 metabolism results in the production of 24,25-$(OH)_2D_2$. On the other hand, only a small amount of 24-OHD_2 is metabolized to 24,25-$(OH)_2D_2$ with hydroxylation at C-26 to yield 24,26-$(OH)_2D_2$ being the major metabolic pathway. These data may aid in explaining differences in biological activity and toxicity known to exist between vitamin D_2 and vitamin D_3 and represent the first significant deviation of vitamin D_2 metabolism from that of vitamin D_3.

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8-Ketodeoxycoformycin and 8-Ketocoformycin as Intermediates in the Biosynthesis of 2′-Deoxycoformycin and Coformycin†

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ABSTRACT: An enzyme has been isolated from cell-free extracts of *Streptomyces antibioticus* that can catalyze the reduction of 8-ketodeoxycoformycin (8-ketodCF) and 8-ketocoformycin (8-ketoCoF) to the naturally occurring nucleoside analogues 2′-deoxycoformycin (dCF) and coformycin (CoF), respectively. The partially purified reductase requires NADPH as the cofactor and stereospecifically reduces the 8-keto group of both ketonucleoside substrates to a hydroxyl group with the *R* configuration at C-8. This is the same configuration of the hydroxyl group as that of the dCF and CoF isolated from *S. antibioticus*. The reduction proceeds at the nucleoside level, and ATP is not required. The reductase is stereospecific for the NADPH cofactor in that it transfers the *pro-S* but not the *pro-R* hydrogen from C-4 of NADPH to the 8-keto group. The apparent K_m for 8-ketodCF and 8-ketoCoF were 250 and 150 μM, respectively. These in vitro results, which show that 8-ketodCF and 8-ketoCoF may be intermediates in the biosynthesis of dCF and CoF, support and extend our earlier results from in vivo studies which established that adenosine and C-1 of D-ribose are the carbon–nitrogen precursors of dCF. A possible mechanism for the formation of dCF is presented.

2′-Deoxycoformycin (dCF)[1] and coformycin (CoF) are nucleoside antibiotics produced in trace amounts by *Streptomyces antibioticus* (Ryder et al., 1975; Woo et al., 1974). The heterocyclic moiety of these compounds, which contains a 1,3-diazepine ring, is similar to that of a purine ring except that an additional CH_2 is located between N-1 and C-6 (Figure 1A).

dCF has been used in the treatment of T-cell acute lymphoblastic leukemia [for review see *Cancer Treatment Symposia* (1984)], chronic lymphoblastic leukemia (Grever et al.,

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[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PRPP, 5-phosphoribose 1-pyrophosphate; Tris, tris(hydroxymethyl)aminomethane. Other names for 2′-deoxycoformycin (dCF) include covidarabine (CoV) and pentostatin (USAN). The systematic name is (8*R*)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol. The systematic name for coformycin (CoF) is (8*R*)-3-(β-D-ribofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol.

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