Measurement of 25,26-Dihydroxyvitamin D: Importance of the Configuration of the C-25 Hydroxyl Group†

Daniel D. Bikle,* Bernard P. Halloran, Joanne O. Whitney, and Bruce W. Hollis

ABSTRACT: 25,26-Dihydroxyvitamin D_3 [25,26$(OH)_2D_3$] is chemically synthesized as two stereoisomers: 25(*R*),26-$(OH)_2D_3$ and 25(*S*),26$(OH)_2D_3$. Both the *R* and *S* configurations have been claimed to be the natural form. Previous studies, however, have not considered the possibility that the stereochemical configuration of the C-25 hydroxyl group could affect the binding of 25,26$(OH)_2D_3$ to the rat serum binding protein used in assays for the measurement of this metabolite. In our study, a 50% displacement of radiolabeled 25-hydroxyvitamin D_3 ([^{3}H]25OHD_3) from its initial binding is achieved with 325 fmol/mL 25(*R*),26$(OH)_2D_3$ and 850–1000 fmol/mL 25(*S*),26$(OH)_2D_3$—a difference in potency of approximately 3-fold. The potency of the *R* isomer in displacing [^{3}H]25OHD_3 was similar to that of 25OHD_3 or 24(*R*),25-$(OH)_2D_3$. When 25,26$(OH)_2$D levels were measured in 12 normal subjects with both the *R* isomer and the *S* isomer as standards, the results were 625 ± 360 fmol/mL 25(*R*),26-$(OH)_2D_3$ equiv and 1800 ± 1130 fmol/mL 25(*S*),26$(OH)_2D_3$ equiv. To determine which stereoisomer is biosynthesized, two types of experiments were performed. In the first, radiolabeled 25,26$(OH)_2D_3$ was biosynthesized from [^{3}H]25OHD_3 by using chick renal mitochondria and compared to [^{3}H]25OHD_3 as a ligand in the rat serum binding protein assay, which was used to measure 25OHD_3, 25(*R*),26$(OH)_2D_3$, and 25(*S*),26-$(OH)_2D_3$. Initial binding of radiolabeled 25OHD_3 and 25,26$(OH)_2D_3$ to the binding protein was comparable, as was their displacement by the nonradioactive metabolites, indicating that chick renal mitochondria produce primarily 25-(*R*),26$(OH)_2D_3$. However, when we attempted to confirm this finding by using a chromatographic system that separated the tris(trimethylsilyl) ether [$(Me_3Si)_3$] derivatives of 25(*R*),26-$(OH)_2D_3$ and 25(*S*),26$(OH)_2D_3$, we observed that the $(Me_3Si)_3$ derivative of radiolabeled 25,26$(OH)_2D_3$ could be partially separated from the nonlabeled derivatives of the *R* and *S* isomers. These results suggest that radiolabeled 25,26$(OH)_2D_3$ cannot be used to identify the nonradiolabeled natural form of 25,26$(OH)_2D_3$ by this approach. When nonradiolabeled 25,26$(OH)_2D_3$ was purified from the blood of a vitamin D intoxicated cow, it formed two $(Me_3Si)_3$ derivatives in approximately equimolar amounts, each of which comigrated on high-performance liquid chromatography with one of the $(Me_3Si)_3$ derivatives of chemically synthesized 25(*R*),26$(OH)_2D_3$ and 25(*S*),26$(OH)_2D_3$. Thus, both stereoisomers of 25,26$(OH)_2D_3$ appear to circulate in this animal model. These data suggest that both stereoisomers can be biosynthesized and that knowledge of the ratio of the circulating forms is important for accurate measurement of 25,26$(OH)_2$D by existing techniques.

The vitamin D metabolite 25,26-dihydroxyvitamin D [25,26$(OH)_2$D][1] has remained an enigma since its identification by Suda et al. in 1970. No unique biologic role has been identified for this metabolite, although it is capable of stimulating intestinal calcium transport (Suda et al., 1970), calcium binding protein (Thomasset et al., 1978), bone calcium mobilization (idem), and bone mineralization (Queille et al., 1978) when administered in vivo. Bilateral nephrectomy blocks these biologic responses (Lam et al., 1975), suggesting that 1α-hydroxylation of 25,26$(OH)_2$D may be required for biologic activity. Although the kidney produces 25,26$(OH)_2$D (Tanaka et al., 1978), nephrectomized humans appear to have normal circulating levels of 25,26$(OH)_2$D (Horst et al., 1979). Thus, there may be nonrenal sources of production as well.

25,26$(OH)_2D_3$ can exist as two stereoisomers with the C-25 configuration in either the *R* or *S* position. It is unclear whether the stereoisomers differ in their biologic activity (Thomasset & Redel, 1980), although it is possible that only the *R* form is metabolized to the 25-hydroxyvitamin D_3 26,23-lactone (Ishizuda et al., 1982), as biologic studies suggest (Hollis et al., 1980). Several efforts to determine the configuration of C-25 in naturally occurring 25,26$(OH)_2D_3$ have been made, but with disparate results. By using radiolabeled 25-hydroxyvitamin D_3 (25OHD_3) as substrate, Redel et al. (1978) determined that chick kidney homogenates produced the *R* isomer. In subsequent studies Redel and colleagues (1979) found that a human patient given radiolabeled vitamin D_3 also produced the *R* isomer. This assignment was challenged by Partridge et al. (1981), who indicated, on the basis of X-ray crystallography, that the assignment of the *R* and *S* configurations of the chemically synthesized standards used by Redel and colleagues had been reversed. Partridge and his coworkers indicated that they were able to purify enough 25,26$(OH)_2D_3$ from 72 mL of human blood to confirm its identity as 25(*S*),26$(OH)_2D_3$. More recently, Ikekawa et al. (1983) observed that chick renal homogenates in vitro and rats in vivo produce both the *R* and *S* isomers of 25,26$(OH)_2D_3$ from radiolabeled 25OHD_3 and that both isomers were present in 25,26$(OH)_2D_3$ isolated from sera (1 L) of 250 rats given massive doses of vitamin D_3.

