Isolation and Identification of 1,25-Dihydroxycholecalciferol. A Metabolite of Vitamin D Active in Intestine*

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ABSTRACT: A metabolite of vitamin D_3, thought to be the "tissue-active" form of the vitamin in the intestine, has been isolated from chicken intestines in pure form as the monotrimethylsilyl ether derivative.

The structure of this metabolite has been identified as 1,25-dihydroxycholecalciferol by means of mass spectrometry, ultraviolet absorption spectrophotometry, and specific chemical reactions.

It is now generally accepted that vitamin D must be metabolically "activated" before it can produce its characteristic physiologic effects (Lund and DeLuca, 1966; Morii *et al.*, 1967; DeLuca, 1967; Ponchon *et al.*, 1969). As a direct consequence of this concept Blunt *et al.* (1968a,b) isolated and identified 25-hydroxycholecalciferol (25-HCC),[1] a metabolite of vitamin D which is more active in all respects than the parent vitamin. Continued experiments suggested that 25 hydroxylation of vitamin D is the initial reaction in the functional metabolism of vitamin D (Ponchon and DeLuca, 1969a; Ponchon *et al.*, 1969). This reaction takes place in the liver (Horsting and DeLuca, 1969) and is strongly controlled by product inhibition suggesting it to be of importance in the regulation of vitamin D metabolism and function.

The synthesis of [^{3}H]25-HCC (Blunt and DeLuca, 1969) made possible an examination of the question of whether 25-HCC is further metabolized before it functions. Cousins *et al.* (1970a,b) demonstrated clearly that 25-HCC is metabolized to more polar metabolites which appear in the target tissues before the physiological responses to 25-HCC ensue. Suda *et al.* (1970a,b) isolated two such polar metabolites, *i.e.*, 21,25-DHCC and 25,26-DHCC from the plasma of pigs. The former preferentially stimulates bone mineral mobilization while the latter has preferential activity on intestinal calcium transport. A metabolite derived from vitamin D_3 which is more polar than 25-HCC has also been found in the intestine (Haussler *et al.*, 1968; Lawson *et al.*, 1969; Ponchon and DeLuca, 1969b). This metabolite acts more rapidly than 25-HCC in initiating intestinal calcium transport (Haussler *et al.*, 1971; Kodicek *et al.*, 1970; Myrtle and Norman, 1971; Omdahl *et al.*, 1971) but is less active in the cure of rickets and does not appear to be more active than 25-HCC in the mobilization of bone mineral (Omdahl *et al.*, 1971) suggesting it to be the metabolically active form in the intestine. Actinomycin D inhibits the conversion of 25-HCC to this metabolite (Tanaka and DeLuca, 1971; Gray and DeLuca, 1971). Of great interest is that this metabolite but not 25-HCC will initiate intestinal calcium transport in the presence of actinomycin D providing strong evidence that it represents the metabolically active form in the intestine (Tanaka *et al.*, 1971).

This metabolite has now been isolated from chicken intestines in pure form as its monotrimethylsilyl ether derivative and identified as 1,25-DHCC. A preliminary report of this work has appeared (Holick *et al.*, 1971).

General Procedures

Radioactive determinations were carried out with a Packard Tri-Carb Model 3375 liquid scintillation counter equipped with an automatic external standardization system. Samples were dried in counting vials with a stream of air and dissolved in toluene-counting solution (2 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl]benzene per l. of toluene).

Ultraviolet spectra were recorded with a Beckman DB-G recording spectrophotometer, while mass spectrometric determinations were carried out with an A.E.I. MS-9 mass spectrometer, using a direct probe inlet at temperatures of 118–130° above ambient. All solvents used were of reagent grade and those utilized for chromatography in the later stages of the isolation of the metabolite were triply distilled before use.

