Biochemistry

 Volume 7, Number 10 October 11, 1968

25-Hydroxycholecalciferol. A Biologically Active Metabolite of Vitamin D_3*

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ABSTRACT: Milligram quantities of a polar metabolite of vitamin D_3 have been isolated in pure form from the plasma of pigs fed 250,000 IU of vitamin D_3 per day for 26 days. This metabolite is the major biologically active component of the peak IV metabolite fraction reported earlier. A combination of ultraviolet spectra, gas–liquid partition chromatography and mass spectrometric and nuclear magnetic resonance spectrometric analyses have unequivocally established its structure as 25-hydroxycholecalciferol. Its biological activity has been set at 1.4 that of vitamin D_3 in its ability to cure rickets in rats.

It has long been assumed that unaltered vitamin D_3 (I) is responsible for its metabolic and physiologic manifestations. The first demonstration of biologically active metabolites of vitamin D appeared from this laboratory in 1964 (Norman *et al.*, 1964). It was, however, the work of Lund and DeLuca (1966) which first demonstrated unambiguously that a polar metabolite of vitamin D designated as peak IV was possibly the metabolically active form. Thus they were able to show large amounts of the peak IV metabolite fraction in bone, liver, and blood serum of rats. Upon estimation of its biological activity it was evident that it was at least as active and perhaps more active than vitamin D itself. Furthermore, it was the predominant form of vitamin D in the target tissues (intestine and bone) after truly physiologic doses (10 IU) of the vitamin (DeLuca, 1967) The metabolite was also demonstrated in human plasma (DeLuca *et al.*, 1967; Avioli *et al.*, 1967), chicks (Drescher *et al.*, 1968), and porcine plasma (present report). Other experiments have shown that vitamin D is converted into this material before its action in promoting intestinal absorption of calcium can be observed (DeLuca, 1967). Finally, the metabolite stimulates the intestinal absorption of calcium and mobilization of bone much as does vitamin D itself (Morii *et al.*, 1967) providing strong evidence that it is in fact the metabolically active form of the vitamin. Finally, that this metabolite is the primary or only form associated with intestinal nuclei, the believed site of vitamin D action, has been unequivocally demonstrated by Stohs and DeLuca (1967).

The biologically active component of the peak IV metabolite fraction has now been isolated in milligram quantities in pure form from the plasma of pigs maintained on high vitamin D_3 rations. Its chemical structure has unambiguously been shown to be 25-hydroxycholecalciferol (II) and its biological activity shown to be greater than that of vitamin D_3 itself. A preliminary report of this work has recently appeared (Blunt *et al.*, 1968).

Methods and Results

General Procedures. Ultraviolet spectra were recorded with a Beckman DB-G spectrophotometer. Nuclear magnetic resonance spectra were recorded in $CDCl_3$ solutions in a Varian Associates Model HA-100 spectrometer coupled to a time-averaging computer, the latter being necessary because of the small amount of metabolite isolated. Tetramethylsilane was used as the internal standard. Data are recorded as δ in parts per million, relative to tetramethylsilane ($\delta = 0$). Gas–liquid partition chromatography was performed in an F & M Model 402, using 4 ft × 0.25 in. glass columns packed with 3% W-98 on 80–100 mesh Diatoport S. High-resolution mass spectra were recorded with an

* From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received May 27, 1968.* Supported by Research Grant No. AMO-5800-07 from the U. S. Public Health Service. Published with the approval of the director of the Wisconsin Agriculture Experimental Station.

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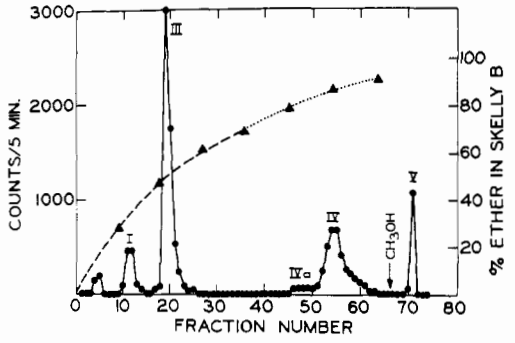

FIGURE 1: Silicic acid chromatography of vitamin D and its metabolites. Peaks III and I are vitamin D and its esters respectively, peaks IVa and V are unidentified, and peak IV is 25-hydroxychlolecalciferol.

