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Isolation and Identification of 24,25-Dihydroxycholecalciferol, a Metabolite of Vitamin D_3 Made in the Kidney†

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ABSTRACT: A metabolite of vitamin D_3 found in normocalcemic and hypercalcemic animals has been isolated in pure form from chicken kidney homogenates. It has been identified as 24,25-dihydroxycholecalciferol by means of mass spectrometry, ultraviolet absorption spectrophotometry, and specific chemical reactions.

It is now clear that vitamin D_3 must be converted first in the liver to 25-hydroxycholecalciferol (25-OHD_3)[1] (Blunt *et al.*, 1968; Ponchon *et al.*, 1969; Horsting and DeLuca, 1969) and then in the kidney to 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$) (Fraser and Kodicek, 1970; Gray *et al.*, 1971; Holick *et al.*, 1971a,b; Lawson *et al.*, 1971) before it can stimulate either intestinal calcium transport (Boyle *et al.*, 1972a) or bone calcium mobilization (Holick *et al.*, 1972). Of great importance is the observation that the 25-OHD_3–1-hydroxylase in the kidney is tightly regulated either directly or indirectly by blood serum calcium levels. Boyle *et al.* (1971) have demonstrated in young rats that the synthesis of 1,25-$(OH)_2D_3$ is maximal in hypocalcemic rats and is almost completely repressed in normocalcemic animals. Furthermore, they showed that the normocalcemic animals produced a metabolite proposed to be 21,25-dihydroxycholecalciferol (21,25-$(OH)_2D_3$) (Suda *et al.*, 1970a). Similarly Omdahl and DeLuca (1971) reported that the inhibition of intestinal calcium transport due to dietary strontium is the result of a block of the kidney hydroxylase which produces 1,25-$(OH)_2D_3$ from 25-OHD_3. They also found that instead of synthesizing 1,25-$(OH)_2D_3$ from 25-OHD_3 these animals make a new metabolite designated as peak Va. This metabolite is made exclusively by the kidney (Boyle *et al.*, 1972b; Omdahl *et al.*, 1972) and can be generated *in vitro* from 25-OHD_3 with kidney mitochondria from chickens that are fed a high calcium diet or a strontium diet.

It is the purpose of this report to establish firmly the structure of the chicken *in vitro* and porcine *in vivo* peak Va metabolite as 24,25-dihydroxycholecalciferol (24,25-$(OH)_2D_3$) based on ultraviolet absorption spectrophotometry, mass spectrometry, and its sensitivity to periodate treatment.

General Procedures

Radioactive determinations were carried out with a Packard Tri-Carb Model 3375 liquid scintillation counter equipped with an automatic external standard system. Samples were dried in 15 × 45 mm glass vial inserts with a stream of air and dissolved in 4 ml of toluene counting solution (2 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)benzene] per 10 of toluene).

Ultraviolet absorption spectra were recorded with a Beckman DB-G recording spectrophotometer while mass spectrometric determinations were carried out with an AEI MS-9 mass spectrometer using a direct probe inlet at temperatures of 120–150° above ambient. Gas–liquid chromatography was carried out with an F & M Model 402 high-efficiency gas chromatograph equipped with a 0.25 in. × 4 ft glass column packed with 3% SE-30 on gas chrom Z, 100–120 mesh (Applied Science Labs, Inc., State College, Pa.). The column temperature was 250° and an outlet flow rate of 80 ml/min was maintained. All solvents used were redistilled before use.

In Vitro Preparation of Peak Va. Chickens (50 one-day old white Leghorn cockerel chicks obtained from Northern Hatcheries, Beaver Dam, Wis.) were kept in cages at 38° and fed *ad libitum* for 2 weeks as previously described (Omdahl *et al.*, 1971). At the end of the second week the chicks were switched to a high calcium diet (3% calcium) and given 0.25 μg of vitamin D_3 orally each day for an additional 12 days. The animals were sacrificed, and the kidneys were removed and homogenized in three volumes of buffer solution (pH 7.4) containing 14 mM Tris-OAc, 0.19 M sucrose, 1.87 mM MgOAc, 5 mM succinate, and 0.4 mM NADP. A total of 450 ml of homogenate was obtained and this was incubated as 6-ml aliquots in 75 erlenmeyer (250 ml) flasks at 37° for 90 min. Each flask contained 4.2 μg of [26,27-^{3}H]25-OHD_3 (specific activity 57,000 dpm/μg) (Suda *et al.*, 1971) in 25 μl of 95% ethanol. The homogenate was extracted with $CHCl_3$ and MeOH as previously described (Lund and DeLuca, 1966).