Isolation. Chickens (1450, 1-day old, White Leghorn cockeral chicks obtained from Northern Hatcheries of Beaver Dam, Wis.) were kept in cages at 38° and fed *ad libitum* for 4 weeks as previously described (Omdahl *et al.*, 1971). At the end of week 4 the chicks were starved for 12 hr and then each was given orally 2.5 μg of [1,2-^{3}H]vitamin D_3 (specific activity 3.2×10^5 dpm/μg) in 0.2 ml of vegetable oil (Wesson oil, a mixture of cottonseed and soybean oil). Twenty-four hours later they were killed by decapitation and the whole small intestine was excised, flushed with chilled distilled H_2O, and immediately frozen on Dry Ice. The chick intestines (12.5 kg) were divided into three batches and each was processed in a similar fashion. The first batch (5.5 kg) was homogenized in two parts MeOH (w/v) for 1 min. One part $CHCl_3$ (5.5 l.) was then added to the homogenate and the mixture allowed to stand at 4° for 3 days. The mixture was filtered and an additional 5.3 l. of $CHCl_3$, 0.5 l. of distilled H_2O, and 0.2 l. of saturated NaCl was added to the filtrate to effect a phase separation. The lower phase was separated from the aqueous phase. The aqueous phase was reextracted with an additional

* From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706. *Received March 12, 1971.* Supported by a grant from the U. S. Public Health Service No. AMO-5800-10, the Harry Steenbock Research Fund, the Philips-Duphar Co., and the Wisconsin Alumni Research Foundation.

† Supported by National Institutes of Health Training Grant No. GM00236-BCH from the National Institute of General Medical Sciences.

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[1] Abbreviations used are: 25-HCC, 25-hydroxycholecalciferol; 21,25-DHCC, 21,25-dihydroxycholecalciferol; 25,26-DHCC, 25,26-dihydroxycholecalciferol; 1,25-DHCC, 1,25-dihydroxycholecalciferol.

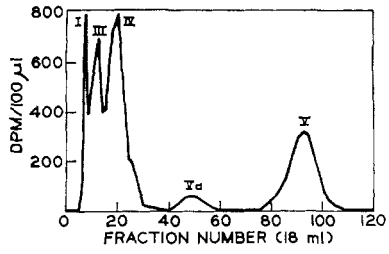

FIGURE 1: Sephadex LH-20 column (3 × 70 cm packed in 65:35 $CHCl_3$–Skellysolve B) profile of intestinal lipid extract from chicks given [1,2-^{3}H]vitamin D_3 orally 24 hr earlier.

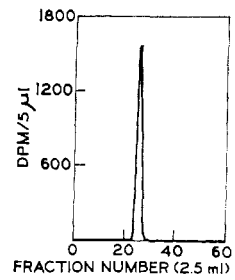

FIGURE 3: Bio-Beads S-X8 column (1 × 150 cm packed in 65:35 $CHCl_3$–Skellysolve B) profile of intestinal peak V isolated from Sephadex LH-20 (MeOH) column (Figure 2).

4 l. of $CHCl_3$–0.5 l. of MeOH. The residue from the initial extraction was reextracted with 6 l. of MeOH and 3 l. of $CHCl_3$. The combined $CHCl_3$ phases (25 l.) was mixed with 16 l. of tap H_2O, 1.5 l. of MeOH, and 0.4 l. of saturated NaCl. This mixture was allowed to stand at 4° for 24 hr. The $CHCl_3$ phase was concentrated to 210 ml on a rotary flash evaporator. This yellow oily residue was partitioned between 1.79 l. of Skellysolve B (predominantly *n*-hexane, bp 67–69°) and 2 l. of 9:1 MeOH–H_2O (v/v) according to the procedure of Suda *et al.* (1970a). The aqueous methanol phase (lower phase) was removed and extracted twice with a mixture of 1.5 l. of $CHCl_3$–0.5 l. of tap H_2O. The $CHCl_3$ phases were combined and evaporated to yield 10 g of a yellow residue. The other two batches 4 and 3.0 kg of chicken intestines were similarly extracted to yield 12 g of lipid residue.

