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Isolation and Identification of 1,25-Dihydroxyvitamin D_2†

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ABSTRACT: The chemical synthesis of [3α-^{3}H]vitamin D_2 of high specific activity has been described. With the use of this radioactive material, the existence of a polar metabolite believed to be the active form of vitamin D_2 in the rat and chick has been demonstrated. It has been isolated in pure form from an in vitro chick kidney mitochondrial system and identified as 1,25-dihydroxyvitamin D_2 by means of mass spectrometry, ultraviolet absorption spectrophotometry, and specific derivative synthesis. Its antirachitic activity equals that of 1,25-dihydroxyvitamin D_3 in the rat.

The recent extensive study of the metabolism of vitamin D_3 has provided overwhelming evidence to support the theory that it must be converted in the liver to 25-hydroxyvitamin D_3 (25-OH-D_3)[1] (Blunt et al., 1968; Ponchon et al., 1969; Horsting and DeLuca, 1969) and then in the kidney to 1,25-dihydroxyvitamin D_3 (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., 1972) or bone calcium mobilization (Holick et al., 1972a).

More complete elucidation of the metabolism of vitamin D_2 on the other hand has awaited the synthesis of a radioactive vitamin D_2 of sufficiently high specific activity in order to detect low levels of metabolites at physiological dose levels. However, Suda et al. (1969) were able to isolate and identify 25-hydroxyvitamin D_2 (25-OH-D_2) from mammalian blood using a vitamin D_2 of low specific activity. Drescher et al. (1969) demonstrated the formation of this metabolite in the rat and chick and pointed out the inability of the chick to raise the blood concentration of 25-OH-D_2 to the steady-state level seen for 25-OH-D_3. Beyond these observations little is known concerning the functional metabolism of vitamin D_2.

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[1] Abbreviations used are: 25-OH-D_3, 25-hydroxyvitamin D_3; 25-OH-D_2, 25-hydroxyvitamin D_2; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1,25-$(OH)_2D_2$, 1,25-dihydroxyvitamin D_2; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3.

This paper reports the synthesis of a radioactive vitamin D_2 of high specific activity; the use of it in the isolation and identification of 1,25-dihydroxyvitamin D_2 (1,25-$(OH)_2D_2$) and a demonstration of the formation of this metabolite in vivo and in vitro.

Materials and Methods

Chemicals. Crystalline ergosterol was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and sodium borodeuteride from Stohler Isotope Chemicals, Rutherford, N.J. Sodium boro[^{3}H]hydride (6.0 Ci/mmol) was a generous gift of New England Nuclear, Boston, Mass. Water dispersible vitamin D_2, used to augment the pig diet mix, was purchased from General Biochemicals, Chagrin Falls, Ohio. Hydroxyalkoxypropyl Sephadex was prepared from Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) and Nedox 1114 (a gift of Ashland Chemical Co., Columbus, Ohio) by the method of Ellingboe et al. (1970). The reagent bis(trimethylsilyl)trifluroracetamide containing 1% trimethylchlorosilane used in the preparation of trimethylsilyl derivatives is a commercial product (Regisil, RC-2 of Regis Chemical Co., Morton Grove, Ill.).

General Procedures. Radioactive determinations were carried out with a Nuclear Chicago Isocap 300 liquid scintillation counter. Chromatographic samples were dried in 15 × 45 mm glass vial inserts with a stream of air and redissolved 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/l. of toluene).

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

Gas chromatography was carried out with a Hewlett-Packard Model 402 gas chromatograph fitted with a Gas-Chrom Z 3% SE-30 column (flow rate of carrier gas, 80 ml/min, oven temperature was 240°).

High-pressure liquid chromatography was performed on a Du Pont 830 LC (Du Pont Instruments, Wilmington, Delaware) fitted with two 25 cm × 2.1 mm i.d. Zorbax-SIL columns in series. Using a 5:95 2-propanol–Skellysolve B solvent system, a pressure of 2000 psi gave a flow rate of 0.25 ml/min.

Synthesis of [3α-^{3}H]Ergosterol. Crystalline ergosterol was oxidized to 4,7,22-ergostatrien-3-one by the method of Shepherd et al. (1955). The product which showed a λ_{max} (ethanol) 240 nm was recrystallized from acetone and then acetylated to give 3β-acetoxyergosta-3,5,7,22-tetraene (Heilbron et al., 1938). This enol acetate exhibited λ_{max} (ethanol) at 331, 316, and 303 nm.

The reduction of the enol acetate by sodium borohydride was achieved using the modified procedure of Callow et al. (1966). 5 mg of enol acetate was refluxed in freshly distilled 1,4-dioxane with 50 μl of water and 5 mg of sodium borohydride. A complete reaction occurred in 2 hr as could be detected by the disappearance of enol acetate absorption (at λ_{max}(ethanol) 331, 316, and 303 nm) and the appearance of the characteristic 5,7-diene absorption of ergosterol (λ_{max} (ethanol) 294, 283, and 272). The reaction product was extracted with ether, and the ether layer washed in turn with 0.1 *N* hydrochloric acid, 2% (w/v) sodium bicarbonate, and water.