A.E.I. MS-9 mass spectrometer coupled to a Scientific Data Systems Sigma-7 computer.

All radioactivity measurements were made in a Packard Tri-Carb liquid scintillation counter, Model 3000, and automatic external standardization was used to determine efficiency. Radioactive samples were counted in a scintillator solution which consisted of 2 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene) per l. of toluene.

Preparation of Extract Containing Labeled Vitamin D_3 and Its Metabolites. To determine the optimum time after dosing a hog with vitamin D_3 so as to recover the greatest amount of peak IV metabolites, a 50-lb hog was given a dose of 0.5 mg of random-labeled [^{3}H]vitamin D_3 (Norman and DeLuca, 1963) (5.7 mCi/mmole) in 1 ml of ethanol intravenously. A preliminary experiment on serial blood samples taken at various times revealed that optimum concentrations of peak IV metabolites appeared at 24 hr after dosing. Accordingly, the same hog was given a further 2-mg dose of [^{3}H]vitamin D_3 in 1 ml of ethanol intravenously 72 hr after the original 0.5-mg dose. Twenty-four hours later, the blood was removed under Nembutal anesthesia. Blood (1.2 l.) was collected, giving 800 ml of serum.

To provide a convenient means for extracting the vitamin D and its metabolites from large volumes of plasma a method involving the precipitation of serum proteins with ammonium sulfate was investigated. Aliquots (0.5 ml) of the radioactive serum were saturated to varying degrees with $(NH_4)_2SO_4$. The protein precipitates were removed by centrifugation and extracted with methanol–chloroform (Bligh and Dyer, 1959) and the whole extracts were counted. The results showed that 65% saturation with $(NH_4)_2SO_4$ effectively precipitates virtually all of the radioactivity.

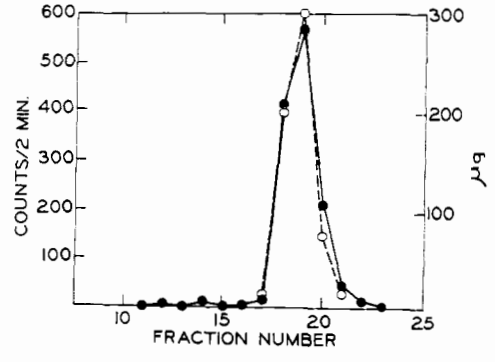

FIGURE 2: Partition chromatography of peak IV from Figure 1.

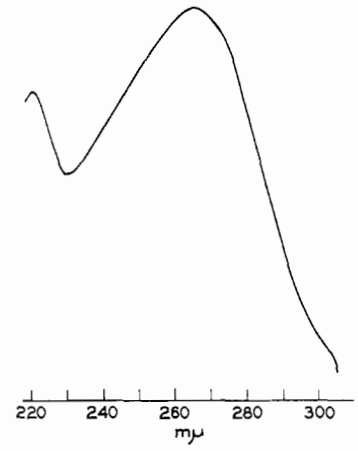

FIGURE 3: Ultraviolet spectrum of 25-hydroxycholecalciferol in ether.

The remainder of the serum was either extracted directly with methanol–chloroform or precipitated by 65% saturation with $(NH_4)_2SO_4$, followed by extraction of the precipitate. The original dose (2.6%) of [^{3}H]D_3 was recovered as peak IV metabolites representing 42% of the radioactivity in the plasma. Of this, 60% was 25-hydroxycholecalciferol.