The resulting yellow lipid residue (1 g) was dissolved in 1.5 ml of 65:35 $CHCl_3$–Skellysolve B (petroleum ether fraction redistilled at 67–69°) and applied to a 2 × 60 cm glass column packed with 60 g of Sephadex LH-20 (a hydroxypropyl ether

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[1] Abbreviations used are: 25-OHD_3,-25-hydroxycholecalciferol; 21,25-$(OH)_2D_3$, 21,25-dihydroxycholecalciferol; 25,26-$(OH)_2D_3$, 25,26-dihydroxycholecalciferol; 1,25-$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; 24,25-$(OH)_2D_3$, 24,25-dihydroxycholecalciferol.

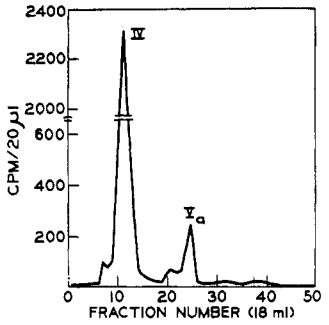

FIGURE 1: Sephadex LH-20 column (2 × 60 cm packed in 65:35 $CHCl_3$–Skellysolve B) profile of lipid extract from kidney homogenates incubated with [26,27-^{3}H]-25-OHD_3.

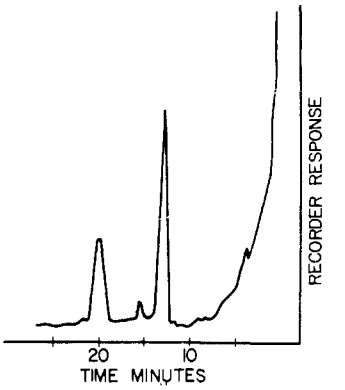

FIGURE 3: Gas–liquid chromatographic profile of the tristrimethylsilyl ether derivative of a mixture of peak Va from chick kidney homogenates and hog plasma.

derivative of Sephadex G-25, Pharmacia Corp., Piscataway, N. J.) according to the procedure of Holick and DeLuca (1971). Fractions (40–18 ml) were collected and 20 μl of each fraction was used for tritium determinations (Figure 1). The peak Va region (tubes 23–27) was combined and dried under N_2 to yield 20 μg of the metabolite in 30 mg of yellow lipid. This residue was dissolved in 50 μl of 65:35 $CHCl_3$–Skellysolve B and applied to a 1 × 150 cm glass column packed with Bio-Beads SX-8 (polystyrene resin produced by Bio-Rad Corp., Richmond, Calif.) to a height of 140 cm in the same solvent according to the procedure of Holick *et al.* (1971b). Fractions (40–2.0 ml) were collected and the peak tubes (29–32) were combined, dried under N_2, and redissolved in 100 μl of 1:1 $CHCl_3$–Skellysolve B. The sample was applied to a 1 × 60 cm glass column packed with 20 g of Sephadex LH-20 and 60 fractions (5.5 ml) were collected. The peak fractions (37–45) (see Figure 2) were combined, dried under N_2, and redissolved in 100 μl of MeOH. This sample was applied to a 1 × 60 cm glass column packed with 17 g of Sephadex LH-20 in methanol and 22 fractions (1.95 ml) were collected. Fractions 15–18 which contained the metabolite were used for mass spectrometry and ultraviolet absorption spectrophotometry.

Gas–liquid chromatography of the tristrimethylsilyl ether derivative revealed two peaks corresponding to pyro and isopyro forms of the metabolite (Figure 3). The gas–liquid chromatogram also illustrated the purity of the isolated material. That the isolated compound contains the ^{3}H label was demonstrated by the close correlation between radioactivity and optical density at λ_{max} 265 nm (absorption maximum of the vitamin D *cis*-triene system) in all fractions on column chromatography as shown previously for the Va material isolated from hog blood (see Figures 3 and 4 of Suda *et al.*, 1970a).

