Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Morgan, T. E., and Hanahan, D. J. (1966), *Biochemistry 5*, 1050.
Pagé, M., and Godin, C. (1969), *Can. J. Biochem. 47*, 401.
Rauderath, K. (1963), Thin Layer Chromatography, Academic, New York, N. Y.
Richardson, S. H., Hulten, H. O., and Green, D. E. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 821.
Rosenberg, S., and Guidotti, G. (1968), *J. Biol. Chem. 243*, 1985.
Rosenberg, S., and Guidotti, G. (1969), *J. Biol. Chem. 244*, 5118.
Schatz, G., and Saltzgaber, J. (1969), *Biochim. Biophys. Acta 180*, 186.
Schnaitman, C. A. (1969), *Proc. Nat. Acad. Sci. U.S., 63*, 412.
Shapiro, A. L., and Maizel, J. V. (1969), *Anal. Biochem. 29*, 505.
Shapiro, A. L., Vinuela, E., and Maizel J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Ward, K. A., and Pollak, J. K. (1969), *Biochem. J. 114*, 41.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Yu, B. P., DeMartinis, F. D., and Masoro, E. J. (1968a), *Anal. Biochem. 24*, 523.
Yu, B. P., DeMartinis, F. D., and Masoro, E. J. (1968b), *J. Lipid Res. 9*, 492.

21,25-Dihydroxycholecalciferol. A Metabolite of Vitamin D_3 Preferentially Active on Bone*

T. Suda, H. F. DeLuca, H. K. Schnoes, G. Ponchon, Y. Tanaka, and M. F. Holick

ABSTRACT: A metabolite of vitamin D_3 has been isolated in pure form from the plasma of pigs given large doses of vitamin D_3. It has been identified as 21,25-dihydroxycholecalciferol by means of mass spectrometry, ultraviolet absorption spectra, and behavior on gas–liquid chromatography.

This metabolite is 0.5 as active as vitamin D_3 in the rat in the cure of rickets and in intestinal calcium transport, but is more active than vitamin D_3 in the mobilization of bone mineral.

The successful identification of vitamin D_3 (cholecalciferol) by Windaus and collaborators essentially brought to a close the search for dietary antirachitic factors (Windaus *et al.*, 1936; Schenk, 1937). A new search in the vitamin D field made its appearance with the successful demonstration in this laboratory of biologically active metabolites of vitamin D_3 (Lund and DeLuca, 1966). One group was identified almost immediately as esters of vitamin D and long chain fatty acids (Lund *et al.*, 1967; Fraser and Kodicek, 1966) but these appeared to be of minor functional importance (DeLuca, 1967). However another metabolite fraction (peak IV) more polar than vitamin D contained at least one metabolite more effective than vitamin D_3 in the cure of rickets, in the stimulation of intestinal calcium transport, and in the mobilization of bone mineral (Lund and DeLuca, 1966; Morii *et al.*, 1967). In addition it acted more rapidly than vitamin D_3 itself. The peak IV fraction was subsequently subfractionated into seven radioactive components, one of which retained the ability to cure rickets (Ponchon and DeLuca, 1969; Blunt *et al.*, 1968b) (peak IV). This substance was subsequently isolated in pure form and identified as 25-hydroxycholecalciferol (Blunt *et al.*, 1968a,b). It was successfully synthesized chemically and shown to be the circulating active form of the vitamin (DeLuca, 1969).

Synthesis of [^{3}H]-25-hydroxycholecalciferol made possible the demonstration that this metabolite is further metabolized to more polar metabolites in intestine, bone, and kidney (Cousins *et al.*, 1970; R. J. Cousins and H. F. DeLuca, 1970, unpublished data). Haussler *et al.* (1968) and Lawson *et al.* (1969) have also shown a metabolite of vitamin D_3 in intestinal nuclei more polar than 25-hydroxycholecalciferol. Thus the identification of these metabolites appeared important to a thorough understanding of the mechanism of vitamin D action. One of these polar metabolites has now been isolated in pure form, identified as 21,25-dihydroxycholecalciferol, and shown to have a marked action on mobilization of bone mineral while having a small but significant effect on intestinal calcium transport. In addition it is one-half as active as vitamin D_3 in curing rickets in rats. It is the purpose of this communication to report these results.

Methods and Results

General Procedures. All radioactive determinations were carried out by means of a Packard Tri-Carb Model 3003 liquid scintillation counter equipped with an automatic

* From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received March 13, 1970.* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported by the H. Steenbock Research Fund of the Wisconsin Alumni Research Foundation, National Institutes of Health Grant, AMO-5800-08, National Institutes of Health Training Grant GM00236 BCH from the National Institute of General Medical Sciences, and the N. V. Philips Duphar Company of the Netherlands.