Chromatography. The yellow residue (10 g) was dissolved in 15 ml of 65:35 $CHCl_3$–Skellysolve B and applied to a 3 × 70 cm glass column packed with 110 g of Sephadex LH-20 (an hydroxypropyl ether derivative of Sephadex G-25, Pharmacia Corp., Piscataway, N. J.) with 3 cm of Celite on the top of the bed in the same solvent according to the procedure of Holick and DeLuca (1971). Fractions (100 18 ml) were collected and 100 µl of each fraction used for tritium determinations (Figure 1). The remaining two batches of chicken intestinal extract were chromatographed in a similar fashion. The peak V region from all three batches was combined and dried on a flash evaporator to yield 11 µg of the metabolite (based on specific activity of vitamin D_3 and assuming 50% loss of tritium (Kodicek *et al.*, 1969; Omdahl *et al.*, 1971)) in 319 mg of yellow lipid. This residue was dissolved in 1 ml of MeOH and applied to a 1 × 150 cm glass column packed to a height of 148 cm with Sephadex LH-20 in MeOH (Suda *et al.*, 1970a). Fractions (80 2.5 ml) were collected and 5 µl of each fraction was used for tritium determination to reveal the elution position of the metabolite (see Figure 2). The peak tubes (39–41) were combined and dried under N_2 to give 11 µg of the metabolite in 68 mg of yellow lipid. The residue was dissolved in 0.1 ml of MeOH and reapplied to the same column. Peak V (11 µg) was recovered in 31 mg of lipid. This sample was dissolved in 0.15 ml of 65:35 $CHCl_3$–Skellysolve B and applied to a 1 × 150 cm glass column packed with Bio-Beads S-X8 (polystyrene resin produced by Bio-Rad Corp., Richmond, Cal.) to a height of 148 cm in the same mixed solvent. The fractions containing the metabolite were combined (see Figure 3), and the same chromatography was repeated three more times. This time only the peak tubes which contained the least amount of lipid were combined to provide 8 µg of the metabolite in less than 1 mg of lipid residue. This residue was dissolved in 0.1 ml of 65:35 $CHCl_3$–Skellysolve B and applied to a 1 × 100 cm Sephadex LH-20 column packed in the same mixed solvent.

The peak fractions were collected and dried under N_2. The metabolite (8 µg) was dissolved in 10 µl of pyridine and reacted with 10 µl of TBT (a special combination TMS-imidazole, bis-TMS-acetamide, and trimethylchlorosilane, Pierce Chemical Co., Rockford, Ill.) at 22° for 10 min. The reaction mixture was evaporated under N_2, dissolved in 0.1 ml of 1:1 $CHCl_3$–Skellysolve B, and applied to a 1 × 60 cm Sephadex LH-20 column packed and developed in the same mixed solvent as previously described (Holick and DeLuca, 1971). The tritrimethylsilyl ether derivative of the metabolite which was recovered from this column was dissolved in 50 µl of pyridine and desilylated by adding 100 µl of 3.6 × 10^{-4}% HCl–MeOH (v/v) and reacting it for 3 hr at 50° then an additional 1.5 hr at 60°. The solvent was removed under N_2, dissolved in 0.1 ml of 65:35 $CHCl_3$–Skellysolve B, and applied to a 1 × 60 cm Sephadex LH-20 column packed in and developed with a solvent of 65:35 $CHCl_3$–Skellysolve B. Fractions (27 3.4 ml) were collected followed by an additional 33 5.2-ml fractions as shown in Figure 4. The ditrimethylsilyl ether, monotrimethylsilyl ether, and the unsilylated products were chromatographed separately on a 1 × 60 cm MeOH Sephadex LH-20

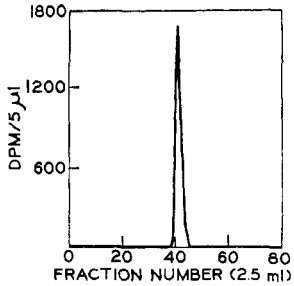

FIGURE 2: Sephadex LH-20 column (1 × 150 cm packed in MeOH) profile of intestinal peak V isolated from Sephadex LH-20 (65:35 $CHCl_3$–Skellysolve B) columns (Figure 1).

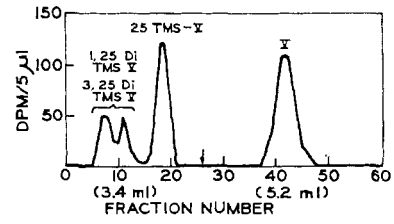

FIGURE 4: Sephadex LH-20 column (1 × 60 cm packed in 65:35 $CHCl_3$–Skellysolve B) profile of the products obtained from the partial desilylation reaction.