Our interest in this problem originated from the observation that the stereoisomers of 25,26$(OH)_2D_3$ differed in their affinities for the rat serum binding protein used in their assay. This observation has implications not only for the measurement of 25,26$(OH)_2$D but also for the assessment of its biologic

† From the Department of Medicine, Veterans Administration Medical Center and University of California, San Francisco, California 94121 (D.D.B. and B.P.H.), the Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94121 (J.O.W.), and the Department of Nutrition, Case Western Reserve, Cleveland, Ohio 44106 (B.W.H.). *Received June 14, 1984.* Work performed at the Veterans Administration Medical Center and University of California, San Francisco, CA, was supported by NIH Grant AM 28116, NIH Research Resource Grant RR 01614, and funds from the Veterans Administration.

* Address correspondence to this author at the VA Medical Center, San Francisco, CA 94121.

[1] Abbreviations: 24,25$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; 25OHD_3, 25-hydroxyvitamin D_3; $(Me_3Si)_3$, tris(trimethylsilyl); HPLC, high-performance liquid chromatography.

0006-2960/84/0423-6920$01.50/0

activity. This paper discusses these observations and our efforts to determine the naturally occurring configuration of 25,26-(OH)$_2$D$_3$.

Materials and Methods

Vitamin D Metabolites and Derivatives. Chemically synthesized, nonradioactive 24(*R*),25(OH)$_2$D$_3$, 25(*R*),26(OH)$_2$D$_3$, and 25(*S*),26(OH)$_2$D$_3$ were gifts from M. R. Uskokovic (Hoffmann-La Roche, Nutley, NJ). 25OHD$_3$ was a gift from J. C. Babcock (Upjohn Co., Kalamazoo, MI). The concentrations of these metabolites were determined by ultraviolet spectroscopy. Radiolabeled [23,24-^{3}H]25OHD$_3$ (sp ac. 80 Ci/mmol) was obtained from Amersham (Arlington Heights, IL) and was used as substrate for the preparation of radiolabeled 25,26(OH)$_2$D$_3$. Radiolabeled 25,26(OH)$_2$D$_3$ was biosynthesized from a preparation of renal mitochondria from chicks raised for 2.5 weeks on a 3% calcium/0.65% phosphorus diet containing 20 μg of vitamin D$_3$/kg. Incubation conditions and initial purification have been described previously (Bikle et al., 1980). The identity of [^{3}H]25,26(OH)$_2$D$_3$ was established on the basis of cochromatography with chemically synthesized 25,26(OH)$_2$D$_3$ (the *R* or *S* configuration) on Whatman SG81 paper (H. Reeve Angel & Co., Clifton, NJ) eluted with ethyl acetate/chloroform/benzene (40:40:20), on a Sephadex LH-20 column eluted with hexane/chloroform/methanol (9:1:1), and on a Zorbax Sil HPLC column (Du Pont, Wilmington, DE) eluted with hexane/2-propanol (90:10 or 96:4) and by complete cleavage with periodate, which resulted in a new metabolite that was identified by chromatography on SG81 paper as a single peak (Bikle, 1980).

Bovine 25,26(OH)$_2$D$_3$ was prepared from 3 L of plasma from a cow given two injections of vitamin D$_3$ (30 × 10^6 international units) 5 and 3 weeks before blood was drawn. 25,26(OH)$_2$D$_3$ was purified from a methanol/dichloromethane extract by a combination of two chromatographic steps using a Sephadex LH-20 column [eluted with hexane/chloroform (65:35) and then hexane/methanol/chloroform (9:1:1)], followed by four purification steps using HPLC. Two of these steps used a μPorasil column (Waters, Milford, MA) eluted first with hexane/2-propanol in a ratio of 90:10 and then in a ratio of 95:5, one step used a C 18 μBondapak column (Waters) eluted with methanol/water (75:25), and the final step used a Zorbax Sil column eluted with dichloromethane-/2-propanol (96:4). Each purification step used a chromatographic system calibrated with chemically synthesized 25,26(OH)$_2$D$_3$, and only the peak from the bovine sample that migrated in the same elution position as the standard 25,26-(OH)$_2$D$_3$ was collected. None of these chromatographic steps resolves 25(*R*),26(OH)$_2$D$_3$ from 25(*S*),26(OH)$_2$D$_3$. The (Me$_3$Si)$_3$ derivatives of 25,26(OH)$_2$D$_3$ were produced by incubating the metabolites at 60 °C for 2 h in pyridine/hexamethyldisilazane with a few drops of trimethylchlorosilane to trigger the reaction (Whitney et al., 1979).

Analyses. 25,26(OH)$_2$D levels in normal sera were determined by the extraction and purification procedures described by Horst et al. (1979) with radiolabeled [^{3}H]25,26(OH)$_2$D$_3$ to monitor recovery (60–70%). The 25,26(OH)$_2$D peak from the final purification with HPLC was assayed in multiple dilutions with a 1/20 000 dilution of vitamin D deficient rat serum diluted in 0.05% BSA/0.05 M barbital acetate, pH 8.6, in a final volume of 1 mL. Chemically synthesized 25-(*R*),26(OH)$_2$D$_3$ and 25(*S*),26(OH)$_2$D$_3$ were used as standards, and radiolabeled 25OHD$_3$ was used as radioligand (Arnaud & Meger, 1983).