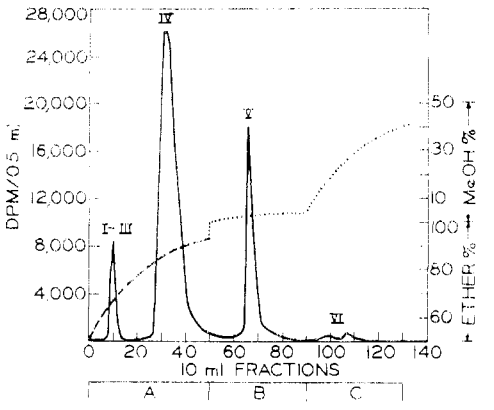

FIGURE 1: Silicic acid column profile of a plasma extract from chicks given [1,2-^{3}H]vitamin D_3 intravenously 20 hr earlier.

external standardization system. Samples were evaporated to dryness with a stream of air, dissolved in toluene-counting solution (2 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene per l. of toluene), and counted.

Ultraviolet spectra were recorded with a Beckman DB-G recording spectrophotometer. Samples in this case were dissolved in ethanol. A molar extinction coefficient of 18,000 was used for vitamin D_3, 25-hydroxycholecalciferol, and 21,25-dihydroxycholecalciferol (Blunt *et al.*, 1968b).

Gas–liquid partition chromatography was carried out in an F & M Model 402 using a 4 ft × 0.25 in. glass column packed with 3% SE-30 on 100–120 mesh Gas Chrom Z.

Mass spectra were obtained with an MS-902 mass spectrometer, using direct probe introduction with source temperature of 115–145° above ambient.

Acetylation Reactions. Acetates of the 21,25-dihydroxycholecalciferol and model compounds were prepared by treating 1–10 μg of the compound with 5 μl of acetic anhydride in 5 μl of pyridine and allowing the mixture to stand at room temperature for 2–3 hr. Gas chromatograms of each were obtained by injecting a fraction of the total mixture into the gas chromatograph, while the remainder was transferred directly to the probe for mass spectrometry.

Partial acetylation of the metabolite and model compounds was affected by treating 1–5 μg of material with 5 μl of acetic anhydride for 10 min at 0°. Methanol or ethanol (30–50 μl) was then added to stop the reaction. Samples were subjected to gas–liquid chromatography and mass spectrometry as described above.

Preparation of Silyl Ether Derivatives. Trimethylsilyl ether derivatives of the metabolite and model compounds were prepared by treating 1–5 μg of material with a mixture of hexamethyldisilazane and trimethylchlorosilane in pyridine (volume ratio 2:1:7) and allowing it to stand at room temperature for several hours. Portions of the solution were used directly for mass spectrometry and gas chromatography.

Isolation of Metabolites. Eight pigs of mixed breed weighing 230–288 lb were fed a stock ration to which was added water-dispersible vitamin D_3 at a level of 31,000 IU/lb of feed (Vitaplus Corp., Madison, Wis.). This supplied 250,000 IU of vitamin D_3 daily per pig. After 28 days the pigs were slaughtered and their blood was collected. It was immediately mixed with 0.1 volume of 0.1 M sodium oxalate to prevent clotting. Plasma was separated from the cells by means of a DeLaval blood separator (DeLaval Co.). The 14.1 l. of plasma thus obtained was made 70% saturated with $(NH_4)_2SO_4$ and allowed to stand at 4° for 7 days. The precipitate was collected by centrifugation at 25,000 rpm for 25 min in a Sharples AS-16-P centrifuge. The protein precipitate (8.4 l.) was extracted with 25.2 l. of methanol–chloroform (2:1) with a portable mixer, Model F (Mixing Equipment Co., Rochester, N.Y.), and allowed to stand for 17 hr. The protein precipitate was reextracted for 4 hr with the same volume of methanol–chloroform (2:1) and filtered. To the combined one-phase extracts were added an additional 13.2 l. of chloroform, 4 l. of tap water, and 200 ml of saturated NaCl. The phases were allowed to separate. The chloroform phase was mixed with 18.4 l. of tap water, 2.7 l. of methanol, and 120 ml of saturated NaCl, and allowed to stand at 4° for 10 days. The chloroform phase was concentrated to 68 ml with a rotary flash evaporator. This black oily residue was partitioned with 332 ml of Skelly B (redistilled petroleum ether, bp 67°) and 400 ml of 90% methanol–10% water in a separatory funnel. The upper phase contained less polar metabolites of vitamin D_3 (peaks I–III) and the lower phase contained the more polar (peaks IV–VII) metabolites. After separation of the phases, 300 ml of chloroform and 100 ml of tap water were added to the lower phase. The chloroform phase contained the polar metabolites and was drawn off. The aqueous phase was reextracted with 200 ml of chloroform. The combined chloroform extracts were then taken to dryness with the flash evaporator and dissolved in 20 ml of chloroform.