TABLE I: Stimulation of Intestinal Calcium Absorption in Rachitic Chicks by 25-HCC and 1,25-DHCC.

Compound	Dosage (μg)	t_a (hr)[a]	% ^{45}Ca Absorption[b]
25-HCC	0.125	24	54.5 ± 6.8[c] (4)
1,25-DHCC	0.065	8.5	54.6 ± 3.9 (5)
Control (0.2 ml of oil)		8.5	18.2 ± 2.3 (7)

[a] t_a represents: time of assay following dose. [b] Net per cent ^{45}Ca absorption for compound at time t_a. [c] Plus or minus the standard error of the mean. Numbers in parentheses show the number of animals in each group. The intestinal calcium absorption measurement was performed as described by (Omdahl *et al.*, 1971).

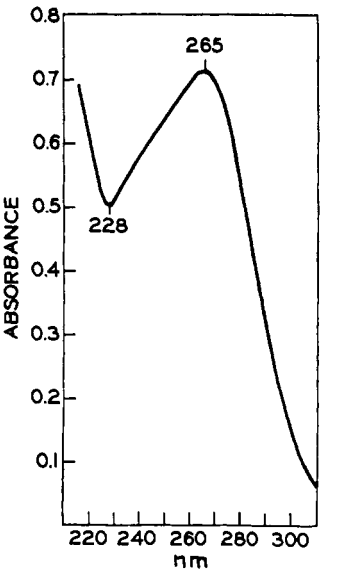

FIGURE 5: Ultraviolet absorption spectrum of the intestinal peak V metabolite.

column as the last purification step before mass spectrometry and ultraviolet spectrophotometry.

Chemical Modifications of the Metabolite. CATALYTIC REDUCTION OF 25-HCC AND OF THE METABOLITE. To 25-HCC (*ca.* 10 μg, specific activity 1.8×10^5 dpm/0.25 μg) in 2 ml of methanol a large excess of PtO_2 catalyst was added and the mixture shaken under a hydrogen atmosphere (3 atm) for 3 hr at room temperature. The solution was decanted from the catalyst and solvent removed under a stream of N_2. The residue was dissolved in 1:1 $CHCl_3$–Skellysolve B and applied to a 1×60 cm Sephadex LH-20 column packed in the same mixed solvent. The identity of the material as 25-hydroxyhexahydrocholecalciferol was confirmed by mass spectrometry (mol wt 406). The metabolite (8000 dpm *ca.* 25 ng) was reduced under the same conditions as above. The major component was recovered after Sephadex LH-20 chromatography. A sample of the 25-hydroxyhexahydrocholecalciferol (3200 dpm) and the reduced metabolite (1500 dpm) were combined and cochromatographed on a 1×60 cm 65:35 $CHCl_3$–Skellysolve B Sephadex LH-20 column. All radioactivity (4780 dpm) was recovered as a single peak (Figure 9).

PERIODATE REACTION OF THE METABOLITE AND MODEL COMPOUNDS. The metabolite (8000 dpm) dissolved 20 μl of methanol was treated with 10 μl of a 5% aqueous solution of $NaIO_4$. After 12 hr the solution was evaporated to dryness under N_2 and the residue redissolved in 0.2 ml of 65:35 $CHCl_3$–Skellysolve B. Original metabolite (4000 dpm) not treated with $NaIO_4$ was added and the mixture applied to a 1×60 cm Sephadex LH-20 column in the same mixed solvent. All radioactivity (12,000 dpm) was recovered as a single peak corresponding to unchanged metabolite.

Periodate cleavage of model compounds (such as 24,25-dihydroxylanosterol and 5β-cholestane-3β,4α,7α-triol) under the same conditions (*i.e.*, 1 μg of compound in 20 μl of MeOH + 10 μl of 5% $NaIO_4$) went to completion within 4 hr at room temperature, as determined by mass spectrometry.

ACETYLATION OF 1,25-DIHYDROXYCHOLECALCIFEROL-25-TRIMETHYLSILYL ETHER. The monosilyl ether derivative of the metabolite (*ca.* 0.2 μg) was allowed to stand at room temperature in a mixture of pyridine (5 μl) and acetic anhydride (5 μl) for 2 hr. The mass spectrum of the product gave the expected molecular weight (mol wt 572) and fragmentation pattern (*m/e* 512, 452, 131) for the diacetylmonotrimethylsilyl ether derivative of the metabolite.