The structural identity of the (Me$_3$Si)$_3$ derivatives of chemically synthesized 25(*R*),26(OH)$_2$D$_3$ and 25(*S*),26-(OH)$_2$D$_3$ was established at high resolution by mass spectroscopy by using a direct insertion probe and a modified Kratos MS-902 mass spectrometer. The (Me$_3$Si)$_3$ derivatives were separated with an HPLC system (Waters) equipped with two linked μPorasil columns that were equilibrated for 2 h and then eluted with either hexane/dichloromethane/acetonitrile (96:4:04) or hexane/dichloromethane (85:15).

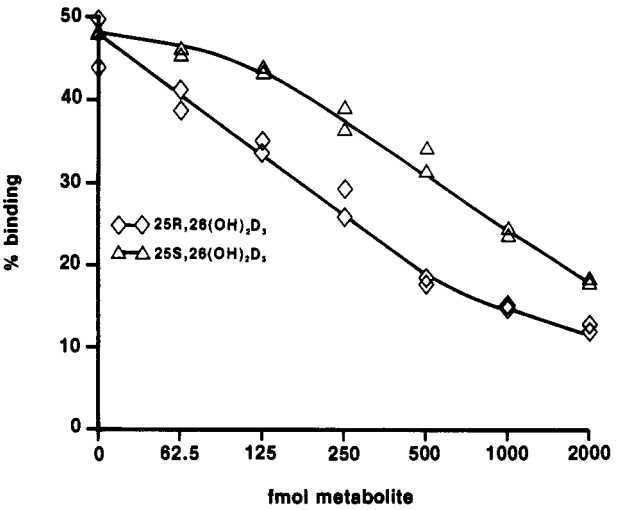

FIGURE 1: Displacement of [^{3}H]25OHD$_3$ by chemically synthesized 25(*R*),26(OH)$_2$D$_3$ and 25(*S*),26(OH)$_2$D$_3$ in the rat serum binding protein assay. Incubation volume was 1 mL.

Results

25(*R*),26(OH)$_2$D$_3$ and 25(*S*),26(OH)$_2$D$_3$ differed in their ability to displace [^{3}H]25OHD$_3$ from rat serum in the competitive binding protein assay commonly used to measure these metabolites (Figure 1). A total of 325 fmol/mL *R* isomer and 850 fmol/mL *S* isomer was required for 50% displacement of [^{3}H]25OHD$_3$. 25(*R*),26(OH)$_2$D$_3$ was similar in potency to 25OHD$_3$ and 24(*R*),25(OH)$_2$D$_3$ in this regard (Figure 2); all three were 2.5–3 times more potent than 25(*S*),26(OH)$_2$D$_3$. When measurements of 25,26(OH)$_2$D in the sera of 12 normal subjects were compared to the *R* and *S* isomers of 25,26-(OH)$_2$D$_3$ as standards, there was a mean concentration (±SD) of 625 ± 360 fmol/mL 25(R),26(OH)$_2$D$_3$ equiv or 1800 ± 1130 fmol/mL 25(*S*),26(OH)$_2$D$_3$ equiv.

Radiolabeled 25,26(OH)$_2$D$_3$ biosynthesized from chick renal mitochondria was compared directly to radiolabeled 25OHD$_3$ as radioligand in the rat serum protein binding assay (Figure 3). Initial binding of radiolabeled 25,26(OH)$_2$D$_3$ (11 fmol/mL) and radiolabeled 25OHD$_3$ (26 fmol/mL) was essentially the same in this assay [35 ± 3% and 31 ± 2.5% (mean ± range of duplicates), respectively]. The radiolabels were displaced equally well by chemically synthesized 25(*R*),25-(OH)$_2$D$_3$ and 25(*S*),26(OH)$_2$D$_3$, with 50% displacement observed at 320 and 1000 fmol/mL, respectively. The comparable initial binding of [^{3}H]25OHD$_3$ and [^{3}H]25,26(OH)$_2$D$_3$ to the serum binding protein at concentrations below that required for appreciable displacement, as well as the comparable displacement by nonradiolabeled 25,26(OH)$_2$D$_3$, indicates that these radioligands have comparable affinity for the serum binding protein and, therefore, that [^{3}H]25,26(OH)$_2$D$_3$ is mostly in the *R* configuration (the configuration comparable to 25OHD$_3$ in its affinity for the rat serum vitamin D binding protein). We calculated that initial binding of the *S* isomer would be approximately one-third that of [^{3}H]25OHD$_3$ and [^{3}H]25(*R*),26(OH)$_2$D$_3$ and that the *S* isomer would be displaced 50% by approximately one-third the concentration of 25,26(OH)$_2$D$_3$ that was required to displace [^{3}H]25OHD$_3$ and