Radiochemically pure [1,2-^{3}H]vitamin D_3 (specific activity 90,000 dpm/IU) was prepared in this laboratory by means of the method of Neville and DeLuca (1966). Chicks were used as a source of radioactive metabolites because of convenience, low cost, and routine availability of rachitic chicks in our laboratory. Exactly 100 IU (2.5 μg) of [1,2-^{3}H]vitamin D_3 was dosed intravenously to each of 51 chickens, which were maintained on a vitamin D deficient diet (Imrie *et al.*, 1967) for 27 days. After administration, they were fasted and 20 hr later blood was collected by decapitation giving 133 ml of plasma after centrifugation. This was extracted with methanol–chloroform (2:1), and ^{3}H-labeled polar metabolites were partitioned in a separatory funnel as described above. The metabolites partitioned in methanol were then extracted with chloroform. The chloroform was evaporated and the residue was dissolved in 5 ml of chloroform. This ^{3}H-labeled chicken plasma extract was first applied to a silicic acid column (Ponchon and DeLuca, 1969) to obtain the ^{3}H-labeled peak V. The column was eluted with an ether–Skelly B–methanol gradient, obtained by running 500 ml of 100% ether from a holding chamber into a 250-ml constant-volume mixing chamber initially containing 250 ml of 50% ether in Skelly B. Following the collection of 50 10-ml fractions, 400 ml of 5% methanol in ether was placed in the holding chamber, and an additional 40 10-ml fractions were collected. After that, 400 ml of 50% methanol in ether was then placed in the holding chamber, and an additional 40 10-ml fractions were collected. Figure 1 shows the radioactivity profile of the column eluate, and the ether–Skelly B–methanol gradient obtained. It can be noted that almost all of the radioactivity due to the less polar metabolites (peaks I–III) had been eliminated by the

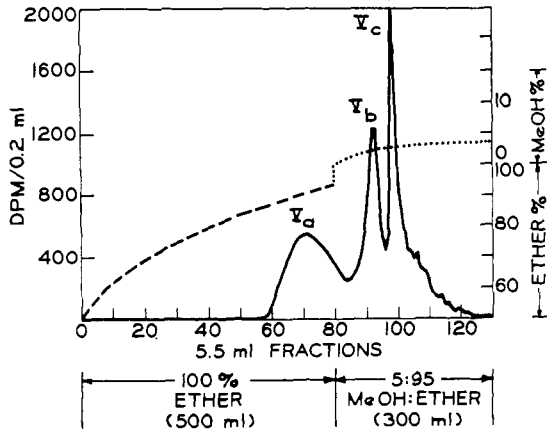

FIGURE 2: Silicic acid column chromatographic profile of plasma peak V from Figure 1 combined with the hog plasma extract.

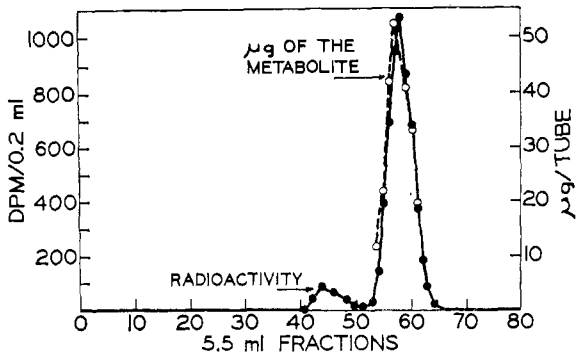

FIGURE 3: Partition column chromatography of peak Va isolated from silicic acid columns (Figure 2).

