1.alpha.,25,26-Trihydroxyvitamin D3: an in vivo and in vitro metabolite of vitamin D3

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actually involved in the two very different processes. Detailed structural work will have to be performed with aldehyde dehydrogenase to determine the groups whose p*Ks* were identified.

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1α,25,26-Trihydroxyvitamin D_3: An in Vivo and in Vitro Metabolite of Vitamin D_3†

Timothy A. Reinhardt, Joseph L. Napoli,* Bikash Praminik, E. Travis Littledike, Donald C. Beitz, John J. Partridge, Milan R. Uskokovič, and Ronald L. Horst

ABSTRACT: A new metabolite of vitamin D_3 has been isolated from the plasma of vitamin D_3 treated cows and has been generated from 25(*S*),26-dihydroxyvitamin D_3 with homogenates of vitamin D deficient chick kidney. This metabolite has been identified as 1,25,26-trihydroxyvitamin D_3 by comigration with synthetic 1,25(*S*),26-trihydroxyvitamin D_3 in four chromatographic systems, ultraviolet spectroscopy, mass spectrometry, and high-pressure liquid chromatography and mass spectrometry of derivatives. 1,25(*S*),26-Trihydroxyvitamin D_3 is one-tenth as effective as 1,25-dihydroxyvitamin D_3 in binding to the chick intestinal cytosol 1,25-dihydroxyvitamin D receptor. Either 25(*S*),26-dihydroxyvitamin D_3 or 1,25-dihydroxyvitamin D_3 can serve as precursor for in vitro production of 1,25,26-trihydroxyvitamin D_3 by chick kidney tissue.

Vitamin D undergoes extensive metabolism. One of its metabolites, 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$],[1] is a hormone that stimulates intestinal absorption of calcium and phosphate (Omdahl & DeLuca, 1973; Napoli & DeLuca, 1979; Stern, 1980). Possible functions of the other characterized metabolites are the subject of controversy. Moreover, the existence of many additional metabolites has been reported, but they remain to be isolated, identified, and studied (Norman, 1979). The extensive modification of this secosterol, which occurs to a large extent in the kidney, is reminiscent of cholesterol metabolism in the adrenal. Therefore, continued investigation of vitamin D metabolism promises to be interesting.

During the development of a protein-binding assay for 1,24,25-$(OH)_3D_3$ (Reinhardt et al., 1981), a previously unidentified vitamin D_3 metabolite, more polar than 1,24(*R*),25-$(OH)_3D_3$, was detected in the plasma of vitamin D_3

† From the Department of Physiopathology, National Animal Disease Center, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Ames, Iowa 50010 (E.T.L. and R.L.H.), Department of Biochemistry, The University of Texas Health Science Center, Dallas, Texas 75235 (J.L.N. and B.P.), Department of Animal Science, Iowa State University, Ames, Iowa 50011 (T.A.R. and D.C.B.), and Chemical Research Department, Hoffmann-La Roche, Inc., Nutley, New Jersey 07110 (J.J.P. and M.R.U.). *Received April 14, 1981.* This investigation was supported in part by National Institutes of Health National Research Service Award AM 06382 (T.A.R.), National Institutes of Health Grant AM 26535 (J.L.N.), by Robert A. Welch Foundation Grant I-797 (J.L.N.), by National Institutes of Health Shared Instrumentation Grant 27506 (UTHSCD), and by U.S. Department of Agriculture Grant 901-15-159 (D.C.B.).

[1] Abbreviations used: 25-OHD_3, 25-hydroxyvitamin D_3; 24(*R*),25-$(OH)_2D_3$, 24(*R*),25-dihydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1,24(*R*),25-$(OH)_3D_3$, 1,24(*R*),25-trihydroxyvitamin D_3; 1,25(*S*),26-$(OH)_3D_3$, 1,25(*S*),26-trihydroxyvitamin D_3; 25(*S*),26-$(OH)_2D_3$, 25(*S*),26-dihydroxyvitamin D_3; HPLC, high-pressure liquid chromatography; Me_3Si, trimethylsilyl; Tris, tris(hydroxymethyl)-aminomethane.

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treated cows. This paper reports the isolation of the metabolite and its identification as 1,25,26-trihydroxyvitamin D_3. This metabolite can be produced in chick kidney homogenates from either 25(*S*),26-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$. A preliminary report of this work has appeared (Reinhardt et al., 1981).

Materials and Methods

General. High-pressure liquid chromatography was performed on a Model LC-204 HPLC (Waters Associates, Milford, MA).[2] Mass spectra were obtained with the solids probe of a Finnigan automated EI/CI, GC/MS system coupled to an INCOS data system. Analyses were performed at 70 eV with an ionizer temperature of 250 °C while the probe was heated from ambient to 320 °C. Ultraviolet spectra were obtained in ethanol by using a Beckman DB recording spectrophotometer.