Isolation of in Vivo Generated Peak Va. The peak Va metabolite was isolated from the blood of eight hogs similar to the procedure of Suda *et al.* (1970a). The purification of the metabolite was conducted in a similar fashion to that described for the purification of the *in vitro* peak Va metabolite. Approximately 200 μg of the metabolite was recovered in pure form for its identification. Purity was established by gas–liquid chromatography, column chromatography, mass spectrometry, and ultraviolet spectrophotometry as described previously (see Figures 3 and 4 in Suda *et al.*, 1970a).

Chemical Modifications of the Metabolites. PERIODATE OXIDATION. The peak Va metabolite (both *in vivo* and *in vitro*) (5 μg) was dissolved in 30 μl of MeOH and treated with 10 μl of a 5% aqueous solution of $NaIO_4$. After 4 hr at 22° 50 μl of MeOH was added to the reaction mixture and the sample was applied to a 0.8 × 30 cm glass column containing 5 g of Sephadex LH-20 in MeOH. Fractions (1 ml) were collected and the product was found in fraction 8.

$NaBH_4$ REDUCTION OF 25,26,27-TRISNORCHOLECALCIFER-24-AL. The periodate cleavage product (3 μg) (*in vivo*) was reduced with an excess of $NaBH_4$ in 30 μl of MeOH. The reaction mixture was extracted with 0.5 ml of diethyl ether–0.2 ml of H_2O (pH 4) and the water phase was reextracted with

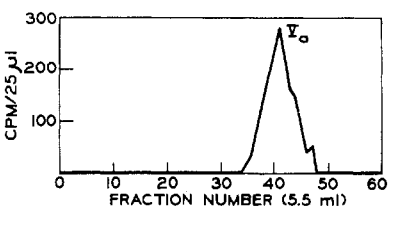

FIGURE 2: Sephadex LH-20 column (1 × 60 cm packed in 1:1 $CHCl_3$–Skellysolve B) profile of *in vitro* peak Va isolated from Bio-Beads SX-8 column.

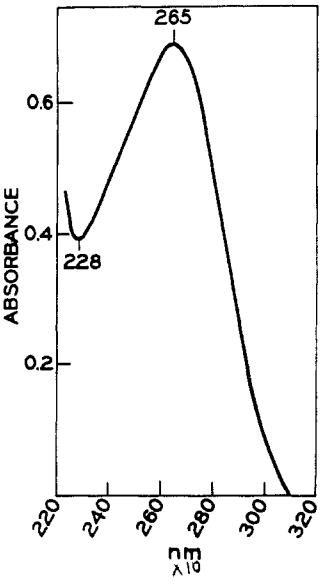

FIGURE 4: Ultraviolet absorption spectrum of the *in vitro* kidney peak Va metabolite.