partition procedure described above. Peak V was eluted as a single peak, and 1,250,000 dpm of radioactivity was recovered as peak V. This radioactive peak V taken from chicken plasma was then mixed with the extract from the hog plasma. The combined extract was then applied in 20 ml of chloroform to a large 150-g, 60-cm, multibore silicic acid column measuring stepwise in diameter 1.0, 2.0, 3.0, and 4.0 cm. The column was eluted with the ether–Skelly B–methanol gradient as described above for chicken plasma extract except that 1 l. of 100% diethyl ether, 1 l. of 5% methanol in ether, and 1 l. of 50% methanol in ether were added successively to the holding chamber. Again, peak V was eluted as a single peak in the 5% methanol region. Peak V was next rechromatographed on a multibore silicic acid (14 g) column as described by Neville and DeLuca (1966) except that the mixing chamber contained 250 ml of 50% diethyl ether in Skelly B and the holding chamber contained 500 ml of 100% diethyl ether. As soon as the holding chamber became empty, it was filled with 300 ml of 5% methanol in diethyl ether. Fractions (5.5 ml) were collected. The elution profile is shown in Figure 2. Clearly the original peak V has been resolved into at least three components, which were designated Va, Vb, and Vc. The peak Va collected in tubes 57–84 was then rechromatographed on a Celite partition column, especially designed for this metabolite by methods described by Johnson (1964) and constructed as follows: 300 ml of 90% methanol–10% water was equilibrated at 4° with 750 ml of 80% Skelly B–20% chloroform. The aqueous methanol plase (15 ml) was mixed with 20 g of Celite and dry packed into a 60 × 1 cm column in 2-cm portions. The upper phase was used as the mobile phase. The column was developed with the mobile phase with 5.5-ml fractions collected. Tubes 53–64 contained the peak Va metabolite. The profile of radioactivity from that column is shown in Figure 3. Optical density at 265 mμ was also measured in each fraction and the calculated micrograms of the peak Va are plotted assuming a molar extinction coefficient of 18,000. As seen in Figure 3, the absorbancy at 265 mμ did not exactly coincide with the radioactivity plot. The mass spectrum of the metabolite indicated the peak Va fraction eluted from the partition column contained a contaminant which had a molecular weight of 426. The contents of tubes 53–64 from the partition column were recombined and then applied in 0.2 ml of methanol to a 60 × 1 cm Sephadex LH-20 column (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) which was developed in methanol. Fractions (1 ml) were collected. Figure 4 shows the profile of radioactivity and 265-mμ absorbing material which eluted from that column. The absorbancy at 265 mμ now exactly coincides with the radioactivity plot. The ultraviolet spectrum of the material is shown in Figure 5, giving maximum absorption at 265 and minimum absorption at 228 mμ. A total of 150 μg of the metabolite was isolated.

The purity of the isolated metabolite was assessed by gas–liquid chromatography. The metabolite failed to chromatograph in the free form under the conditions generally used for vitamin D_3 and 25-hydroxycholecalciferol. Va (5 μg) was converted into the ditrimethylsilyl ether derivative by means of bistrimethylsilylfluoroacetamide in pyridine (1:1) at 60° for 20 min. The gas–liquid chromatography trace shows the formation of pyro- and isopyrocalciferol forms (Figure 6).

Identification of Va as 21,25-Dihydroxycholecalciferol. The fact that the ultraviolet absorption spectrum (Figure 5) of Va is identical with either vitamin D_3 or 25-hydroxycholecalciferol provides evidence that the *cis* triene system of Va is unchanged from that of vitamin D_3. The formation of the pyro- and isopyrocalciferol forms on gas–liquid chromatography (Figure 6) provides additional evidence for this belief. The typical fragmentation pattern in the mass spectrum (Fig-

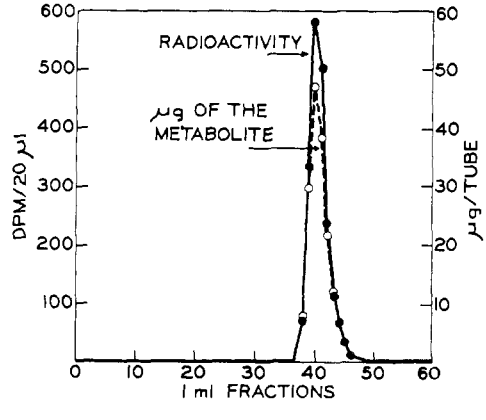

FIGURE 4: Chromatography of peak Va from Figure 3 on a Sephadex-LH20 column. The micrograms per tube were calculated on the basis of a molar extinction coefficient at 265 mμ.