Animals. Jersey cows were treated with vitamin D_3 as described (Horst & Littledike, 1979). After 5 weeks of treatment, blood was collected in heparinized containers. Plasma was prepared by centrifugation and stored at −20 °C until extracted.

Sterols. Synthetic 1,25(*S*),26-$(OH)_3D_3$, 1,24(*R*),25-$(OH)_3D_3$, and 1,25-$(OH)_2D_3$ were provided by Hoffmann-La Roche (Nutley, NJ). 25(*S*),26-Dihydroxyvitamin D_3 was isolated from the plasma of a pig treated with vitamin D_3, and its structure was confirmed by mass spectroscopy (Horst et al., 1981a).

25(*S*),26-$(OH)_2$[23,24-$^3H_2$]D_3 and 1,25(*S*),26-$(OH)_3$-[23,24-$^3H_2$]D_3 were prepared from 25-OH[23,24-$^3H_2$]D_3 and 1,25-$(OH)_2$[23,24-$^3H_2$]D_3, respectively, with chick kidney homogenates. Chick kidney 26-hydroxylase activity was stimulated by treatment of chicks as follows. One-day-old chicks were fed a 1% calcium vitamin D deficient diet for 7 days. On day 8, the diet was changed to a 3% calcium vitamin D deficient diet. On days 11 and 12, the chicks received an intramuscular injection of 26 nmol of vitamin D_3 in 0.1 mL of ethanol. On day 14, kidney homogenates were prepared, and incubations were carried out as described by Tanaka et al. (1978). 1α-Hydroxylated 25(*S*),26-$(OH)_2D_3$ compounds were biosynthesized by using a procedure developed to 1α-hydroxylate 25-OH-D_3 (Tanaka et al., 1975). Briefly, a glass Potter–Elvehjem homogenizer with a Teflon pestle was used to prepare a 20% (w/v) homogenate in buffer A, consisting of 15 mM Tris–acetate, 150 mM sucrose, and 2 mM magnesium acetate, pH 7.4. To the homogenate (3 mL) in a 25-mL Erlenmeyer flask was added buffer A containing 7.5 mM sodium succinate (1.5 mL) and 10–100 pmol of substrate in ethanol (50 μL). The mixture was gassed with oxygen–carbon dioxide (95:5) for 1 min, sealed, and incubated at 37 °C for 1 h with shaking. The reaction was quenched by adding methanol–chloroform (2:1, 17 mL). Chloroform (6 mL) was then added, and the phases were separated. The aqueous phase was extracted with an additional 6 mL of chloroform. The combined chloroform phases were dried under a stream of N_2, and the products were separated by chromatography of the residue.

In vitro generated 1,25(*S*),26-$(OH)_3$[23,24-$^3H_2$]D_3 was purified on a Sephadex LH-20 column (1.8 × 25 cm) eluted with chloroform–hexane–methanol (75:23:2). Forty fractions (10 mL) were collected. The major polar radioactive peak eluting from 280 to 350 mL was pooled, and the solvent was dried under a stream of N_2. This radioactive vitamin D_3 metabolite was homogeneous and comigrated with synthetic 1,25(*S*),26-$(OH)_3D_3$ in the HPLC systems described (Figure 1). Therefore, it was used as a tracer in the purifications of both in vivo and in vitro generated metabolites.

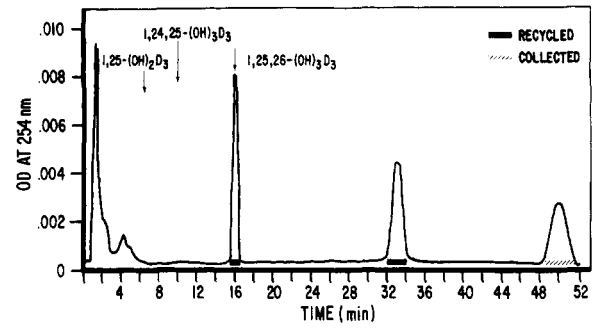

FIGURE 1: Final chromatogram of 1,25,26-$(OH)_3D_3$ purified from bovine plasma. The sample was recycled through the column twice for a total of three passes to confirm homogeneity before collection. The elution positions of synthetic standards are indicated.