Isolation of 25-Hydroxycholecalciferol (II). A 100-lb hog was maintained on normal rations supplemented with 100,000 IU of vitamin D_3 (Delsterol, Vita Plus Corp., Madison, Wis.) daily for 3 days before collection of a blood sample. The serum from this sample was extracted with chloroform–methanol and separated as before (Lund and DeLuca, 1966) into the vitamin D and peak IV fractions which were submitted to assay for vitamin D activity by the rat line test method (U. S. Pharmacopeia, 1955). This assay indicated 4 IU/ml due to vitamin D and 2 IU/ml due to peak IV. The same hog was maintained on a supplemented diet (100,000 IU of D_3 daily) for 4 months (weight gain, 300 lb) after which time serial samples assayed for 10 IU/ml of serum due to vitamin D and 5 IU/ml due to peak IV. Hogs weighing 200 lb and maintained on normal diet supple-

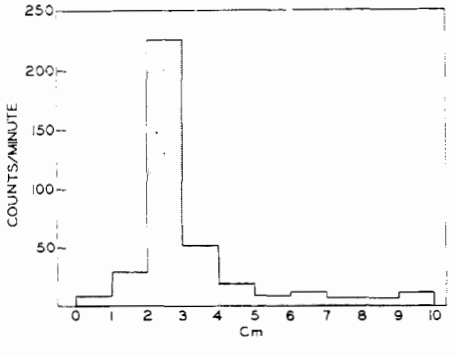

FIGURE 4: Thin-layer chromatography of 25-hydroxycholecalciferol on silica gel G.

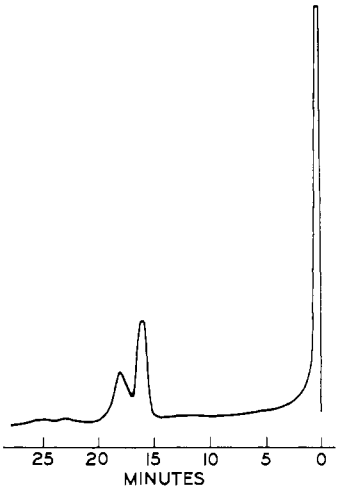

FIGURE 5: Gas–liquid partition chromatogram of 25-hydroxycholecalciferol. The larger peak is 25-hydroxypyrocholecalciferol (VI), while the smaller is its isopyro isomer (IV). Injection and column temperature 240°, helium flow rate 70 cc/min at 50 psi. Sensitivity 10 × 8.

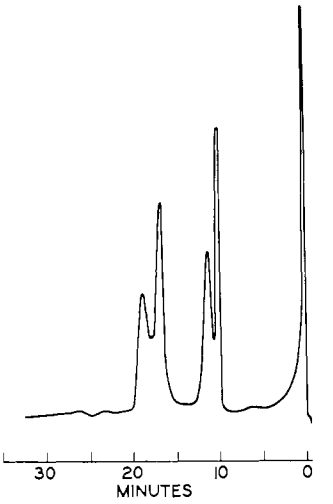

FIGURE 6: Gas–liquid partition chromatography of 25-hydroxycholecalciferol as in Figure 4, but with vitamin D_3 added.

mented with 250,000 IU of D_3 daily for 10 days gave blood serum which assayed for 14 IU/ml of total vitamin D activity, consisting of 6 IU/ml due to vitamin D and 8 IU/ml due to peak IV. Finally, four hogs (210–240 lb) were maintained on the 250,000 IU of D_3 daily supplement for 26 days. Serial samples assayed for 20 IU/ml of serum total D activity, consisting of 8 IU/ml due to vitamin D and 12 IU/ml due to peak IV. The blood collected from these four hogs was treated with 1.6 l. of 0.1 M sodium oxalate, giving 6.8 l. plasma. Sufficient ammonium sulfate was added to the plasma to achieve 65–70% saturation. The precipitate was allowed to form at 4° for 3 days, collected by centrifugation for 25 min at 15,000 rpm in a Sharples AS-16-P centrifuge, and extracted with methanol–chloroform. The total extract was made up to 50 ml with Skelly B. This extract assayed for a total of 100,000 IU of vitamin D activity by a rat line test assay (U. S. Pharmacopeia, 1955).