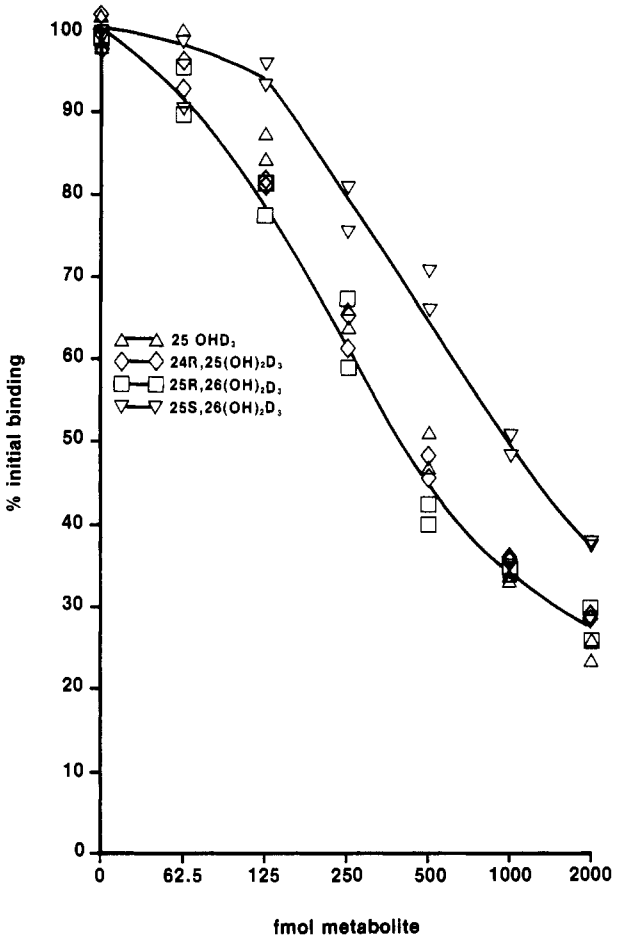

FIGURE 2: Displacement of [^{3}H]25OHD$_3$ by 25OHD$_3$, 24(R),25-(OH)$_2$D$_3$, 25(R),26(OH)$_2$D$_3$, and 25(S),26(OH)$_2$D$_3$ in the rat serum binding protein assay. Data are normalized to 100% initial binding, which was 38.2 ± 0.4, 41.0 ± 0.1, 42.8 ± 0.6, and 42.7 ± 0.1% (mean ± range of duplicates), respectively. The lines are fit to the data for the two stereoisomers of 25,26(OH)$_2$D$_3$. Incubation volume was 1 mL.

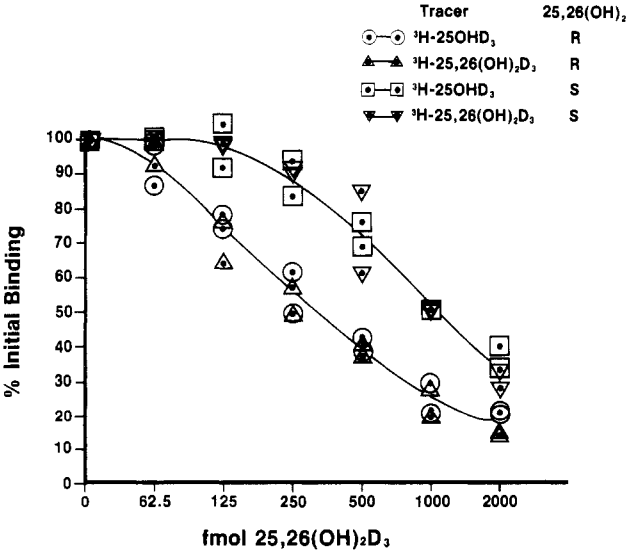

FIGURE 3: Comparison of [^{3}H]25OHD$_3$ and biosynthesized [^{3}H]-25,26(OH)$_2$D$_3$ as radioligands in the rat serum binding protein assay using chemically synthesized 25(R),26(OH)$_2$D$_3$ and 25(S),26(OH)$_2$D$_3$ as displacing standards. The radioligands had comparable initial binding (see text) and were displaced to the same extent by each stereoisomer of 25,26(OH)$_2$D$_3$.

[^{3}H]25(R),26(OH)$_2$D$_3$. To confirm this, we subjected biosynthesized [^{3}H]25,26(OH)$_2$D$_3$ to chromatography with chemically synthesized R and S isomers of 25,26(OH)$_2$D$_3$ as standards, under conditions in which these stereoisomers could be separated. This required the preparation of (Me$_3$Si)$_3$ derivatives.

The mass spectra of the (Me$_3$Si)$_3$ derivatives of the chemically synthesized isomers are shown in Figure 4. They are essentially identical. The molecular ion is at M$^+$· 632, and peaks formed by sequential loss of methyl and trimethylsilanol (Me$_3$SiOH) groups are seen at a mass to charge (m/z) of 617 (M − 15), 542 (M − 90), 527 (M − 90 −15), 452 (M −90 − 90), 437 (M −90 − 90 − 15), 362 (M −90 − 90− 90), and 347 (M −90 − 90− 90 − 15). The large peak at m/z 529 represents cleavage of the 25,26 bond and loss of CH$_2$OMe$_3$Si (M − 103), whereas the peaks at m/z 439 and 349 represent losses of a single Me$_3$SiOH + CH$_2$OMe$_3$Si fragment (M − 90 − 103) and two Me$_3$SiOH + CH$_2$OMe$_3$Si fragments (M − 90 −90 − 103), respectively. The peak at m/z 118 represents cleavage of the uncycled molecule at C-7,8 with loss of Me$_3$SiOH, and is diagnostic of an intact triene system. The small peak at m/z 501 (M − 131) is also diagnostic of vitamin D$_3$ metabolites and has been interpreted (Whitney et al., 1979) as representing fragmentation of the A ring, with the resulting loss of C-2, C-4, and C-3 OMe$_3$Si. Both spectra correspond to spectra for the (Me$_3$Si)$_3$ derivatives of 25,26(OH)$_2$D$_3$. Loss of methyl groups from the S isomer and its fragments seems to be greater than that from the R isomer in these spectra, but this observation needs confirmation.