Isolation of the Vitamin D_3 Metabolites. Plasma (16 L) from vitamin D_3 treated cows was extracted by the procedure of Bligh & Dyer (1959). The chloroform phase was evaporated under reduced pressure, and 1,25(*S*),26-$(OH)_3$[23,24-$^3H_2$]D_3 (0.25 μCi) in ethanol was added to the residue. The residue was dissolved in eluting solvent and applied to a Sephadex LH-20 column (4 × 60 cm) which was eluted with 1.5 L of chloroform–hexane–methanol (50:48:2). The column contents were removed and extracted twice with methanol (2 L each time). From this point on, the metabolite generated from 25,26-$(OH)_2D_3$ by chick kidney homogenates was purified by using these same procedures. The methanol was evaporated under reduced pressure, and the residue was chromatographed on a Sephadex LH-20 column (1.8 × 25 cm) eluted with chloroform–hexane–methanol (75:23:2). The region corresponding to the elution of 1,25(*S*),26-$(OH)_3$-[23,24-$^3H_2$]D_3 was collected (260–350 mL), and the solvent was evaporated under reduced pressure. The residue was further purified by HPLC on a Zorbax ODS column (0.46 × 25 cm) eluted with methanol–H_2O (75:25) at a flow rate of 2 mL/min. The region corresponding to the elution of synthetic 1,25(*S*),26-$(OH)_3D_3$ (24–30 mL) was collected, and the solvent was dried under a stream of N_2. The recovered material was rechromatographed on a Zorbax Sil column (0.46 × 25 cm) eluted with methylene chloride:isopropanol (93:7). The region corresponding to the elution of synthetic, 1,25-(*S*),26-$(OH)_3D_3$ (22-26 mL) was collected and the solvent was dried under a stream of N_2. The final purification and confirmation of homogeneity was done on a Zorbax Sil column (0.46 × 25 cm) developed with hexane–2-propanol (83:17). The major 254-nm-absorbing peak eluted in the same position as synthetic 1,25(*S*),26-$(OH)_3D_3$ and comigrated with the 1,25(*S*),26-$(OH)_3$[23,24-$^3H_2$]D_3 added at the beginning of the purification. The metabolite was recycled twice, for a total of three passes through the column, to confirm homogeneity. The peak (48–58 mL) was then collected and subjected to structural analysis.

Silylations. Compounds were silylated by heating them at 85 °C in 50 μL of TRI-SIL/TBT for 2 h. TRI-SIL/TBT is a mixture of (trimethylsilyl)imidazole, bis(trimethylsilyl)-acetamide, and trimethylchlorosilane (3:3:2) (Pierce Chemical Co., Rockford, IL). Approximately 100 ng of the biological samples were silylated whereas several micrograms of synthetic 1,25,26-$(OH)_3D_3$ were silylated. At the end of the reaction, the mixture was cooled, and methanol (100 μL) was added.

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

Aliquots or the whole sample were purified by HPLC on a Zorbax ODS column (0.46 × 25 cm) developed in methylene chloride–methanol (3:17).

Sodium Metaperiodate Cleavage. Biological samples (~ 100–200 ng) or synthetic 1,25(*S*),26-$(OH)_3D_3$ (1–2 μg) in 50 mL methanol were allowed to react with 50 μL of 5% sodium metaperiodate in water for 30 min at room temperature. The reaction mixture was dried under a stream of nitrogen and chloroform was added to the residue. The chloroform-soluble compounds were transferred to another vessel and evaporated. The sample was dissolved in hexane–methanol–chloroform (78:7:15) and purified by HPLC with a Zorbax ODS column (0.46 × 25 cm) eluted in the same solvent.

Receptor Binding Assay. Chick intestinal cytosol was prepared as described by Reinhardt et al. (1981), and the assay was performed as described by Horst et al. (1981a,b). Briefly, 1,25-$(OH)_2$[23,24-$^3H_2$]D_3 was displaced from the receptor with increasing concentrations of synthetic 1,25-$(OH)_2D_3$, 1,24(*R*),25-$(OH)_3D_3$, 1,25(*S*),26-$(OH)_3D_3$, or 1,25(*S*),26-$(OH)_3D_3$ generated in vitro from 25(*S*),26-$(OH)_2D_3$.

Results

Approximately 500 ng of this new vitamin D_3 metabolite was isolated from 16 L of cow plasma. Additionally, 6 μg of the metabolite were obtained from the incubation of 50 μg of 25,26-$(OH)_2D_3$ with kidney homogenates from vitamin D deficient chicks. Each metabolite was homogeneous, as illustrated in Figure 1, for the material isolated from bovine plasma.

An ultraviolet spectrum was obtained of 1,25(*S*),26-$(OH)_3D_3$ prepared from 25(*S*),26-$(OH)_2D_3$ with chick kidney homogenates. The spectrum was typical of the vitamin D_3 *cis*-triene chromophore with a λ_{max} at 264 nm, and a λ_{min} at 228 nm, and a $\lambda_{max}/\lambda_{min}$ of 1.6. We were unable to obtain a spectrum of the in vivo isolated compound because of its low abundance.