Biological Activity of 1,25-DHCC. Intestinal calcium transport response of the metabolite was measured by the *in situ* gut sac technique as previously described (Omdahl *et al.*, 1971). The results shown in Table I reveal that the isolated metabolite is fully effective in inducing intestinal calcium absorption in chicks 8.5 hr after an oral dose in agreement with previous data (Omdahl *et al.*, 1971).

Results

Identification of the Metabolite as 1,25-DHCC. Ultraviolet absorption maximum (265 nm) and trough at 228 nm (Figure 5), characteristic for the 5,6-*cis*-triene chromophore of the D vitamins, and the molecular ion peak at *m/e* 416 in its mass spectrum (Figure 6); suggested a dihydroxycholecalciferol structure for this compound. The presence of three hydroxyl functions was demonstrated by the formation of a tritrimethylsilyl ether derivative of mol wt 632 (structure III, Figure 7) and of a trimethylsilyl ether diacetate of mol wt 572 (structure I, Figure 7). A mass fragment of *m/e* 131 in the case of the tritrimethylsilyl derivative (Figure 8), the monotrimethylsilyl derivative (II, Figure 7; Figure 6), and the trimethylsilyl ether diacetate (I, Figure 7) established the presence of a hydroxyl function at C-25 (Blunt *et al.*, 1968a; Suda *et al.*, 1969, 1970a). Small peaks at *m/e* 287, 269 (287 − H_2O), and 251 (287 − $2H_2O$) which arise by loss of the entire side chain (C-17–C-20 cleavage) confirm the lack of additional oxygen substituents on the side chain. The other two hydroxyl groups must be located in ring A, since the mass spectra of the metabolite (Figure 6) and of its 25-trimethylsilyl ether derivative (Figure 6; II, Figure 7) exhibited prominent ions at *m/e* 152 and 134 (152 − H_2O) which can only be interpreted as the oxygen analogs of the characteristic ions at *m/e* 136 and 118 (136 − H_2O) observed in the spectra of cholecalciferol, 25-HCC, and other cholecalciferol metabolites (Blunt *et al.*, 1968a; Suda *et al.*, 1969, 1970a,b). The origin of these peaks is graphically illustrated in Figure 6. In the mass spectrum of the tritrimethylsilyl ether compound (III, Figure 7) these peaks appear at *m/e* 296 (152 + 2 silyl groups) and 206 (296 − HOSi-$(CH_3)_3$) (Figure 8). In addition to providing conclusive proof of the presence of two hydroxyl functions in ring A, the peaks at *m/e* 152 and 134 also corroborate the ultraviolet spectral