The (Me$_3$Si)$_3$ derivative of the R isomer of 25,26(OH)$_2$D$_3$ elutes sooner than the derivative of the S isomer when they are chromatographed separately (not shown) or together (Figure 5). When the (Me$_3$Si)$_3$ derivative of radiolabeled 25,26(OH)$_2$D$_3$ biosynthesized by chick renal mitochondria was chromatographed with chemically synthesized 25(R),26-(OH)$_2$D$_3$ and 25(S),26(OH)$_2$D$_3$, one major peak (89% of total), followed by a minor peak (11% of total) or shoulder of radioactivity, emerged, corresponding in degree of separation (9 mL) to that of the chemically synthesized (Me$_3$Si)$_3$ derivatives of the R and S isomers (Figure 5). However, there was a 4-mL phase shift in the elution of radioactive metabolites in comparison to the elution of nonradioactive metabolites, such that the major peak of radioactivity eluted between the nonradioactive derivatives of the R and S isomers. The plotting of radioactivity in the 1-mL fractions collected against the ultraviolet tracing of the nonradioactive peaks was adjusted for the 1-mL volume between the ultraviolet monitor and the fraction collector in this system. The 4-mL phase shift is not an artifact caused by the separation of the ultraviolet monitor and the fraction collector. We have observed a similar separation of radiolabeled and nonradiolabeled (Me$_3$Si)$_3$ derivatives of 1,25(OH)$_2$D$_3$ (the former elutes after the latter under these conditions) (B. Halloran and D. Bikle, unpublished work). If it is assumed that these chromatographic conditions retard the radiolabeled isomer in comparison to the nonradiolabeled isomer, the results confirm the binding results above, which indicate that radiolabeled 25,26(OH)$_2$D$_3$ biosynthesized from [^{3}H]25OHD$_3$ with chick renal mitochondria is predominantly in the R configuration. The ambiguity introduced by the separation of radiolabeled and nonradiolabeled material under these conditions, however, led us to investigate nonradioactive, biologically synthesized 25,26(OH)$_2$D$_3$. Since the circulating levels of 25,26(OH)$_2$D$_3$ are very low in normal humans, we extracted 25,26(OH)$_2$D$_3$ from the blood of a cow given massive doses of vitamin D$_3$. We altered the chromatographic system (see Figure 6 legend) to permit reproducible separation of the (Me$_3$Si)$_3$ derivatives of 25(R),26(OH)$_2$D$_3$