The mass spectra of 1,25(*S*),26-$(OH)_3D_3$ produced by chick kidney homogenates or isolated from the plasma of vitamin D_3 treated cows were like that of synthetic 1,25(*S*),26-$(OH)_3D_3$ (Figure 2). Each spectrum showed the expected molecular ion at *m/e* 432, with three sequential losses of water giving peaks at *m/e* 414, 396, and 378. The peak at *m/e* 381 resulted from the loss of a methyl group from *m/e* 396. The peak at *m/e* 269 arose from dehydration of the A ring and side-chain cleavage. Dehydration of *m/e* 269 produced *m/e* 251. Formal cleavage between carbons 7 and 8 ultimately results in peaks at *m/e* 152 and 134. The peak at *m/e* 152 represented the A ring plus carbons 6 and 7 fragment. Loss of water from *m/e* 152 yielded the base peak at *m/e* 134, which is typical of vitamin D compounds with intact *cis*-triene systems (Okamura et al., 1976).

For confirmation of the existence of a side-chain vicinal diol for both the in vivo and in vitro metabolites, they, as well as synthetic 1,25(*S*),26-$(OH)_3D_3$, were treated with sodium metaperiodate. In each case, HPLC of the crude reaction mixture after periodate treatment demonstrated the disappearance of 1,25,26-$(OH)_3D_3$ and the appearance of a new compound which was less polar than the parent compound. The 27-nor-25-keto product of synthetic 1,25(*S*),26-$(OH)_3D_3$ eluted at 13 mL in the system used (data not shown). The 1,25,26-$(OH)_3D_3$ isolated from in vivo and in vitro sources gave the same results (Figure 3). The identity of the product was confirmed by mass spectroscopy (Figure 4). The molecular ion at *m/e* 400 in both samples indicated at new compound which differed from the original compound by formal loss of methanol. Peaks at *m/e* 382 and 364 resulted from sequential loss of two water molecules. The peaks at *m/e* 269, 251, 152, and the base peak at *m/e* 134 were generated in the same manner as discussed for 1,25(*S*),26-$(OH)_3D_3$. Unfortunately, there was insufficient material to obtain a mass spectrum on the periodate cleavage product of 1,25,26-$(OH)_3D_3$ isolated from bovine blood.

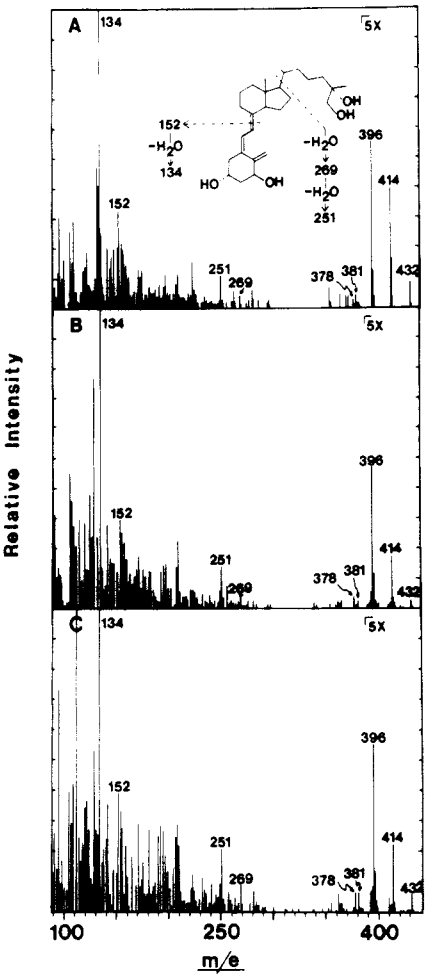

FIGURE 2: Mass spectra of 1,25,26-$(OH)_3D_3$. (A) Synthetic; (B) produced by chick kidney homogenates; (C) isolated from the blood of vitamin D treated cows. The peaks above *m/e* 390 have been increased 5-fold.

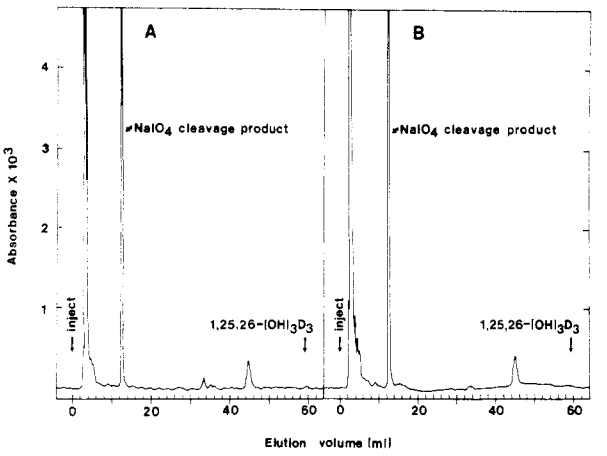

FIGURE 3: High-pressure liquid chromatogram of 1,25,26-$(OH)_3D_3$ sodium metaperiodate cleavage products. (A) Product of 1,25,26-$(OH)_3D_3$ produced by chick kidney homogenates; (B) product of 1,25,26-$(OH)_3D_3$ isolated from the blood of vitamin D treated cows. The chromatography was done with a Zorbax Sil column (0.46 × 25 cm) developed with hexane–methanol–chloroform (79:7:15) at a flow rate of 2.0 mL/min.