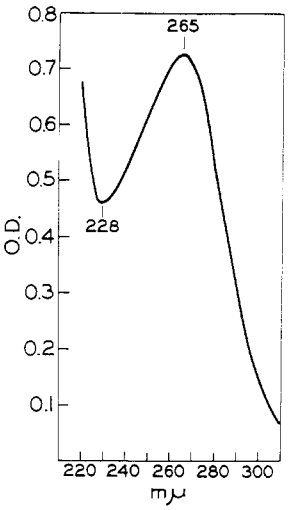

FIGURE 5: Ultraviolet absorption spectrum of the peak Va metabolite isolated from LH20 columns.

ure 7) further corroborated this conclusion. The molecular ion of the metabolite at *m/e* 416—an increase of 32 mass units over the molecular weight of vitamin D_3—suggested the incorporation of two additional oxygen atoms, which could be identified as hydroxyl functions by the formation of a trisilyl ether derivative (mol wt 632, Figure 8). The extra hydroxyl groups of the metabolite are both located in the side chain, since the mass spectrum (Figure 7) retains the peak at *m/e* 271 also observed in the spectrum of vitamin D_3 as well as other vitamin D derivatives, and which corresponds to the loss of the entire side chain (cleavage of C_{17}–C_{20} bond). In addition, peaks of *m/e* 136—comprising ring A and carbons C_6, C_7, and C_{19}—and *m/e* 118 (136 minus H_2O) which are typical for vitamin D systems corroborate the ultraviolet spectral and gas–liquid chromatography evidence for an unchanged conjugated triene system. The metabolite forms a diacetate (mol wt 500) upon treatment with acetic anhydride–pyridine at room temperature for several hours suggesting two primary/secondary and one

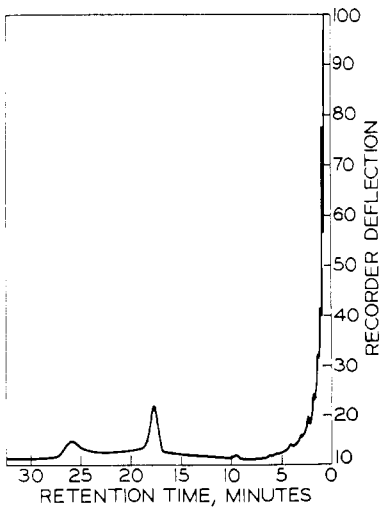

FIGURE 6: Gas–liquid chromatography of the disilyl ether derivative of Va.

TABLE I: Effect of Intrajugular Administration of 2.5 μg of 21,25-Dihydroxycholecalciferol, 25-Hydroxycholecalciferol, or Vitamin D_3 on Calcium Transport and Serum Calcium of Rats on a Low-Calcium Diet.

	12 Hr after Administration[a]	
Group	Calcium Transport [^{45}Ca]Serosal/ [^{45}Ca]Mucosal	Bone Mobilization Serum Ca (mg %)
Control	1.5 ± 0.3 (5)	4.4 ± 0.4 (5)
21,25-Dihydroxycholecalciferol	2.6 ± 0.8 (5)	6.5 ± 0.2 (5)
25-Hydroxycholecalciferol	5.9 ± 1.6 (5)	7.3 ± 0.5 (5)
Vitamin D_3		5.0 ± 0.5 (5)

[a] Plus or minus shows the standard deviation of the mean. Numbers in parentheses show the number of animals in each group.

tertiary hydroxyl group. The location of the latter at C-25 is established by the intense peak at *m/e* 131 (corresponding to $(CH_3)_2C{=}O^+Si(CH_3)_3$) in the spectrum of the tritrimethylsilyl derivative (Figure 8) of the compound. This fragment which is observed for all 25-hydroxy steroid systems we have investigated (*e.g.*, 25-hydroxycholesterol, 25-hydroxycholecalciferol, 25-hydroxyergocalciferol, ecdyson) also establishes the absence of another hydroxyl function at C-26, -27. For the third hydroxyl function then, only C-21, -22, -23, and -24 could be considered, since C-20 is eliminated both by the acetylation results (formation of a diacetate) and mass spectral evidence, *i.e.*, 20-hydroxycholesterol, 20,22-dihydroxycholesterol, and ecdysteron show very abundant fragments due to cleavage of the C-20, -22 bond which is absent in the metabolite spectra. Location of the hydroxyl function at C-22, -23, and -24 is also made highly unlikely by the absence in the spectrum of the trimethylsilyl ether derivative (Figure 8) of any peaks which could be rationalized by the expected bond cleavages α to the *O*-trimethylsilyl grouping. Such cleavages which give rise to prominent peaks are commonly employed for the determination of positions of hydroxyl functions in carbon chains (Eglinton *et al.*, 1968) and a similar fragmentation would be expected for the steroid side chain. A limited number of models which we have investigated confirm this prediction; for example, the spectrum of the trimethylsilyl ether of ecdyson (22,25-di-OH side chain) exhibits the expected peaks at *m/e* 131 (C-24, -25 cleavage) and *m/e* 261 (C-20, -22 cleavage) as well as *m/e* 171 (261 minus 90). The absence of analogous fragments in the spectrum of the metabolite silyl ether thus points to C-21 as the location of the additional hydroxyl. It is well known that a trimethylsilyl derivative of a primary hydroxyl function which *a priori* can give rise to an α-cleavage fragment at *m/e* 103 ($CH_2{=}OSi(CH_3)_3$) usually exhibits a weak to moderate peak at that mass. (For example, 26-hydroxycholesterol ditrimethylsilyl ether shows a rather small peak at *m/e* 103 in its mass spectrum.)