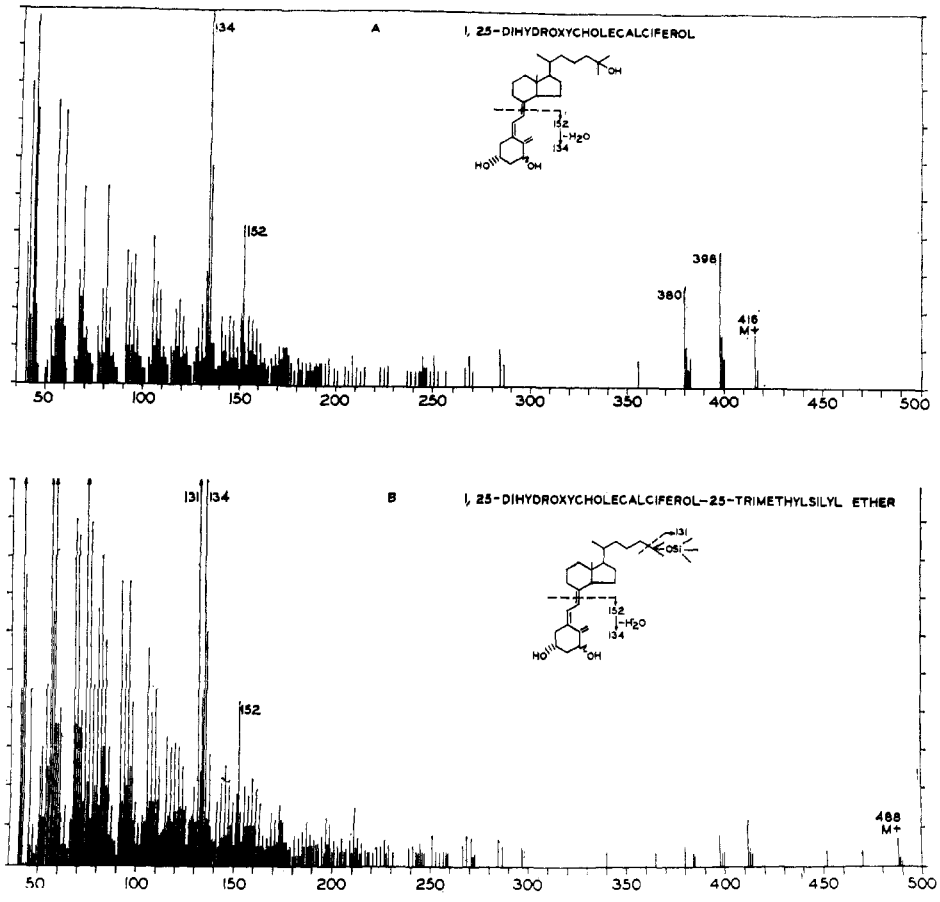

FIGURE 6: Mass spectrum of (A) 1,25-DHCC and (B) 25-monotrimethylsilyl ether derivative of 1,25-DHCC.

data (Figure 5) for the existence of a vitamin D type triene system (Blunt *et al.*, 1968a; Suda *et al.*, 1969, 1970a,b). Since 25-HCC is the immediate precursor of this new metabolite a hydroxyl group at C-3 can be assumed with confidence, and the foregoing results thus allowed only C-1, -2, and -4 as positions for the additional hydroxyl function in ring A. Upon catalytic hydrogenation (PtO_2–H_2) the metabolite yielded a product identical (by cochromatography) (Figure 9) with 25-hydroxyhexahydrocholecalciferol (IV, Figure 7). Since formation of IV (Figure 7) from the metabolite requires hydrogenolysis of an hydroxyl group, the hydroxyl function lost in this process must have been located at either C-1 or C-4 (*i.e.*, allylic to the double-bond system). The failure of the metabolite to react with periodate (under conditions which oxidize model compounds readily, as, for example, 24,25-dihydroxylanosterol and 5β-cholestane-3β,4α,7α-triol) eliminated a C-4 (as well as C-2) hydroxyl group and thus required that the additional hydroxyl group be placed at C-1. The observation that the biosynthesis of this metabolite from 1α-tritiocholecalciferol results in almost complete loss of tritium (Lawson *et al.*, 1969) and in 50% loss of label with 1,2-dititriocholecalciferol as precursor (Omdahl *et al.*, 1971) provides corroborative evidence for a C-1 hydroxy compound. The structure of the intestinal polar vitamin D_3 metabolite is therefore 1,25-DHCC (Figure 10).

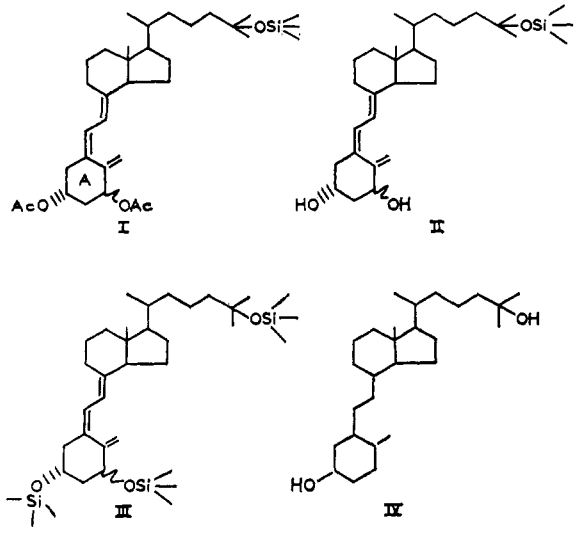

FIGURE 7: Structures of (I) 1,25-DHCC-1,3-diacetyl-25-trimethylsilyl ether, (II) 1,25-DHCC-25-trimethylsilyl ether, (III) 1,25-DHCC-1,3-25-tritrimethylsilyl ether, and (IV) 25-hydroxyhexahydrocholecalciferol.