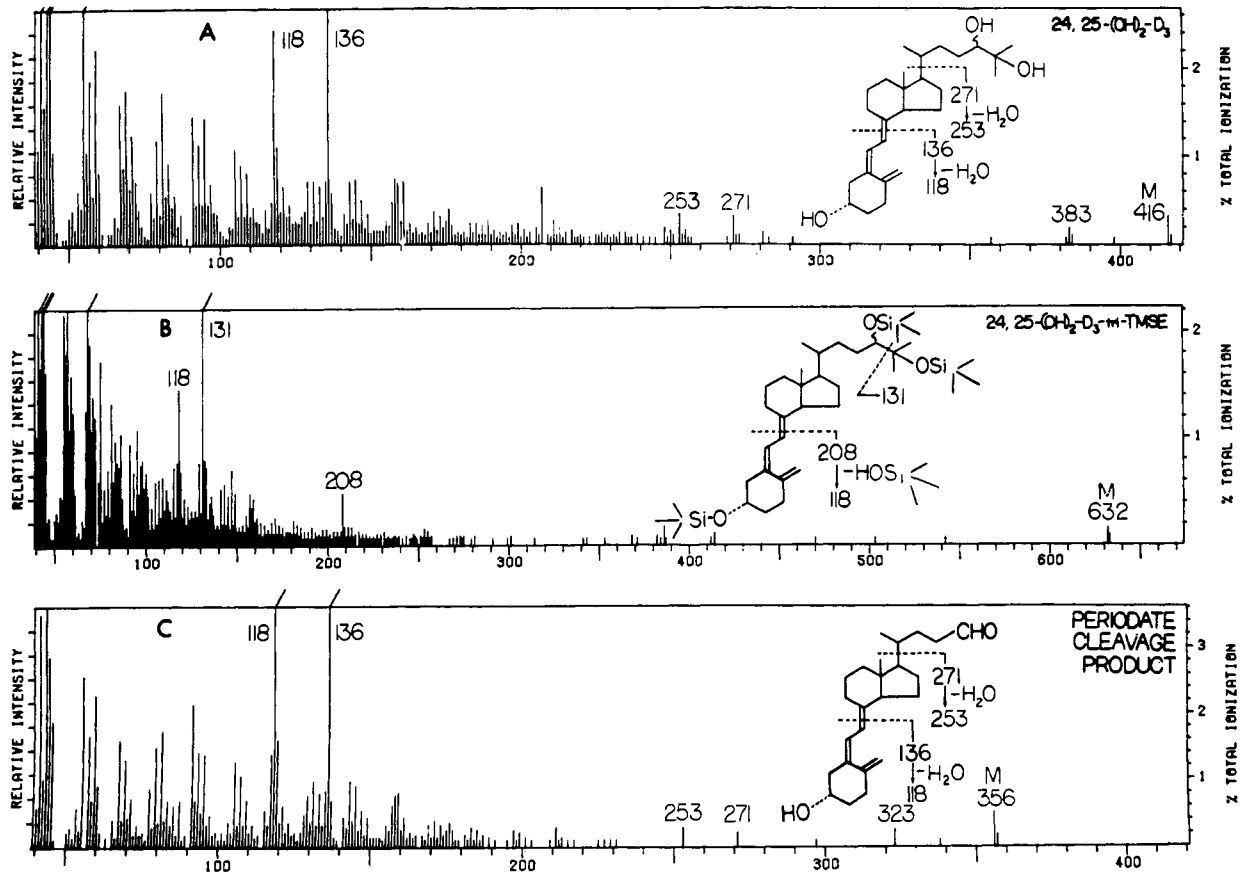

FIGURE 5: Mass spectrum of (A) 24,25-$(OH)_2D_3$, (B) 24,25-$(OH)_2D_3$-3,24,25-tristrimethylsilyl ether, and (C) 25,26,27-trisnorcholecalcifer-24-al.

0.2 ml of diethyl ether. The ether phases were combined and dried under N_2. Part of the sample was used for mass spectrometry and the rest of the sample was dried under N_2 and dissolved in 5 μl of pyridine. To this was added 2 μl of TBT (a special combination of trimethylsilylimidazole, bistrimethylsilylacetamine, and trimethylchlorosilane, Pierce Chemical Co., Rockford, Ill.) and the reaction was allowed to proceed for 15 min at 22°. The reaction mixture was extracted with water–*n*-hexane and the product in the *n*-hexane was used for mass spectrometry.

TRIMETHYLSILYLATION OF 24,25-$(OH)_2D_3$. The peak Va metabolite (1 μg; from either *in vivo* or *in vitro* sources) was dissolved in 15 μl of pyridine and reacted with 7 μl of TBT at 22° for 15 min. The reaction mixture was either used directly for mass spectrometry and gas–liquid chromatography or it was purified on a 0.8 × 30 cm Sephadex LH-20 column developed in MeOH as described above.

CHROMIUM TRIOXIDE OXIDATION OF 24,25-$(OH)_2D_3$. The *in vitro* peak Va metabolite (4 μg) was dissolved in 20 μl of dichloromethane and to this was added 1 μl of a 5% solution of CrO_3–pyridine complex in dichloromethane (w/v) (Collins, 1968) and the reaction was continued for 5 min. MeOH (75 μl) was added to the reaction and the sample was immediately applied to a 0.8 × 30 cm MeOH Sephadex LH-20 column as described above.