About 400,000 dpm of the radioactive extract from the 50-lb hog was combined with 20 ml of the Skelly B extract from the four hogs. This was applied to a silicic acid (25 g) adsorption column for chromatography (Lund and DeLuca, 1966). The column was eluted with an ether–Skelly B gradient, obtained by running 400 ml of 85% ether–Skelly B from a holding chamber into a 250-ml mixing chamber initially containing 250 ml of Skelly B. Following the collection of 36 11-ml fractions, 100% ether was placed in the holding chamber, and an additional 30 11-ml fractions were collected. Methanol was then applied directly to the column. Figure 1 shows the radioactivity profile of the column eluate, and the ether–Skelly B gradients obtained. It is seen that the originally described peak IV has been resolved into at least three components, of which the major one retains the designation peak IV. Peak IV and the minor preceding peak IVa are eluted by high concentrations of ether in Skelly B, whereas peak V is eluted by methanol. Occasionally, a small distinct peak appeared immediately after peak IV, and was labeled peak IVb. It is emphasized that the size of these peaks represents the amount of metabolites 24 hr after a single dose of [^{3}H]D_3 and has no reflection on the amount of metabolites present after 26 days of daily dosage of unlabeled vitamin D_3. Other chromatographic systems developed in this laboratory have also demonstrated the heterogeneity of peak IV, and these will be reported later, together with the biological activity data for the minor peaks (G. Ponchon and H. F. DeLuca, unpublished data). Fractions 51–60 from Figure 1 were combined and applied to a partition column, constructed as follows. Celite (20 g) was mixed with 15 ml of a stationary phase (80% methanol–20% water, equilibrated with an equal volume of Skelly B). About two-thirds of this was packed in a 1-cm diameter column. The sample was applied to the column in a small quantity of the mobile phase (the Skelly B equilibrated with methanol–water) and the column eluted with mobile phase, with 5-ml fraciions being collected. Figure 2 shows the radioactivity profile, and also the weight of the metabolite, as determined from its ultraviolet spectrum in ether (Figure 3).

The radioactive homogeneity of the peak material was further demonstrated on thin-layer chromatography

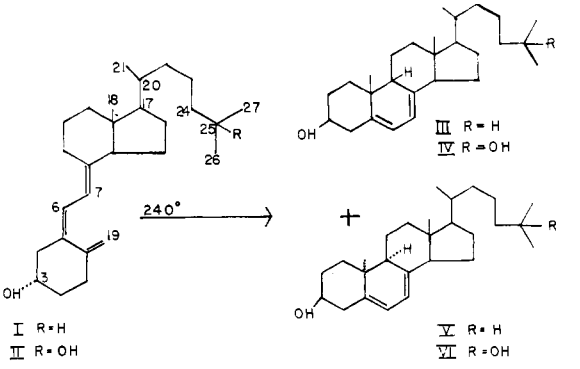

FIGURE 7: Structures for cholecalciferol (vitamin D_3) (I), 25-hydroxycholecalciferol (II), pyrocholecalciferol (V), isopyrocholecalciferol (III), and their respective 25-hydroxy derivatives, VI and IV.

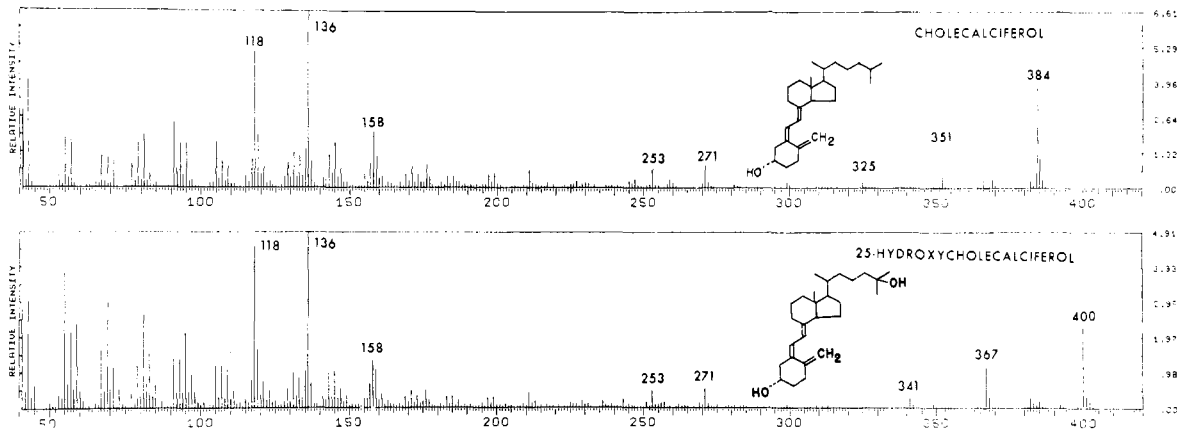

FIGURE 8: Mass spectra of cholecalciferol and 25-hydroxycholecalciferol.