Mass spectrometric studies were performed on the reduction product of enol acetate when sodium borodeuteride was used in place of sodium borohydride; 70% of the total ergosterol synthesized by the procedure was monodeuterated.

This successful incorporation of deuterium into ergosterol was followed by an attempt to synthesize tritiated ergosterol by the same method. Enol acetate (6.4 mg) was reacted with 6.4 mg of sodium boro[^{3}H]hydride (6.0 Ci/mmol) in the presence of 50 μl of water. After a 2-hr reflux the total reaction product contained 4.34 mg of ergosterol (a 75% yield). The product was extracted and redissolved in 50:50 chloroform–Skellysolve B. It was applied to a (1 × 60 cm) glass column containing 16 g of Sephadex LH-20, swelled, packed, and eluted with 50:50 chloroform–Skellysolve B according to the procedure of Holick and DeLuca (1971). Fractions (1.7 ml) were collected and liquid scintillation counting of small aliquots indicated that peaks were eluted in fractions 12–17 and fractions 18–24. The first peak corresponded to 3α-OH-[3β-^{3}H]ergosta-5,7-diene and the second to [3α-^{3}H]ergosterol. The epimer ratio of 3α-OH:3β-OH = 29:71 agreed with that obtained by Dauben and Eastham (1953). The [3α-^{3}H]ergosterol prepared in this manner exhibited a uv absorption (λ_{max} 294, 283, and 272) identical with naturally found ergosterol and cochromatographed with it on thin-layer chromatography (silica gel G; 10% acetone in Skellysolve B according to Norman and Deluca (1963)). Its specific radioactivity was 1.44 Ci/mmol.

Synthesis of [3α-^{3}H]Vitamin D_2 by Irradiation of [3α-^{3}H]Ergosterol. All irradiations were carried out in 400 ml of ether contained in a jacket around a doubly walled, water-cooled, quartz immersion well. A Hanovia high-pressure quartz, mercury-vapor lamp, Model 654A, was ignited for 20 sec before placement in the immersion well. During irradiation the ether was flushed continuously with nitrogen and stirred vigorously.

After the 2.3 mg of [3α-^{3}H]ergosterol was irradiated, the solvent was evaporated under a stream of nitrogen (keeping the temperature below ambient) and the product redissolved in 1:9 chloroform–Skellysolve B. The sample was applied to a 1 × 77 cm glass column containing 20 g of hydroxyalkoxypropyl Sephadex (55% hydroxyalkyl group content), swelled and eluted with 1:9 chloroform–Skellysolve B. Fractions (1.7 ml) were collected.

A combination of liquid scintillation counting, gas–liquid chromatography, and ultraviolet absorption spectroscopy showed that fractions 35–46 contained a peak of 685 μg of previtamin D_2 (λ_{max} in ethanol 260 nm) uncontaminated by tachysterol$_2$ (λ_{max} in ethanol 292, 281, and 270 nm) which appeared in fractions 55–66 (Figure 1). Unchanged ergosterol, which appeared in fractions 71–91, was reirradiated to yield an additional 496 μg of [^{3}H]previtamin D_2.

The total of 1181 μg of previtamin D_2 yielded 730 μg of pure vitamin D_2 after equilibration (in toluene, under N_2, at 0–4°) for 2 weeks and subsequent chromatography through hydroxyalkoxypropyl Sephadex. A 1 × 77 cm column containing hydroxyalkoxypropyl Sephadex swelled and eluted with 1:9 chloroform–Skellysolve B gave vitamin D_2 in fractions at 48 to 60 (1.7 ml fractions).

The [3α-^{3}H]vitamin D_2 gave a single peak on Sephadex (Holick and DeLuca, 1971), hydroxyalkoxypropyl Sephadex, Celite (Suda et al., 1969), and silicic acid column chromatography (Neville and DeLuca, 1966). Its uv absorption

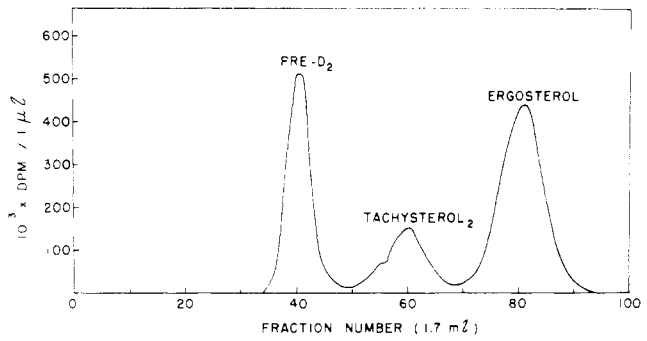

FIGURE 1: Hydroxyalkoxypropyl Sephadex column (1 × 77 cm packed in 1:9 chloroform–Skellysolve B) profile of the irradiation mixture of [3α-H]ergosterol.