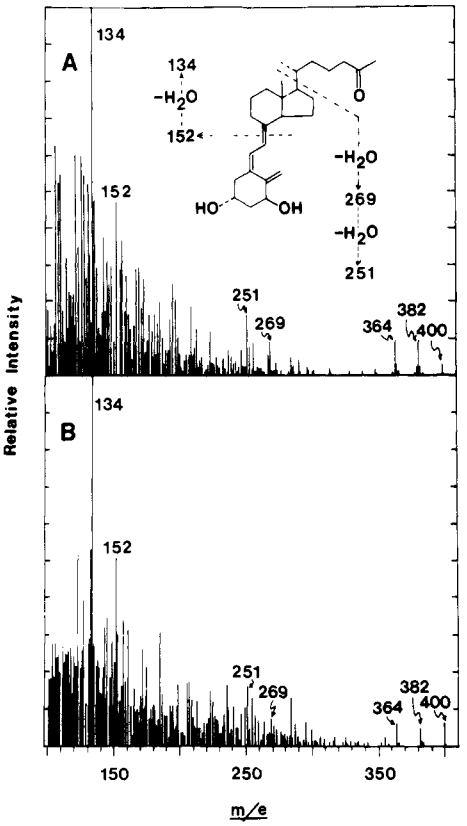

FIGURE 4: Mass spectra of 1,25,26-$(OH)_3D_3$ sodium periodate cleavage products obtained from (A) Synthetic 1,25(*S*),26-$(OH)_3D_3$ and (B) 1,25,26-$(OH)_3D_3$ produced by chick kidney homogenates.

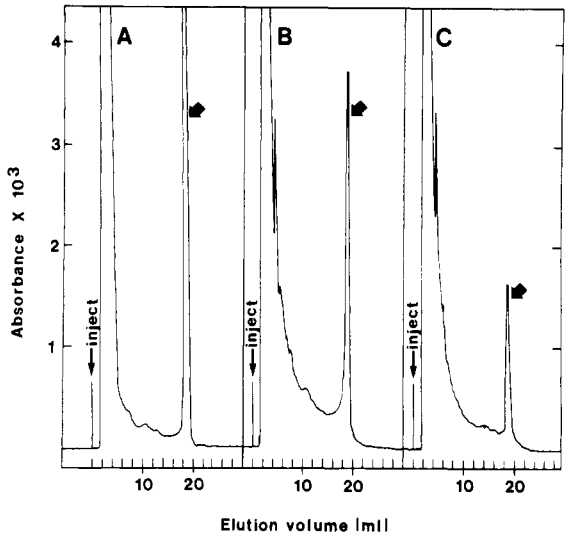

FIGURE 5: Nonaqueous reverse-phase HPLC of $(Me_3Si)_4$-1,25,26-$(OH)_3D_3$. (A) Synthetic 1,25,26-$(OH)_3D_3$; (B) 1,25,26-$(OH)_3D_3$ produced by chick kidney homogenates; (C) 1,25,26-$(OH)_3D_3$ isolated from the blood of vitamin D treated cows. The wide arrow indicates the elution position of the silylated materials. The column used was a 0.46 × 25 cm Zorbax ODS developed with methylene chloride–methanol (3:17) at a flow rate of 2 mL/min.

Additional evidence for the structural assignment was obtained by silylation of this new vitamin D_3 metabolite. After silylation, the reaction mixtures were individually applied directly to HPLC without preliminary purification (Figure 5). The silylating reagent produced nonvolatile byproducts. This, plus the large excess of reagent used, was responsible for the appearance of an intense absorbance at the solvent front. Each silylated sample of 1,25,26-$(OH)_3D_3$ gave rise to a peak which

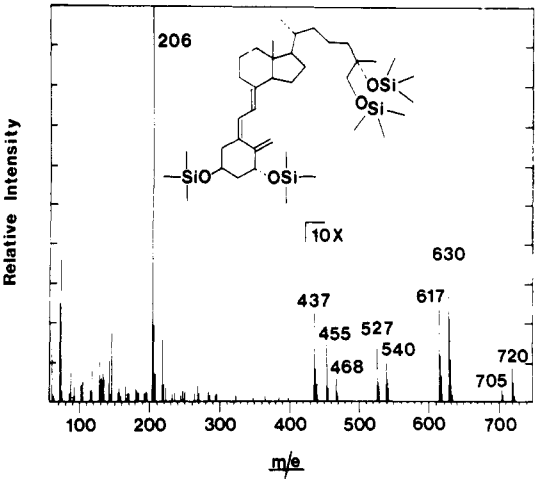

FIGURE 6: Mass spectrum of tetratrimethylsilyl-1,25,26-$(OH)_3D_3$ after purification by HPLC as described in Figure 4. The peaks after *m/e* 400 have been amplified 10-fold.