using silica gel G as support and 4% methanol in chloroform as the developing solvent. Figure 4 shows the location of the radioactivity confined to a very narrow region. Further, visualization (with 50% H_2SO_4 followed by heating) of a plate to which 10 μg of metabolite was applied and developed with 6% methanol in chloroform displayed one spot only, R_F 0.64, compared with 25-hydroxycholesterol, R_F 0.54. Gas chromatography (see later) of the peak material indicates for it a high purity (Figure 5).

The remainder of the 50 ml of Skelly B extract was combined with a further quantity of radioactive extract and applied in two portions to silicic acid columns followed by partition chromatography of the peak IV as just described. In this manner, 1.3 mg of pure metabolite was obtained.

Identification of Peak IV as 25-Hydroxycholecalciferol. The peak IV material exhibited a ultraviolet absorption maximum in diethyl ether at 265 mμ (Figure 3) as for vitamin D. When subjected to gas–liquid partition chromatography, two peaks only appeared (see Figure 5). Vitamin D_3, when chromatographed under the same conditions, also gave rise to two peaks, representing pyro- and isopyrocholecalciferol (V and III) (see Figures 6 and 7), the products of thermal rearrangement of vitamin D_3. Collection of the peaks from the metabolite and from vitamin D_3 and measurement of their ultraviolet spectra showed the first peaks of each pair to have identical spectra (λ_{max} 274, 284, and 296 mμ). Similarly, the second peaks of each pair had identical spectra (λ_{max} 274, 284, and λ_{inf} 294 mμ).[1] Clearly then, the metabolite has a similar structure to that of vitamin D_3, particularly in the region of the triene system. Accordingly, an extinction coefficient of 18,200, as for vitamin D, is assumed for the ultraviolet absorption maximum of the metabolite. The high-resolution mass spectra of the vitamin and its metabolite provided information on the difference between the two compounds (Figure 8). The molecular weight of the metabolite was 400.3343 ($C_{27}H_{44}O_2$), *i.e.*, a cholecalciferdiol. The presence of a peak at *m/e* 271.2053 ($C_{19}H_{27}O$) in the spectra of both compounds indicated the location of the second hydroxyl group of the metabolite on the side chain, since the fragment *m/e* 271 arises by loss of side chain through cleavage of the C_{17-20} bond. Further, a peak at *m/e* 59.0492 (C_3H_7O) in the spectrum of the metabolite, but not in the spectrum of the vitamin, could arise by cleavage of the C_{24-25} bond, with the hydroxyl group attached to C_{25}. A detailed study of the mass spectra of these, and other related compounds, will be the subject of a forthcoming publication. The location of the hydroxyl group at C_{25} was verified by the 100-Mcps nuclear magnetic resonance spectrum of the metabolite (Figure 9), which exhibited a strong singlet peak at δ = 1.20 ppm, as in 25-hydroxycholesterol (Figure 10), and an absence of the doublet at δ = 0.87 ppm (J = 6.5 cps) due to the secondary $C_{26,27}$-methyl groups as found in cholecalciferol (Figure 11). The peaks at δ = 0.58 ppm are due to the tetramethylsilane internal standard. The C_{18}–H_3 resonance is seen at δ = 0.54 ppm, and the doublet due to the C_{21}–H_3 group is at δ = 0.93 ppm (J = 5 cps) for both the metabolite and the vitamin. Further, the downfield regions for the vitamin and the metabolite are shown in Figure 12, and are seen to be identical. The peaks at δ = 4.81 and 5.03 ppm are from

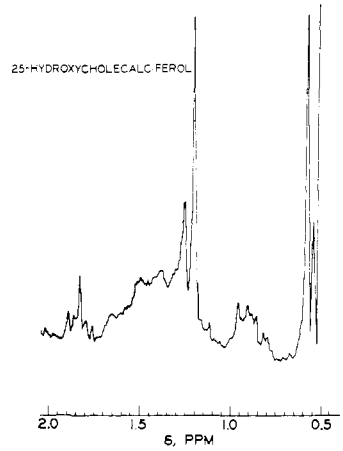

FIGURE 9: Nuclear magnetic resonance spectra (at 100 Mcps) of 25-hydroxycholecalciferol.