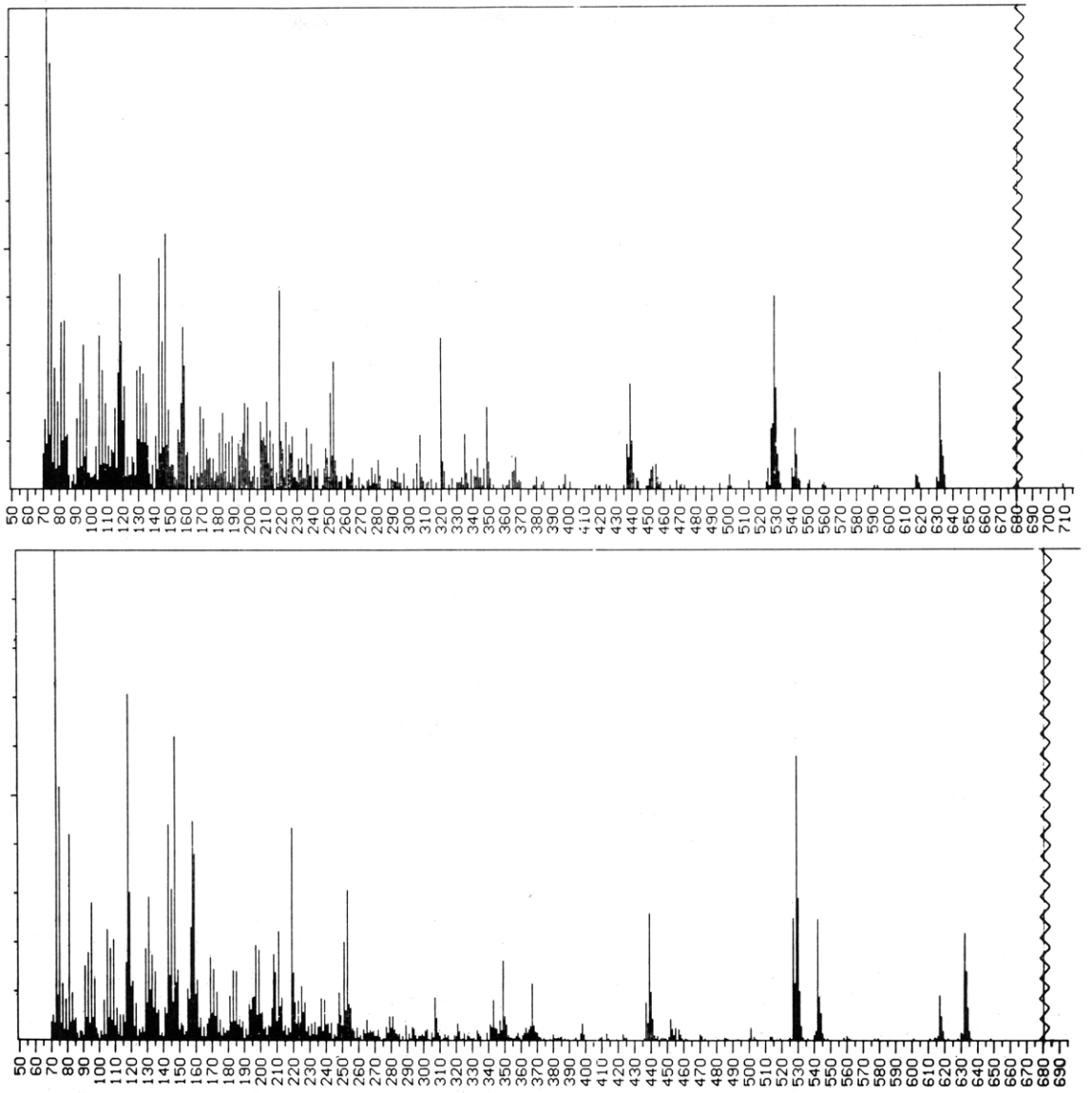

FIGURE 4: High-resolution mass spectra of the $(Me_3Si)_3$ derivatives of the chemically synthesized stereoisomers of 25,26$(OH)_2D_3$.

and 25(*S*),26$(OH)_2D_3$ in a reasonable period off time, without recycling, to enhance the detection of the small amount of bovine 25,26$(OH)_2D_3$ available to us. As in the first chromatographic system, the derivative of the *R* isomer eluted ahead of the derivative of the *S* isomer. The $(Me_3Si)_3$ derivative of bovine 25,26$(OH)_2D_3$ separated into two nearly equal peaks, with elution times comparable to those of the $(Me_3Si)_3$ derivatives of chemically synthesized 25(*R*),26-$(OH)_2D_3$ and 25(*S*),26$(OH)_2D_3$ (Figure 6). When the bovine preparation and chemically synthesized derivatives are combined, the two peaks from the bovine preparation coeluted with the two peaks of the chemically synthesized standards (not shown).

Discussion

25,26$(OH)_2D_3$ was one of the first vitamin D metabolites to be structurally identified (Suda et al., 1970), but the source of its production and its biologic function remain incompletely defined. Investigators have attempted to measure it under a variety of physiologic and pathophysiologic conditions with the expectation that such measurements would shed light on its biologic functions (Horst et al., 1979; Thomasset & Redel, 1980; Ishizuka et al., 1982). These measurements have assumed that 25,26$(OH)_2D_3$ displaces radioligand ([^{3}H]-25OHD_3) from the rat serum binding protein with a potency comparable to that of the 25OHD_3 used as standard. Our data suggest that this assumption is valid only for the *R* isomer of 25,26$(OH)_2D_3$. The apparent affinity of 25(*S*),26$(OH)_2D_3$ is only one-third that of 25(*R*),26$(OH)_2D_3$, 24(*R*),25$(OH)_2D_3$, or 25OHD_3. Therefore, depending on the extent to which 25(*S*),26$(OH)_2D_3$ circulates in blood, the previously reported assays of 25,26$(OH)_2D_3$ may be using an inappropriate standard.

Determining the configuration of the circulating form of 25,26$(OH)_2D_3$ has been a difficult task, and all possible results have been reported: the *R* configuration alone (Redel et al., 1978, 1979), the *S* configuration alone (Partridge et al., 1981), and *R* and *S* together (Ikekawa et al., 1983). Our data support the conclusion reached by Ikekawa et al. (1983) that both isomers can be produced biologically, but neither study resolves the question of which configuration predominates in normal humans not treated with excess vitamin D. Conceivably, the ratio of the two isomers is regulated by the vitamin D status of the animal.

There are several possible explanations for the difficulty encountered in identifying the configuration of 25,26$(OH)_2D_3$ in humans. First of all, the amount of circulating 25,26-$(OH)_2D_3$ is very small, and a relatively large amount is required for structural identification. Levels can be increased by administration of massive doses of vitamin D_3, but such

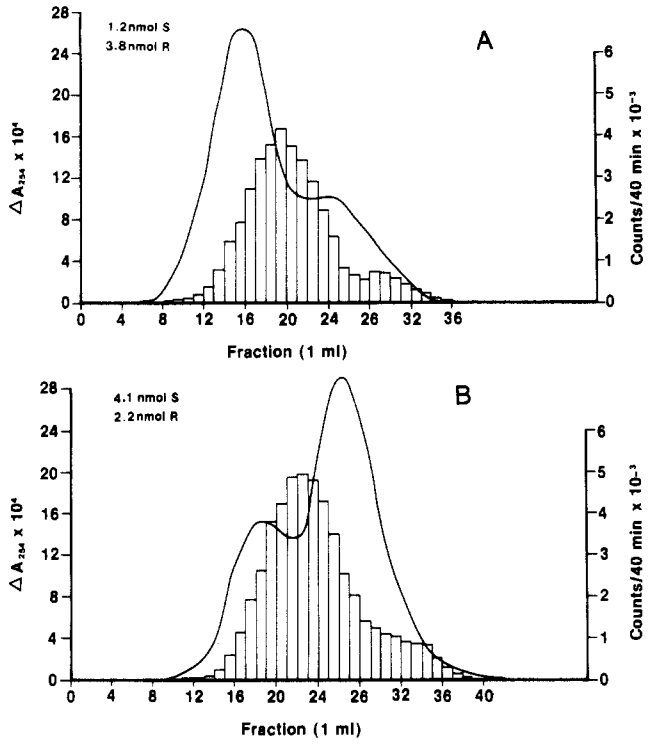

FIGURE 5: Chromatographic separation of two different ratios of chemically synthesized 25(*R*),26(OH)$_2$D$_3$ and 25(*S*),26(OH)$_2$D$_3$ derivatized [with (Me$_3$Si)$_3$] and chromatographed together with biosynthesized, radiolabeled 25,26(OH)$_2$D$_3$. Each separation depicted is the result of two recycles (the third was collected) with a double μPorasil column eluted with hexane/dichloromethane (85:15) at 2 mL/min. Nonradioactive peaks eluted at 63 and 67.5 min. Fractions of 1 mL were collected at the end of the second recycle (at 55 or 56 min) and were counted for radioactivity.