eluted at 18 mL (Figure 5). On the other hand, partially silylated 1,25,26-$(OH)_3D_3$'s eluted before 12 mL in this system. A mass spectrum of the peak that eluted at 18 mL confirmed its identity as $(Me_3Si)_4$-1,25,26$(OH)_3D_3$ (Figure 6). The series of peaks in the high mass region were diagnostic of a compound possessing at least one primary alcohol and three other hydroxyl groups. The molecular ion at *m/e* 720, for example, demonstrated the addition of four Me_3Si groups to 1,25,26-$(OH)_3D_3$. Loss of a methyl group from *tetra*-TMS-1,25,26-$(OH)_3D_3$ produced *m/e* 705. Loss of $(CH_3)_3SiOH$ from *m/e* 720 yielded *m/e* 630. The peak at *m/e* 617 resulted from loss of $(CH_3)_3SiOCH_2$ and, therefore, confirmed the presence of a primary alcohol function. This peak was not observed in synthetic $(Me_3Si)_4$-1,24,25-$(OH)_3D_3$ (J. L. Napoli, unpublished results). Peaks at *m/e* 540, 527, and 468 represented loss from the molecular ion of two $(CH_3)_3SiOH$ groups, $(CH_3)_3SiOH$ and $(CH_3)_3SiOCH_2$, and two $(CH_3)_3$-SiOH groups plus a $(CH_3)_2SiCH_2$ group, respectively. The peak at *m/e* 455 resulted from loss of $(CH_3)_3SiOCH_2$, $(CH_3)_3SiOH$, and a $(CH_3)_2SiCH_2$ from *m/e* 720 whereas that at *m/e* 437 resulted from loss of two $(CH_3)_3SiOH$ groups and a $(CH_3)_2SiOCH_2$ group. The base peak at *m/e* 206 confirmed the presence of a vitamin D_3 like *cis*-triene system. It arose from cleavage between carbons 7 and 8 and loss of $(CH_3)_3$-SiOH to give an A ring plus carbons 6 and 7 fragment. In other words, it is a Me_3Si derivative of the peak at *m/e* 134 that is occurring as the base peak in unsilylated 1α-hydroxyvitamin D_3 metabolites. Insufficient material was available to obtain mass spectra of the $(Me_3Si)_4$-1,25,26-$(OH)_3D_3$ products of 1,25,26-$(OH)_3D_3$ isolated from cow plasma and chick kidney homogenates.

Preliminary evidence indicates that 1,25-$(OH)_2D_3$ as well as 25,26-$(OH)_2D_3$ can serve as precursors in the biosynthesis of 1,25(*S*),26-$(OH)_3D_3$ in chick kidney homogenates (Figure 7). In these experiments, 25,26-$(OH)_2D_3$ was used as substrate when the chicks were vitamin D_3 deficient, and 1,25-$(OH)_2D_3$ was used as substrate when chicks were vitamin D repleted. Clearly, compounds that comigrated with 1,24,25-$(OH)_3D_3$ and 1,25,26-$(OH)_3D_3$ were generated in vitro from 1,25-$(OH)_2D_3$.

1,25,26-Trihydroxyvitamin D_3 was only one-tenth as effective as 1,25-$(OH)_2D_3$ in binding to the chick intestinal receptor (Figure 8). The amount of 1,25-$(OH)_2D_3$ that caused 50% displacement of 1,25-$(OH)_2$-[26,27-$^3H_2$]D_3 was 28 pg compared to concentrations of 90, 282, and 310 pg for 1,24(*R*),25-$(OH)_3D_3$, synthetic 1,25(*S*),26-$(OH)_3D_3$, and

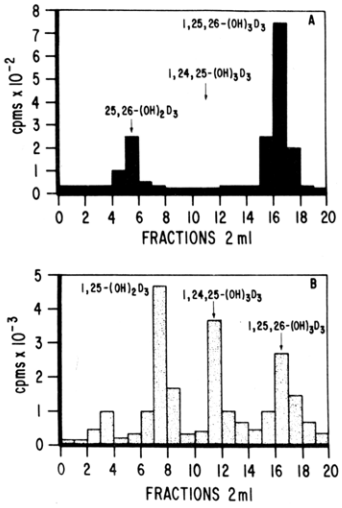

FIGURE 7: HPLC profiles of lipid extracts from chick kidney homogenates after incubation with either 25(*S*),26-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$. (A) Kidney homogenates were prepared from vitamin D deficient chicks and incubated with 25,26-$(OH)_2$[23,24-$^3H_2$]D_3; (B) kidney homogenates were prepared from vitamin D_3 repleted chicks and incubated with 1,25-$(OH)_2$[23,24-$^3H_2$]D_3. Aliquots of the lipid extract from each incubation were applied to a Zorbax Sil column (0.46 × 25 cm) developed in hexane–2-propane (83:17) at a flow rate of 2 mL/min. Fractions (2 mL) were collected, and the amount of radioactivity was determined. Arrows indicate the elution positions of synthetic standards.