[1] These data are identical with those supplied by Professor E. R. H. Jones in a personal communication, but differ from data reported elsewhere (Fieser and Fieser, 1959).

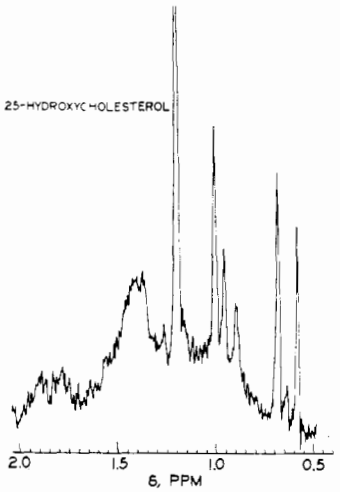

FIGURE 10: Nuclear magnetic resonance spectra (at 100 Mcps) of 25-hydroxychloesterol.

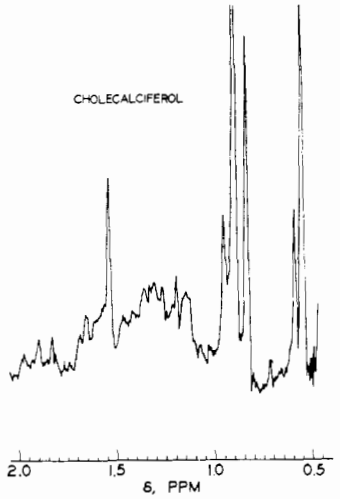

FIGURE 11: Nuclear magnetic resonance spectra (at 100 Mcps) of cholecalciferol.

the $C_{19}H_2$ group, and the doulets at δ = 6.02 ppm (J = 11.5 cps), and δ = 6.24 ppm (J = 10.5 cps) are due to the protons at the 6 and 7 positions.

Thus, a complete identification of the major metabolite as 25-hydroxycholecalciferol has been made.

Biological Activity of 25-Hydroxycholecalciferol. Upon completion of its isolation and identification, the 25-hydroxycholecalciferol was dissolved in Wesson oil and bioassayed in rats for vitamin D activity by the U. S. Pharmacopeia line-test method (1955). Several independent assays were carried out at the Wisconsin Alumni Research Foundation and in our own laboratory. The samples were made up according to their ultraviolet absorbance at 265 mμ assuming an extinction of 18,200. Routinely the 25-hydroxycholecalciferol gave a value of 56–60 IU/μg or 1.4 times that of vitamin D_3.

Discussion

A number of metabolites of vitamin D are now known to exist (DeLuca, 1967). In his early work, Kodicek reported the existence of breakdown products of vitamin D but could find no biologically active metabolites (Kodicek, 1956, 1958). In this laboratory, at least three biologically active metabolites and a number of inactive metabolites were unequivocally demonstrated (Lund and DeLuca, 1966). One of these proved to be an ester of long-chain fatty acids with the 3-OH of the vitamin D_3 (Lund *et al.*, 1967). Fraser and Kodicek (1968) have studied the ester fraction and shown that palmitic, stearic, oleic, and linoleic are the predominant fatty acids found in this combination. However, exactly what is the physiologic importance of the ester fraction remains unknown inasmuch as its concentration remains low regardless of vitamin D dosage. Up to the present time all other metabolites of the vitamin remained unidentified although a sulfate ester of vitamin D has also been reported (Higaki *et al.*, 1965). In addition the existence of glucuronides of vitamin D and its metabolites have been reported but have not been positively identified as yet (Avioli *et al.*, 1967).