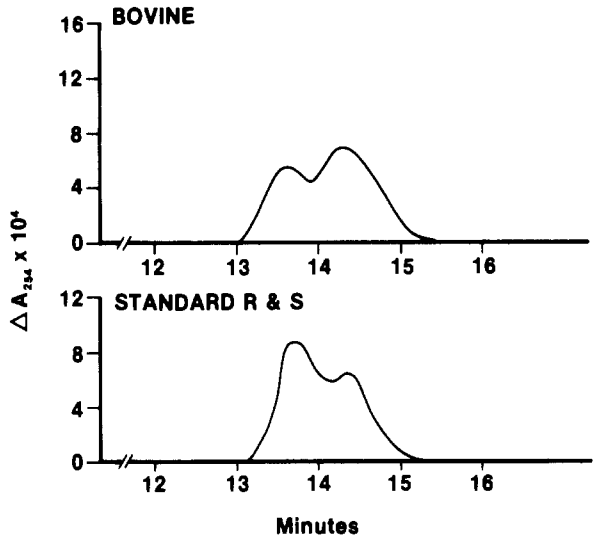

FIGURE 6: Bovine 25,26(OH)$_2$D$_3$ and chemically synthesized 25(*R*),26(OH)$_2$D$_3$ and 25(*S*),26(OH)$_2$D$_3$ were derivatized [with (Me$_3$Si)$_3$] and chromatographed separately on a double μPorasil column eluted with hexane/dichloromethane/acetonitrile (96:4:04) at 4 mL/min. Stereoisomers were separated without recycling in the time given on the abscissa. The bovine 25,26(OH)$_2$D$_3$ preparation resolved into two peaks, which comigrated with the chemically synthesized stereoisomers of 25,26(OH)$_2$D$_3$.

treatment may change the ratio of the *R* and *S* isomers produced. Second, it is difficult to separate the isomers of 25,26(OH)$_2$D$_3$. Separation techniques use derivatization [with Me$_3$Si ethers or α-methoxy α-[[[(trifluoromethyl)phenyl]-acetyl]esters], followed by HPLC. The chromatographic separations tend to require a long time and are difficult to reproduce. We have found that the addition of a small amount of acetonitrile to the eluting solvent helps to overcome these difficulties. Third, the use of radioactive tracers to overcome the difficulty in detecting small amounts of 25,26(OH)$_2$D$_3$ may produce misleading results. We have observed that radiolabeled 25,26(OH)$_2$D$_3$ does not comigrate with nonradiolabeled 25,26(OH)$_2$D$_3$ under the very exacting conditions required to separate 25(*R*),26(OH)$_2$D$_3$ from 25(*S*),26-(OH)$_2$D$_3$. This is not surprising, considering that the substitution of four tritium atoms for four hydrogen atoms increases the molecular weight of 25,26(OH)$_2$D$_3$ by 2% and might be expected to exert a subtle change in its elution pattern. It is possible that the radiolabel we biosynthesized with chick renal mitochondria was not 25,26(OH)$_2$D$_3$, but the sensitivity of this material to periodate and its coelution with 25,26(OH)$_2$D$_3$ in a variety of chromatographic systems makes this possibility remote. Furthermore, the chromatographic separation of isotopes is not unique to 25,26(OH)$_2$D$_3$ (De Ridder & Van Hal, 1976; Cartoni & Ferretti, 1976).

The main purpose of this paper is to point out a potential problem in the measurement of 25,26(OH)$_2$D$_3$. Until suitable means are found to identify the ratio of the *R* and *S* configurations in the 25,26(OH)$_2$D$_3$ sample being measured or until an assay that measures both configurations equivalently is developed, the results of current assays must be interpreted with caution. Moreover, the difference between 25(*R*),26-(OH)$_2$D$_3$ and 25(*S*),26(OH)$_2$D$_3$ in their affinity for the rat serum binding protein suggests that these stereoisomers differ in biologic function. Therefore, for both practical and theoretical reasons, we believe that it is important to distinguish the two stereoisomers of 25,26(OH)$_2$D$_3$ and that our techniques will be useful in this regard.

Acknowledgments

We thank Dr. H. R. Conrad, Ohio Agricultural Research and Development Center, for the use of the cow in preparing bovine 25,26(OH)$_2$D$_3$, Elaine Gee and Scott Munson for technical assistance, Mary Jean Moore for editorial assistance, and Stephen Braitman for secretarial assistance.

Registry No. 25OHD$_3$, 19356-17-3; 25(*R*),26(OH)$_2$D$_3$, 42737-58-6; 25(*S*),26(OH)$_2$D$_3$, 42737-59-7; 25,26(OH)$_2$D$_3$, 29261-12-9.

References

Aksnes, L., & Aarskog, D. (1982) *J. Clin. Endocrinol. Metab. 55*, 94–101.

Arnaud, S. B., & Meger, J. (1983) in *Assay of Calcium-Regulating Hormones* (Bikle, D. D., Ed.) pp 65–83, Springer-Verlag, New York.

Bikle, D. D. (1980) *Biochim. Biophys. Acta 615*, 208–222.

Cartoni, G. P., & Ferretti, I. (1976) *J. Chromatogr. 122*, 287–291.