Identification of the in Vitro Peak Va Metabolite as 24,25-$(OH)_2D_3$. The ultraviolet absorption spectrum for the *in vitro* metabolite showed a λ_{max} at 265 nm and a λ_{min} at 228 nm (Figure 4) (Blunt *et al.*, 1968) demonstrating the presence of the 5,6-*cis*-triene chromophore characteristic for the D vitamins. The mass spectrum of the metabolite (Figure 5) showed a molecular ion at *m/e* 416 suggesting the incorporation of an additional oxygen function into its parent 25-OHD_3. Furthermore, the peaks at *m/e* 271 and 253 (271 − H_2O) as well as at *m/e* 136 and 118 (136 − H_2O) mimic those for both vitamin D_3 and 25-OHD_3 requiring that the additional oxygen function be in the side chain. The trimethylsilyl ether derivative of the metabolite (Figure 4) displayed a molecular ion at *m/e* 632 demonstrating the presence of three hydroxyl functions in the molecule while the strong peak at *m/e* 131 firmly established the presence of a C-25 hydroxyl function (Blunt *et al.*, 1968; Suda *et al.*, 1970a).

Upon treatment with periodate, 95% of the tritium in the metabolite on C_{26} and C_{27} was lost after the sample was evaporated under N_2 to dryness. The mass spectrum of the product showed a molecular ion peak at *m/e* 356 which could only result from the cleavage of the C_{24}–C_{25} bond to yield the corresponding 24 aldehyde derivative (Figure 4). Treatment of the metabolite with CrO_3–pyridine complex in CH_2Cl_2 (Collins, 1968) gave a mixture of mono- and diketones (mol wt 414,412) as well as the cleavage product of mol wt 356. These results require that the additional hydroxyl is vicinal to the C_{25}-OH and on C_{24}.

Because it has been reported that the peak Va metabolite generated *in vivo* from animals on a high calcium diet comigrated on a liquid–liquid partition chromatographic system (Boyle *et al.*, 1971) with the proposed 21,25-$(OH)_2D_3$ isolated from porcine plasma (Suda *et al.*, 1970a), it was of interest to reexamine its structure in light of the results obtained for the structure of the *in vitro* peak Va metabolite. The peak Va