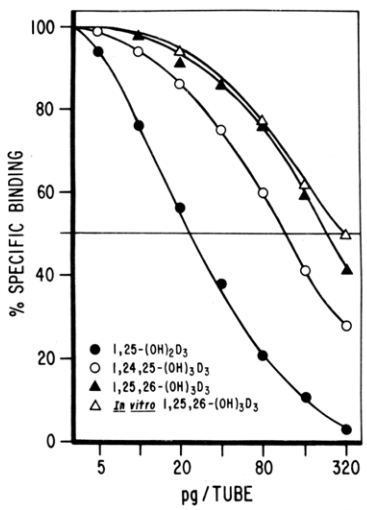

FIGURE 8: Binding assay comparing competition of synthetic 1,25-(*S*),26-$(OH)_3D_3$, in vitro generated 1,25,26-$(OH)_3D_3$, or 1,24-(*R*),25-$(OH)_3D_3$ with 1,25-$(OH)_2D_3$ for the chick intestinal cytosolic 1,25-$(OH)_2D_3$ specific receptor.

1,25,26-$(OH)_3D_3$ generated in vitro from 25,26-$(OH)_2D_3$, respectively.

Discussion

This paper describes the isolation and structural characterization of 1,25,26-$(OH)_3D_3$ produced both in vivo and in vitro. Comigration of the metabolite isolated from the plasma of vitamin D_3 treated cows with the metabolite produced from 25,26-$(OH)_2D_3$ by vitamin D deficient chick kidney homogenates as well as with synthetic 1,25(*S*),26-$(OH)_3D_3$ provided strong evidence that the new metabolite is 1,25,26-$(OH)_3D_3$. This conclusion is reinforced by the mass spectra which indicate that the biologically generated compounds are 1-hydroxylated vitamin D_3 derivatives with intact *cis*-triene systems and two side-chain hydroxyl groups. Further confirmation of these conclusions was provided by the HPLC and mass spectroscopy of derivatives. The periodate cleavage data clearly indicated that vicinal diols are present in the side chain and pinpoint the location at the 25- and 26-carbons. These structural assignments were strongly supported by the observation that hydroxylation of 1,25-$(OH)_2D_3$ by chick kidney homogenates from vitamin D repleted chicks and hydroxylation of 25(*S*),26-$(OH)_2D_3$ by chick kidney homogenates from vitamin D deficient chicks apparently produce the same metabolite. Thus, the new metabolite is conclusively identified as 1,25,26-$(OH)_3D_3$.

These data do not permit determination as to whether the configuration of the 25-carbon is *R* or *S*. However, it has recently been shown that natural 25,26-$(OH)_2D_3$ possesses the 25(*S*) absolute configuration (Partridge et al., 1980, 1981a). Since 25(*S*),26-$(OH)_2D_3$ is metabolized to 1,25,26-$(OH)_3D_3$, this metabolite should also possess the 25(*S*) absolute configuration. The receptor binding assay supports this notion. 1,25,26-Trihydroxyvitamin D_3 generated in vitro from 25-(*S*),26-$(OH)_2D_3$ was indistinguishable from synthetic 1,25-(*S*),26-$(OH)_3D_3$ in the receptor assay. This assay has discriminated between side-chain hydroxyl isomers of vitamin D_3 metabolites such as 1,24(*R*)-$(OH)_2D_3$ and 1,24(*S*)-$(OH)_2D_3$ (Napoli et al., 1979) and also 1,24(*R*),25-$(OH)_3D_3$ and 1,24(*S*),25-$(OH)_3D_3$ (Eisman & DeLuca, 1977). Therefore, it is probable, but not certain, that naturally occurring 1,25,26-$(OH)_3D_3$ has the 25(*S*) configuration.

Additionally, the receptor binding assay data show that 1,25(*S*),26-$(OH)_3D_3$ is 3–4 times less potent than 1,24-(*R*),25-$(OH)_3D_3$. In our hands, 1,24(*R*),25-$(OH)_3D_3$ is 3 times more potent than 1,25-$(OH)_2D_3$. Previous work suggested a difference of greater than 10-fold between 1,24-(*R*),25-$(OH)_3D_3$ and 1,25-$(OH)_2D_3$. This difference could be attributed to our use of a semipurified receptor compared to the crude cytosol used by Eisman & DeLuca (1977). These results indicate that previous work evaluating the binding affinities of vitamin D_3 metabolites by using crude chick cytosol as a source of 1,25-$(OH)_2D$ receptor must be reevaluated.