The stereochemistry of the 1-hydroxy function cannot be specified as yet. Treatment of 1,25-DHCC with triphenylboroxine did not result in a cyclic boronate, suggesting a 1α,3β configuration for the two hydroxyl functions. On the other hand, Bell and Kodicek (1970) using 1-tritiocholecalciferol (83–85% 1α-^{3}H, 15–17% 1β-^{3}H) report at least 96% loss of label suggesting that epimerization at C-1 may have occurred. This steriochemical problem, therefore, will only be resolved by synthesis or by definitive degradation experiments.

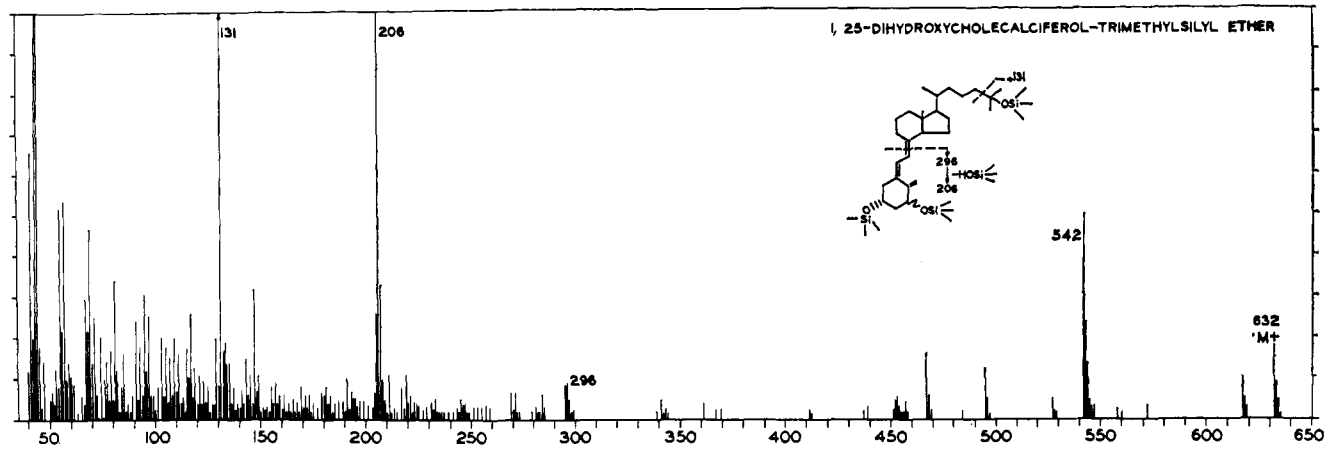

FIGURE 8: Mass spectrum of tritrimethylsilyl ether derivative of 1,25-DHCC.

Discussion

The present report establishes conclusively 1,25-DHCC (Figure 10) as the chemical structure of the polar vitamin D metabolite (designated by different laboratories as peak V, 4B, or P) from intestine which is biologically active in intestine. This structure, as well as 1-oxo-25-HCC, were suspected structures when Lawson *et al.* (1969) and Bell and Kodicek (1970) demonstrated that during its biosynthesis from [1-^{3}H]-vitamin D_3 (85% 1α) the 1-^{3}H is lost. More recently attention was focused on the 1,25-dihydroxy compound by the work of Fraser and Kodicek (1970) which demonstrated that kidney homogenates are able to synthesize the metabolite from 25-HCC by a reaction requiring reduced pyridine nucleotide and oxygen. The 1,25-DHCC structure has now been established by unequivocal physical measurements and known chemical reactions on the metabolite isolated from the intestines of chicks given [1,2-^{3}H]vitamin D_3.