This report demonstrates unequivocally the existence of 25-hydroxycholecalciferol as a biologically active metabolite of vitamin D_3. First, the triene structure of the parent vitamin was shown in the metabolite by its ultraviolet spectrum which was identical with that of vitamin D_3. This structure was supported by the generation of pyro and isopyro forms (VI and IV) on gas–liquid partition chromatography of the metabolite and by the downfield region of the nuclear magnetic resonance spectrum. The mass spectrum demonstrated a molecular weight of 400, or a calciferdiol. Fragmentation analysis clearly showed the extra oxygen in the side chain, probably at the 25 position. Unequivocal proof for 25-hydroxycholecalciferol was provided by its nuclear magnetic resonance spectrum, in which the doublet due to the 26,27-methyl groups coupled with the 25-H was shifted to a singlet corresponding to a substitution of OH for H at the 25 position.

The biological significance of the 25-hydroxycholecalciferol is still under study; however, there is a strong possibility that it represents the metabolically active form of vitamin D. A complete study of its biological activities is currently nearing completion and will be the subject of a forthcoming report. It is nevertheless clear that on a weight basis the 25-hydroxy compound is more effective in curing rickets in rats than is the un-

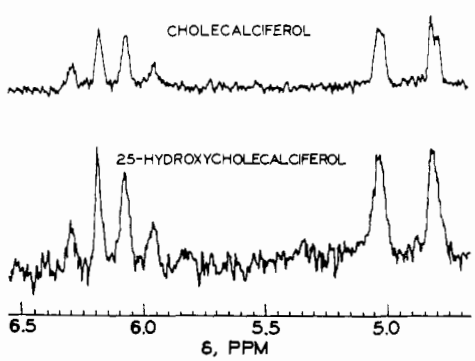

FIGURE 12: Nuclear magnetic resonance spectra (at 100 Mcps) of cholecalciferol and 25-hydroxycholecalciferol.

altered vitamin. In previous work it was shown that the peak IV metabolites from rats mimicked the action of vitamin D in raising serum calcium (bone mobilization) and in increasing intestinal transport of calcium (Morii *et al.*, 1967). Furthermore, the metabolite acted more rapidly than did vitamin D itself. The 25-hydroxycholecalciferol represents the biologically active component of the peak IV metabolite fraction previously used and although not tested directly as yet, it seems almost certain to possess these properties. Other experiments have shown that the peak IV metabolites appear in the target tissues before any biochemical changes due to vitamin D are known to occur and that after truly physiological doses of the vitamin, the peak IV fraction is the predominant form of the vitamin in the body. Finally, the major if not sole form of vitamin D in at least one of the believed subcellular sites of vitamin D action is in the peak IV fraction. Although the evidence for the 25-hydroxycholecalciferol as the metabolically active form of the vitamin is highly suggestive, final proof of such a role must rest with the isolation of the biochemical system in which it functions.

Of greatest importance to the successful isolation was the achievement of high levels of metabolite in the plasma of the pigs. Clearly, large single doses of vitamin D_3 were not sufficient. However, chronic doses of large amounts of vitamin D resulted in metabolite activities as high as 12 IU/ml of plasma. Modification of the silicic acid chromatography to extend the diethyl ether gradient also resulted in unexpected purification over that achieved with all other solvent systems attempted. Finally, the partition column developed for the metabolite effected a high degree of purification resulting in essentially pure material. Purity was established by gas–liquid partition chromatography, by thin-layer chromatography, by ultraviolet spectra, and by nuclear magnetic resonance data.

Acknowledgment

We gratefully acknowledge the excellent technical assistance of Mrs. Yoko Tanaka, the assistance of E. Joe Dahmer and E. B. Olson in handling the pigs, the generous gift of pigs and use of swine facilities by E. J. Briskey and R. H. Grummer of the Meat and Animal Science Department, University of Wisconsin, nuclear magnetic resonance spectral determinations by Miss Martha Petrie of the University of Wisconsin, Chemistry Department, vitamin D assays by Mr. Lloyd Hein of the Wisconsin Alumni Research Foundation, Madison, Wis., the generosity of Professor Burlingame, University of California, Berkeley, Calif., for the use of the mass spectrometer, and the assistance fo Dr. Smith and Mr. Simoneit in recording and calculating the mass spectrometer results.

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