A C-21 hydroxyl group was confirmed by the following

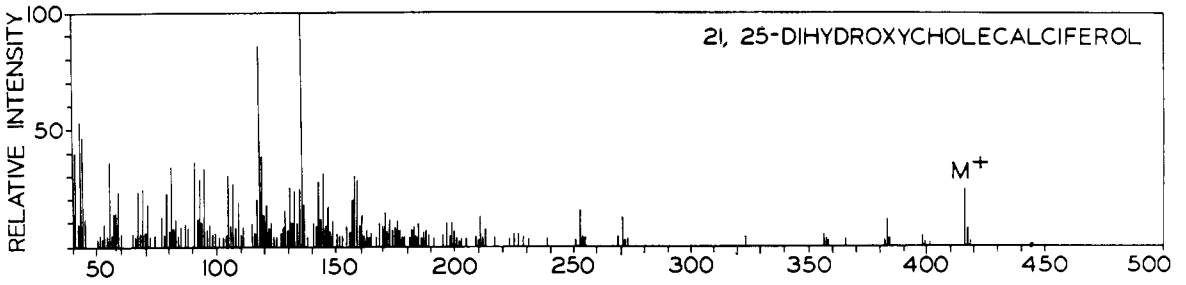

FIGURE 7: Mass spectrum of 21,25-dihydroxycholecalciferol.

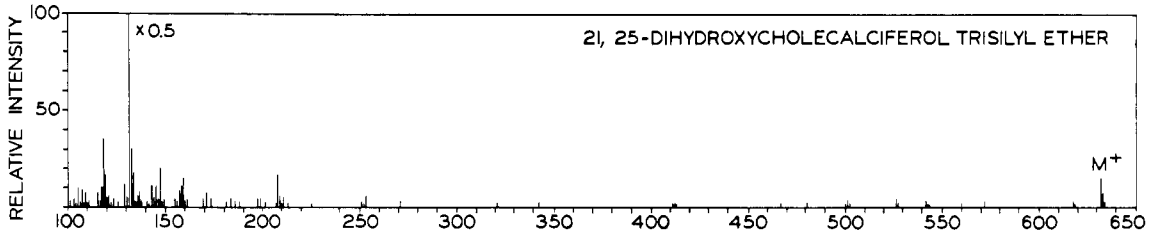

FIGURE 8: Mass spectrum of tritrimethylsilyl ether derivative of 21,25-dihydroxycholecalciferol.

experiments. Under conditions (pyridine, acetic anhydride, at 0° for 10 min) where no appreciable acetylation occurs (as evidenced by mass spectrometry and gas–liquid chromatography) with vitamin D_3, cholesterol, 24-hydroxy-27-norcholesterol, 25-hydroxy-27-norcholesterol (all possessing secondary OH groups), both the metabolite and 26-hydroxycholesterol yield a mixture of unacetylated and monoacetylated material (ratio roughly 60:40) as determined by mass spectrometry and (for 26-hydroxycholesterol) gas chromatography. This constitutes evidence for a primary hydroxyl function in the metabolite which together with the silylation results defines the structure of the compound as 21,25-dihydroxycholecalciferol (21,25-DHCC) (Figure 9).

Biological Activity of 21,25-Dihydroxycholecalciferol. Initially, the line test assay carried out as previously described (U. S. Pharmacopoeia, 1955) revealed the biological activity of Va to be less than 10 units/μg. This low value agreed with previous work (Ponchon and DeLuca, 1969) which showed peak V to lack antirachitic activity when given orally. When it was given intravenously, however, 21,25-DHCC was approximately 0.5 as active as vitamin D_3 or approximately 20 IU/μg as compared to 60 IU/μg for 25-HCC (Blunt *et al.*, 1968c).