De Ridder, J. J., & Van Hal, H. J. M. (1976) *J. Chromatogr. 121*, 96–99.

Hollis, B. W., Roos, B. A., & Lambert, P. W. (1980) *Biochem. Biophys. Res. Commun. 95*, 520–528.

Horst, R. L., Shepard, R. M., Jorgensen, N. A., & DeLuca, H. F. (1979) *J. Lab. Clin. Med. 93*, 277–285.

Ikekawa, N., Koizumi, N., Ohshima, E., Ishizuka, S., Takeshita, T., Tanaka, Y., & DeLuca, H. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5286–5288.

Ishizuka, S., Ishimoto, S., & Norman, A. W. (1982) *FEBS Lett. 138*, 83–87.

Lam, H.-Y., Schnoes, H. K., & DeLuca, H. F. (1975) *Steroids 25*, 247–256.

Markestad, T. (1983) *J. Clin. Endocrinol. Metab. 57*, 755–759.

Partridge, J. J., Shiuey, S.-J., Chadha, N. K., Baggiolini, E. G., Blount, J. F., & Uskokovic, M. R. (1981) *J. Am. Chem. Soc. 103*, 1253–1255.
Queille, M. L., Miravet, L., Bordier, P., & Redel, J. (1978) *Biomedicine 28*, 237–242.
Redel, J., Bazely, N., Tanaka, Y., & DeLuca, H. F. (1978) *FEBS Lett. 94*, 228–230.
Redel, J., Bazely, N., Mawer, E. B., Hann, J., & Jones, F. S. (1979) *FEBS Lett. 106*, 162–164.
Suda, T., DeLuca, H. F., Schnoes, H. K., Tanaka, Y., & Holick, M. F. (1970) *Biochemistry 9*, 4776–4780.
Tanaka, Y., Shepard, R. A., DeLuca, H. F., & Schnoes, H. K. (1978) *Biochem. Biophys. Res. Commun. 83*, 7–13.
Thomasset, M., & Redel, J. (1980) *J. Steroid Biochem. 13*, 803–807.
Thomasset, M., Redel, J., Marche, P., Laborde, K., & Cuisinier-Gleizes, P. (1978) *Steroids 32*, 577–587.
Whitney, J. O., Shackleton, C. H. L., Edmonds, C. G., Burlingame, A. L., & Piel, C. F. (1979) in *Stable Isotopes, Proceedings of the Third International Conference* (Klein, E. R., & Klein, P. D., Eds.) pp 47–61, Academic Press, New York.

CORRECTIONS

Slow Interconversion of Acid and Base Forms of Methemerythrin and Some Implications for Methemerythrin Reactivity, by Z. Bradić and R. G. Wilkins*, Volume 22, Number 23, November 8, 1983, pages 5396–5401.

Page 5399. In Table I, the column heading k_{obsd}(slow) (M^{-1} s^{-1}) should read $10^3 k_{obsd}$(slow) (s^{-1}).

Purification of the Messenger RNA Cap-Binding Protein Using a New Affinity Medium, by Nancy R. Webb, Ravi V. J. Chari, Gia DePillis, John W. Kozarich, and Robert E. Rhoads*, Volume 23, Number 2, January 17, 1984, pages 177–181.

Page 179. In column 1, line 1 should read as follows: eluted with 70 μM m^7GTP and 100 mM KCl in buffer A.

Static and Time-Resolved Fluorescence Studies of Fluorescent Phosphatidylcholine Bound to the Phosphatidylcholine Transfer Protein of Bovine Liver, by Theo A. Berkhout, Antonie J. W. G. Visser, and Karel W. A. Wirtz*, Volume 23, Number 7, March 27, 1984, pages 1505–1513.

Page 1512. In the Appendix, eq 4a and 4b should read as follows:

$$\phi_1/\phi = 6[6F_\perp(\rho)]^{-1} \quad (4a)$$

$$\phi_2/\phi = 6[5F_\perp(\rho) + F_\parallel(\rho)]^{-1} \quad (4b)$$

Protein Folding Kinetics from Magnetization Transfer Nuclear Magnetic Resonance, by Christopher M. Dobson* and Philip A. Evans, Volume 23, Number 19, September 11, 1984, pages 4267–4270.

Pages 4268 and 4269. The units of the enthalpies of activation and unfolding should read kcal mol^{-1}, not kJ mol^{-1}. The values in kJ mol^{-1} are thus the following: unfolding enthalpy = 460 ± 25 kJ mol^{-1}; activation energy of unfolding = 255 ± 8 kJ mol^{-1}; activation energy of folding = −167 ± 17 kJ mol^{-1}.

Interaction of Purified Nicotinamidenucleotide Transhydrogenase with Dicyclohexylcarbodiimide, by Donna C. Phelps and Youssef Hatefi*, Volume 23, Number 19, September 11, 1984, pages 4475–4480.

Page 4477. In footnote *a* to Table I, nanomoles should read micromoles.

Kinetic Isotope Effects on the Oxidation of Reduced Nicotinamide Adenine Dinucleotide Phosphate by the Flavoprotein Methylenetetrahydrofolate Reductase, by Maria A. Vanoni and Rowena G. Matthews*, Volume 23, Number 22, October 23, 1984, pages 5272–5279.

Page 5276. In footnote *a* to Scheme I, the last two lines should read as follows: $K_m(\text{NADPH}) = (k_{-1}/k_1)(1 + C_f + C_r)/(1 + R_f + C_r) = (k_{-1}/k_1)({}^DV - 1)/({}^DV/K - 1)$ (Klinman & Matthews, 1984).