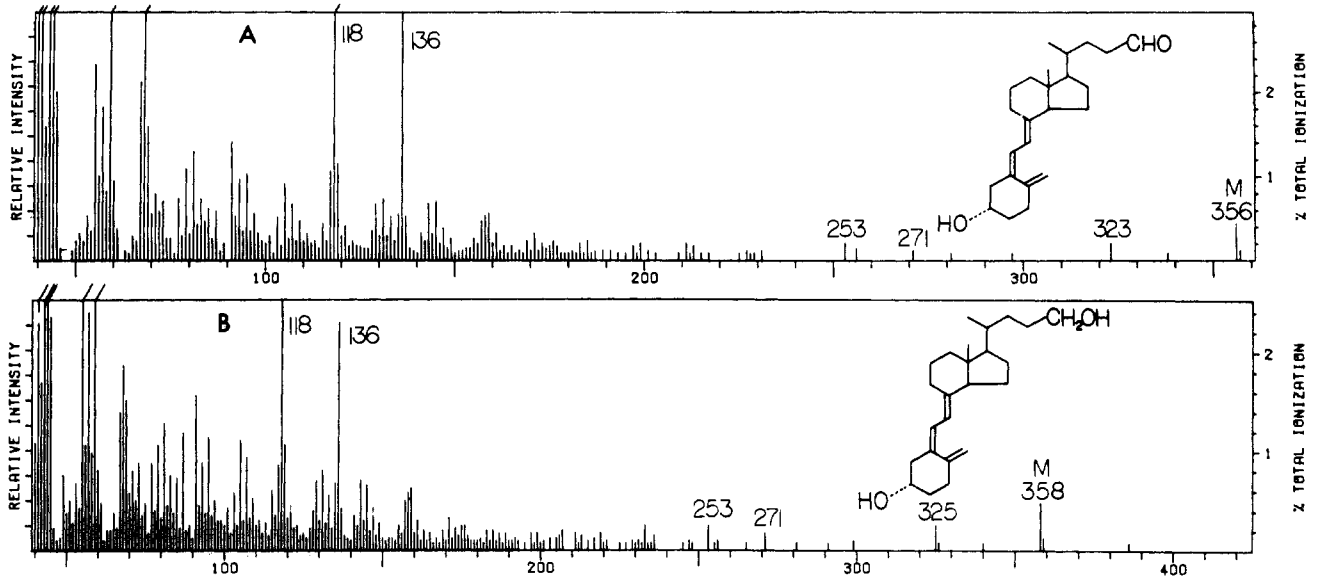

FIGURE 6: Mass spectrum of (A) 25,26,27-trisnorcholecalcifer-24-al and (B) 25,26,27-trisnor-24-hydroxycholecalciferol.

metabolite isolated from porcine plasma was obtained in pure form. The ultraviolet absorption spectrum showed a λ_{max} at 265 nm and a λ_{min} at 228 similar to that reported by Suda *et al.* (1970a) and was identical with that reported for the *in vitro* peak Va in Figure 3. The mass spectra of both the porcine peak Va and its tristrimethylsilyl ether derivative were essentially identical with those reported for the proposed 21,25-$(OH)_2D_3$ (Suda *et al.*, 1970a) and the gas–liquid chromatography trace of the tristrimethylsilyl ether derivative showed the characteristic pyro and isopyro peaks. However, when the metabolite was treated with periodate, the mass spectrum of the resulting product (Figure 6) was identical with that obtained for the periodate treated *in vitro* peak Va (Figure 5) showing a molecular ion peak at *m/e* 356 (resulting from cleavage of C_{24}–C_{25} bond) and fragment peaks at *m/e* 271, 253 (271 − H_2O), 136, and 118 (136 − H_2O). $NaBH_4$ reduction of the product yielded the corresponding alcohol with a molecular ion peak at *m/e* 358 with fragments at *m/e* 271 and 253 (271 − H_2O) representing a cleavage of C_{17}–C_{20} bond (loss of the entire side chain) and *m/e* 136 and 118 (136 − H_2O) (representing ring A plus C_6, C_7, and C_{19}) (Figure 6). The reduction product forms a ditrimethylsilyl ether (mol wt 502) upon treatment with TBT suggesting the presence of two hydroxyl functions in the molecule, one of them being in ring A presumably at C_3 and the other in the side chain. These results clearly demonstrate that the porcine peak Va is 24,25-$(OH)_2D_3$ and not 21,25-$(OH)_2D_3$ as proposed by Suda *et al.* (1970a).

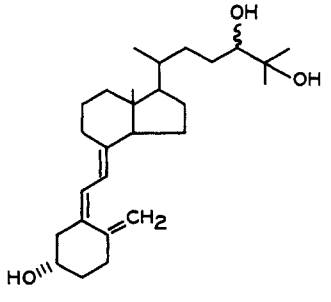

FIGURE 7: 24,25-Dihydroxycholecalciferol (24,25-$(OH)_2D_3$).

Discussion

It appears that 1,25-$(OH)_2D_3$ (Frolik and DeLuca, 1971) is the hydroxylated form of vitamin D_3 responsible for inducing intestinal calcium transport (Boyle *et al.*, 1972a) and bone calcium mobilization (Holick *et al.*, 1972). Furthermore the biosynthesis of this hormone from 25-OHD_3 is closely controlled in the kidney by a direct or indirect action of serum calcium concentrations (Boyle *et al.*, 1971). When the animal is hypocalcemic the need for calcium is interpreted in some way by the kidney resulting in the "turning on" of the 25-OHD_3–1-hydroxylase system. The ultimate goal of this process is to supply the intestine and bone with 1,25-$(OH)_2D_3$ in order to increase the flux of calcium through the intestine and from the bone to raise the serum calcium concentration. Similarly when the animal is normocalcemic or hypercalcemic there is little need for a supply of calcium and therefore the kidney "shuts down" the 25-OHD_3–1-hydroxylase system. Instead the kidney produces another metabolite designated as peak Va. This metabolite was first demonstrated in both hogs and chickens that had received excess amounts of vitamin D_3 (Suda *et al.*, 1970a). Similarly Holick and DeLuca (1971) noted that intestinal lipid extracts from chickens that received 2.5 μg of vitamin D_3 24 hr before sacrifice contained three metabolites more polar than 25-OHD_3 designated as peak V_{t_1}, Va, and 1,25-$(OH)_2D_3$ whereas intestinal lipid extracts from chickens that received 0.25 μg of vitamin D_3 only contained 1,25-$(OH)_2D_3$. More recently Boyle *et al.* (1972b), Omdahl and DeLuca (1971), and Omdahl *et al.* (1972) have shown that the peak Va is made in the kidney from animals maintained on a high calcium diet or on a strontium-supplemented diet.