Bone mineral mobilization response to 21,25-DHCC intravenously was tested as previously described (Blunt *et al.*, 1968c). In addition, intestines were removed from these rats and calcium transport was measured by the everted sac technique as previously described (Blunt *et al.*, 1968c). The results show that at 12 hr after administration, 21,25-DHCC was almost but not quite as effective as 25-hydroxycholecalciferol but more effective than vitamin D_3 in stimulating the rise in plasma calcium at the expense of bone. However it was less than one-third as active in the stimulation of intestinal calcium transport. Thus the 21,25-DHCC (Table I) appears to act preferentially on the bone mobilization system.

Discussion

In previous work in this laboratory, 25-hydroxycholecalciferol (25-HCC) (Blunt *et al.*, 1968a,c) and 25-hydroxyergocalciferol (Suda *et al.*, 1969a,b) were isolated and identified. Further biological evidence established these metabolites as the circulating active forms of vitamin D_3 and D_2, respectively (Blunt *et al.*, 1968b; Suda and DeLuca, 1970; DeLuca, 1969; Trummel *et al.*, 1969; Olson and DeLuca, 1969). Further experiments with newly synthesized [^{3}H]25-HCC demonstrated that 25-HCC is rapidly metabolized to more polar metabolites in intestine, bone, and kidney (Cousins *et al.*, 1970; Cousins and DeLuca, 1970, unpublished data). Ponchon and DeLuca (1969), Haussler *et al.* (1968), and Lawson *et al.* (1969) have also reported metabolites of vitamin D_3 more polar than 25-HCC but as yet these remain unidentified. Continued experiments in this laboratory have shown that at least some of these polar metabolites appear in blood making their isolation from the plasma of pigs given large doses of vitamin D_3 feasible. By highly refined silicic acid column chromatography the peak V fraction of blood has now been resolved into at least three major components (Va, Vb, and Vc) (Figure 2), which may suggest that the metabolites from more than one

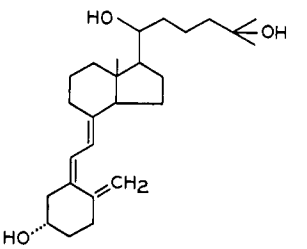

FIGURE 9: Structure of 21,25-dihydroxycholecalciferol.

tissue are represented in the peak V fraction of blood. The isolation of the first of these, Va, in pure form has now been accomplished and it has been identified as 21,25-dihydroxycholecalciferol. However the origin of this metabolite cannot be decided on the basis of the presently available data. Experiments are now in progress to delineate exactly its origin.

The isolation of the 21,25-dihydroxy metabolite in pure form proved to be a much more difficult isolation than that of the 25-HCC. Because the concentration of Va in blood is very much lower than that of 25-HCC, it was necessary to eliminate the bulk of contaminating lipids by two liquid–liquid partitions. Besides this, refined silicic acid chromatography and a newly devised partition column were needed. Although highly purified at this stage, the Va still contained a major contaminant with a mass of 426. By means of an LH 20 column system the contaminant could be removed and Va obtained in pure form. Its purity was shown by gas–liquid chromatography of the ditrimethylsilyl ether. It may be interesting to note that the unsilylated Va will not volatalize under the gas–liquid chromatography conditions used.

It should be noted that the radioactive label used to follow the peak V components during purification was obtained from the blood of chicks given only 100 IU of [1,2-^{3}H]vitamin D_3. Thus the metabolite isolated (21,25-DHCC) certainly represents a physiologic one. However some metabolites of vitamin D_3 from the pigs may not have been labeled and hence missed. In any case the Va can be considered as formed at least in both chicks and pigs.

Identification of Va rested on ultraviolet absorption spectra, gas–liquid chromatography behavior, and mass spectrometry of it and its derivatives. A molecular ion of 416 mass units demonstrates a vitamin D_3 molecule with two additional oxygens or hydroxyl groups. A mass fragment of 271 (minus side chain) shows that the two added hydroxyls are found on the side chain. A 131 fragment of the tritrimethylsilyl ether derivative fixes one hydroxyl on the 25 position as does a mass of 59 fragment in the unsilylated Va. It also shows that the added OH cannot be on the 26, 27 positions. Because an OH on any carbon but the 21 would produce cleavages of the side chain at adjacent bonds (because they are secondary and tertiary alcohols) giving characteristic fragments, the second hydroxyl must be on the 21 position. Acetylation studies confirmed this belief in that the Va contain one primary alcohol function. That the vitamin D_3 triene structure is intact in Va is clearly demonstrated by (a) the characteristic ultraviolet absorption spectra (Figure 5) and (b) the formation of pyro- and isopyrocalciferol derivatives on gas–liquid chromatography (Figure 6). Thus the structure is firmly established as 21,25-dihydroxycholecalciferol (21,25-DHCC) (Figure 9).