The isolation of this metabolite proved to be the most difficult yet performed in this laboratory. Chemical purifications such as saponification were avoided because of fear of losses and isomerization. Even silicic acid column methods were not used nor were the powerful partition methods previously developed for polar vitamin D metabolites (Suda *et al.*, 1970-a,b) because of 10–20% losses suffered during the use of those methods. Total purification depended heavily on liquid–gel partition chromatography (Holick and DeLuca, 1971) and gel filtration using Sephadex LH-20 and Bio-Beads S-X8. However, complete purification was not achieved until the chromatographic properties of the metabolite were changed in a specific fashion. The relative difficulty of removing the silyl group from the tertiary hydroxyl (C-25) of the metabolite was used. The resulting 25-trimethylsilyl ether derivative chromatographed sufficiently different to separate it from the contaminating lipids.

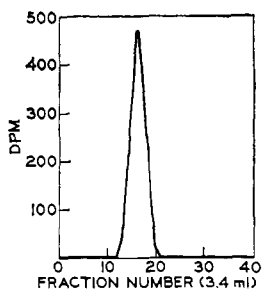

FIGURE 9: Sephadex LH-20 column (1 × 60 cm packed in 65:35 $CHCl_3$–Skellysolve B) profile for the cochromatography of 25-hydroxyhexahydrocholecalciferol with reduction product of intestinal peak V metabolite.

The ability of the isolated metabolite (Table I) to stimulate intestinal calcium transport leaves no doubt that it represents the polar metabolite (peak V in our laboratory, 4B in that of Haussler and Norman, and P in that of Kodicek) believed to be the metabolically active form of vitamin D in the intestine. It is now clear that the 1,25-DHCC acts more rapidly than does 25-HCC in initiating calcium transport (Haussler *et al.*, 1971; Myrtle and Norman, 1971; Omdahl *et al.*, 1971). Of great importance is the fact that the 1,25-DHCC can initiate intestinal calcium transport in the presence of actinomycin D while 25-HCC cannot (Tanaka *et al.*, 1971). Actinomycin D and cycloheximide both block the conversion of 25-HCC into 1,25-DHCC illustrating that 25-HCC interacts with the genetic machinery to induce the formation of an enzyme or factor necessary for the appearance of 1,25-DHCC in the intestine. The 1,25-DHCC then probably acts by a mechanism not involving transcription of DNA. The data further show that the 1,25-DHCC or a further metabolite thereof must be the metabolically active form of vitamin D in the intestine.

It is not known whether 1,25-DHCC is the metabolically active form of vitamin D in inducing bone mineral mobilization. It is active in this system but does not appear to act faster nor does it appear more active than 25-HCC (Omdahl *et al.*, 1971). Furthermore the 25-HCC does induce bone mineral mobilization in isolated cultures of embryonic bone (Trummel *et al.*, 1969) which appear unable to produce the 1,25-DHCC. Only additional experiments can clarify this point, however.

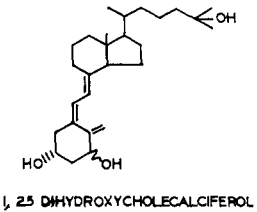

FIGURE 10: 1,25-Dihydroxycholecalciferol (1,25-DHCC).

It must be emphasized that the exact sterochemical position of the hydroxyl at C-1 has not been established. It is likely that the position is α since 1-α-^{3}H is totally lost in its biosynthesis and since our attempt to form a boronate ester of the isolated metabolite failed. Experiments are now in progress to delineate the exact stereochemical position of the 1-hydroxy function of 1,25-DHCC.

The elucidation of the structure of this important metabolite opens many possible avenues of investigation. The exact metabolic sequence involved in the vitamin D initiation of intestinal calcium transport can now be approached more directly. Furthermore, if synthesis is successful, this compound could be useful in some types of vitamin D resistant diseases such as renal osteodystrophy and familial hypophosphatemia.

Acknowledgment

We are especially grateful to J. Omdahl and M. Garabedian for their help in this project and we thank C. Frolik, J. Knutson, R. Hallick, T. Chen, I. Boyle, Y. Tanaka, M. Horsting, E. B. Olson, and R. Gray for their assistance in some of this work. Also we are indebted to Dr. M. Sunde of the Department of Poultry Science for the use of facilities used in the animal portion of these experiments and to Professor Charles Sih of the Department of Pharmaceutical Chemistry for providing us with several steroid model compounds.

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