The peak Va was isolated in pure form both from chick kidney homogenate and hog plasma. The purity of both materials was established as follows: the optical density at 265 nm (ultraviolet maximum for the *cis*-triene of vitamin D) correlated with the radioactivity in each fraction as shown in Figures 3 and 4 of Suda *et al.* (1970a). This establishes that the radioactivity isolated is associated with the substance having

the vitamin D absorption spectrum. This was confirmed by gas–liquid chromatography as shown in Figure 3 of this paper and Figure 6 of Suda *et al.* (1970a) which reveals a pyro and isopyro peak characteristic of vitamin D compounds on gas–liquid chromatography (Blunt *et al.*, 1968). These results also show the chemical purity of the isolated material. The mass spectra (Figure 5) also attest to the purity and reveals the expected vitamin D fragments at *m/e* of 271, 253, 136, and 118 (Blunt *et al.*, 1968). When the isolated material is treated with periodate the ^3H from 26,27-^3H-labeled metabolite is lost and the molecular ion shows a loss of the 25, 26, and 27 carbons (Figure 6), again providing evidence that the radioactivity is contained in the isolated compound which proved to be the 24,25-$(OH)_2D_3$.

The peak Va metabolite generated from chicken kidney homogenates has been isolated in pure form and identified as 24,25-$(OH)_2D_3$ (Figure 7). The identification of this metabolite was based on its ultraviolet absorption spectrum, mass spectrum of it and its derivatives, and its sensitivity to periodate treatment. The ultraviolet absorption spectrum (Figure 3) and the mass spectrum of the metabolite (Figure 4) left little doubt that the 5,6-*cis*-triene system had remained intact. A molecular ion peak at *m/e* 416 demonstrated that an additional oxygen or hydroxyl group had been incorporated into its precursor 25-OHD_3. Mass fragments at *m/e* 271 (loss of side chain) and 253 (271 − H_2O) made it clear that the additional oxygen function was in the side chain. Of great importance was the fact that the metabolite was sensitive to periodate treatment yielding a product with a molecular weight 356 which could only be accounted for if the extra hydroxyl was vicinal to the C_{25}-OH and on C_{24}. A C_{26} hydroxyl was automatically ruled out based on comparison of the mass spectrum of the peak Va metabolite and its tristrimethylsilyl ether derivative with those reported for 25,26-$(OH)_2D_3$ (Suda *et al.*, 1970b).

Because 24,25-$(OH)_2D_3$ cochromatographed with porcine plasma peak Va which had been identified as 21,25-$(OH)_2D_3$ it was necessary to reevaluate its structure. Although Suda *et al.* (1970a) presented a strong argument for the proposed 21,25-$(OH)_2D_3$ structure, it was based on the supposition that it behaved as the model compounds. Unfortunately the 24,25-$(OH)_2D_3$ does not behave according to the models used for the rate of acetylation for primary and secondary alcohols or for the mass spectrum of the corresponding trimethylsilyl ether derivative. For example, the mass spectrum of the tristrimethylsilyl ether derivative of 24,25-$(OH)_2D_3$ does not show a peak for the loss of 131 mass units (Figure 5) (corresponding to α cleavage between the vicinal hydroxyls), whereas a model compound such as 24,25-dihydroxylanosterol tristrimethylsilyl ether showed a relatively strong peak due to this cleavage. However, when the porcine peak Va metabolite was treated with periodate the product (mol wt 356) could only result from the transformation of the 24,25-dihydroxy compound to the corresponding 24-aldehyde derivative leaving little doubt that the structure is in fact 24,25-$(OH)_2D_3$ and not 21,25-$(OH)_2D_3$.

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