Of major interest is the biological activity of the 21,25-DHCC. It is not appreciably effective orally in the cure of rickets in rats, but when injected intrajugularly, it is one-half as active as vitamin D_3 and one-third as active as 25-HCC in this system. Thus it does not appear to be readily absorbed from the intestine accounting for the lack of biological activity when it was tested orally in its crude form previously (Blunt *et al.*, 1968b,c; Ponchon and DeLuca, 1969). Most interesting however is that it is very effective in the mobilization of bone mineral and has some effectiveness on intestinal calcium transport. Thus this indicates that this metabolite preferentially carries out the well-known effect of vitamin D on bone. It is therefore exciting to consider that vitamin D_3 itself is the storage form of the vitamin, 25-HCC, the circulating active or hormonal form in the blood and, in the target tissues, it is converted to specific active forms. Clearly much more evidence is needed before such an hypothesis is tenable, but preliminary work in progress in this laboratory is substantially in support of this possibility.

Acknowledgment

We wish to thank, at the University of Wisconsin, Dr. Charles Sih, Department of Pharmacy, and Dr. Stanley Beck, Department of Entomology, for some of the compounds used in the mass spectral analysis and we wish to thank Dr. E. J. Briskey, Department of Meat and Animal Science, for the pigs and use of swine facilities.

References

Blunt, J. W., DeLuca, H. F., and Schnoes, H. K. (1968a), *Chem. Commun. 14*, 801.

Blunt, J. W., DeLuca, H. F., and Schnoes, H. K. (1968b), *Biochemistry 7*, 3317.

Blunt, J. W., Tanaka, Y., and DeLuca, H. F. (1968c), *Proc. Natl. Acad. Sci. U. S. 61*, 1503.

Cousins, R. J., DeLuca, H. F., Suda, T., Chen, T., and Tanaka, Y. (1970), *Biochemistry 9*, 1453.

DeLuca, H. F. (1967), *Vitam. Horm. 25*, 315.

DeLuca, H. F. (1969), *Fed. Proc. 28*, 1678.

Eglinton, G., Henneman, D. H., and McCormick, A. (1968), *Org. Mass Spectrom. 1*, 593.

Fraser, D. R., and Kodicek, E. (1966), *Biochem. J. 100*, 67.

Haussler, M. R., Myrtle, J. F., and Norman, A. W. (1968), *J. Biol. Chem. 243*, 4055.

Imrie, M. H., Neville, P. F., Snellgrove, A. W., and DeLuca, H. F. (1967), *Arch. Biochem. Biophys. 120*, 525.

Johnson, M. J. (1964), *in* Manometric Techniques, Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Ed., Minneapolis, Minn., Burgess, p 233–261.

Lawson, D. E. M., Wilson, P. W., and Kodicek, E. (1969), *Biochem. J. 115*, 269.

Lund, J., and DeLuca, H. F. (1966), *J. Lipid Res. 7*, 739.

Lund, J., DeLuca, H. F., and Horsting, M. (1967), *Arch. Biochem. Biophys. 120*, 513.

Morii, H., Lund, J., Neville, P., and DeLuca, H. F. (1967), *Arch. Biochem. Biophys. 120*, 508.

Neville, P., and DeLuca, H. F. (1966), *Biochemistry 5*, 2201.

Olson, E. B., and DeLuca, H. (1969) *Science 165*, 405.

Ponchon, G., and DeLuca, H. F. (1969), *J. Nutr. 99*, 157.

Schenck, F. (1937), *Naturwissenschaften 25*, 129.

Suda, T., and DeLuca, H. F. (1970), *J. Nutr.* (in press).

Suda, T., DeLuca, H. F., Schnoes, H. K., and Blunt, J. W. (1969a), *Biochem. Biophys. Res. Commun. 35*, 182.

Suda, T., DeLuca, H. F., Schnoes, H. K., and Blunt, J. W. (1969b), *Biochemistry 8*, 3515.

Trummel, C., Raisz, L. G., Blunt, J. W., and DeLuca, H. F. (1969), *Science 163*, 1450.

U. S. Pharmacopoeia (1955), 14th Revision, Easton, Pa., Mack, p 889.

Windaus, A., Schenck, F., and Von Werder, F. (1936), *Z. Physiol. Chem. 241*, 100.