A role for 1,25,26-$(OH)_3D_3$ in calcium and phosphate metabolism is not immediately obvious. However, the data of Thomasset et al. (1978) suggest that 1,25,26-$(OH)_3D_3$ does possess intestinal-calcium transport and bone-calcium mobilization activity. They dosed 25,26-$(OH)_2D_3$ to vitamin D deficient rats and determined that there was a lag in the onset of its activity and that bilateral nephrectomy abolished its activity altogether. Vitamin D deficient rats have an induced 1α-hydroxylase which is located exclusively in kidney (Tanaka & DeLuca, 1974). Moreover, their 24(*R*)-hydroxylase is suppressed. Therefore, it is highly probable that the active form of 25,26-$(OH)_2D_3$ is 1,25,26-$(OH)_3D_3$.

Unlike other animals, cattle are known to increase production of 1,25-$(OH)_2D_3$ as well as 25,26-$(OH)_2D_3$ in response to vitamin D treatment (Horst & Littledike, 1979; Reinhardt & Conrad, 1980). It is, therefore, noteworthy that 1,25,26-$(OH)_3D_3$ has been found in vitamin D_3 treated cows. This finding, plus the probable in vivo activity of 1,25,26-$(OH)_3D_3$ indicated by the data of Thomasset et al. (1978) and by the receptor protein binding data reported here, makes important an understanding of the effects of 1,25,26-$(OH)_3D_3$ on calcium and phosphate metabolism.

This work has established the identity of a new vitamin D_3 metabolite as 1,25,26-$(OH)_3D_3$ and has highlighted new pathways of vitamin D_3 metabolism.

Added in Proof

The absolute configuration of 1α,25,26-trihydroxyvitamin D_3 isolated from bovine plasma has been conclusively shown to be 25*S* by HPLC comparison of the natural product to

synthetic 25(S)- and 25(R)-1α,25,26-trihydroxyvitamin D_3 (Partridge et al., 1981b).

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Nonrandom Substitution of 2-Aminopurine for Adenine during Deoxyribonucleic Acid Synthesis in Vitro†

Reynaldo C. Pless,‡ Lore M. Levitt, and Maurice J. Bessman*

ABSTRACT: The incorporation of the deoxyribonucleotide of 2-aminopurine [(AP)] for deoxyadenylate into deoxyribonucleic acid (DNA) in vitro has been examined by using five highly purified DNA polymerases: calf thymus polymerase α, *Escherichia coli* polymerase I, and the polymerases induced by T4 phage mutant L56 (mutator phenotype), wild-type T4 phage, and T4 phage mutant L141 (antimutator phenotype). On a template of gapped salmon sperm DNA, the overall incorporation of (AP) relative to the incorporation of adenine decreases in this series of enzymes, in line with the increasing 3′-exonucleolytic activity associated with these polymerases. The nearest-neighbor distributions for (AP) and for adenine in the newly synthesized DNA were determined to test for potential sequence selectivity in the incorporation of (AP). In polymerizations in which d(AP)TP fully replaced dATP, the L141 polymerase, and to a lesser degree the wild type T4 polymerase, synthesized a DNA in which the distribution for (AP) was distinctly skewed compared to the nearest-neighbor distribution observed for adenine; incorporation of (AP) was relatively favored after guanine and disfavored after adenine and thymine. These sequence effects were less pronounced in syntheses in which both dATP and d(AP)TP were present. When dGTP was replaced by dITP, or dTTP by dUTP, adenine was still incorporated to the normal extent after the analogue, but the incorporation of (AP) was reduced after these analogues, which form weaker base pairs. The results indicate that incorporation of (AP) is disfavored with all polymerases tested and that a greater bias exists with those polymerases containing an active 3′-exonuclease. This bias against (AP) incorporation is alleviated after strong base pairs, and particularly following guanine, possibly due to stabilizing vertical stacking interactions.

Benzer's (Benzer, 1961) elegant analysis of the rII cistron of bacteriophage T4 demonstrated that spontaneous mutations were not distributed randomly throughout the gene and that certain loci ("hot spots") mutated at much higher frequencies than others. He suggested, at that time, that one of the factors which might contribute to this nonrandomness was the thermodynamic stability of the deoxyribonucleic acid (DNA)[1] in a particular region, and he reasoned that less stable regions

† From the McCollum-Pratt Institute and Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218. *Received February 18, 1981.* This is Contribution No. 1110 from the McCollum-Pratt Institute. This work was supported by Grant No. GM18649 from the National Institutes of Health.

‡ Present address: Department of Chemistry, The Ohio State University, Columbus, OH 43210.

[1] Abbreviations used: (AP), 2-aminopurine; (AP)dR, 2-aminopurine deoxyriboside; d(AP)MP and d(AP)TP, the mono- and triphosphate of (AP)dR; DNA, deoxyribonucleic acid; Tris, tris(hydroxymethyl)aminomethane.

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