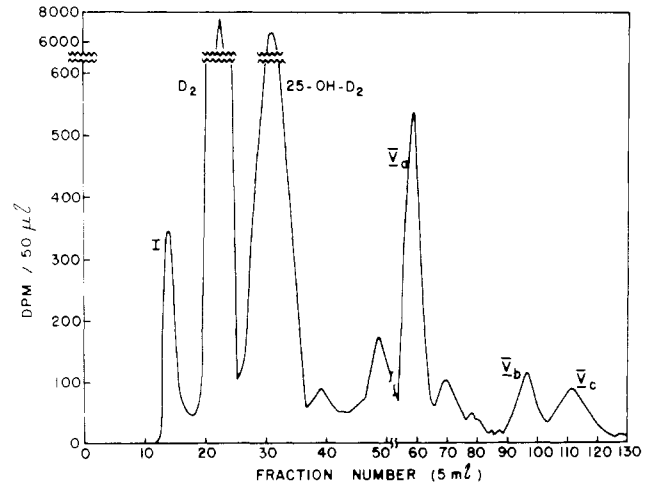

FIGURE 2: Sephadex LH-20 column (2 × 60 cm packed in 65:35 chloroform–Skellysolve B) profile of a lipid extract of the plasma of 10 rats each given 100 IU of [3α-^{3}H]vitamin D_2 24 hr before sacrifice.

characteristics (λ_{max} 265; λ_{min} 230; λ^{max}:λ_{min} 1.8:1) and constant specific radioactivity of 1.2 Ci/mmol indicated that the product was pure.

In Vitro Preparation of 25-OH-[3α-^{3}H]Vitamin D_2. Male albino rats obtained from the Holtzman Company, Madison, Wis., were fed a low vitamin D stock ration. Ten rats weighing approximately 400 g were each given 100 IU of [3α-^{3}H]vitamin D_2 (1.2 Ci/mmol) intrajugularly in 50 μl of ethanol; 24 hr later, blood was drawn by cardiac puncture using a heparinized syringe giving 57 ml of plasma. This was extracted with 2:1 methanol–chloroform as described by Blunt et al. (1968) and mixed with the lipid extract obtained from the blood of six pigs given 500,000 IU of water dispersible vitamin D_2 per day for 26 days according to the procedure of Suda et al. (1969).

The combined lipid extract was applied in several batches to 2 × 60 cm columns of Sephadex LH-20, swelled and eluted with 65:35 chloroform–Skellysolve B (Holick and DeLuca, 1971). Figure 2 shows the chromatographic profile of one of these columns illustrating the many radioactive metabolites obtained from [3α-^{3}H]vitamin D_2. The peak of radioactivity contained by fractions 26–36 (5 ml) of such columns is 25-OH-[3α-^{3}H]D_2. The 25-OH-[3α-^{3}H]D_2 was purified by passage through two columns in succession; (1) a 1 × 100 cm column containing 26 g of hydroxyalkoxypropyl Sephadex, swelled and eluted with 1:9 chloroform–Skellysolve B, yielded 25-OH-D_2 in fractions 44–52 (5 ml); and (2) a 1 × 150 cm column containing 30 g of Sephadex

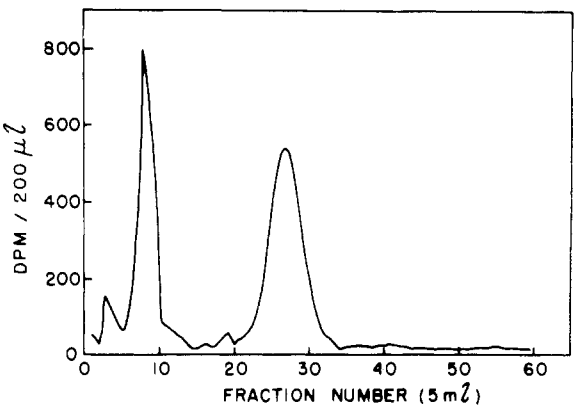

FIGURE 3: Sephadex LH-20 column (1 × 60 cm packed in 65:35 chloroform–Skellysolve B) profile of part of the lipid extract from a mitochondrial incubation of 25-OH-[3α-^{3}H]vitamin D_2.

LH-20, swelled and eluted with pure methanol, yielded relatively pure 25-OH-D_2 in fractions 46–50 (1.7 ml).

25-OH-[3α-^{3}H]D_2 thus obtained was only partially pure, the main contamination coming from an unidentified sterol (mol wt 402, main fragment at *m/e* 384). Complete purification of a part of the material (on a high-pressure liquid chromatograph Du Pont 830 LC) gave the mass spectrum shown in Figure 6A. Thus the 25-OH-D_2 before passage through the high-pressure liquid chromatograph was only 85% radiochemically pure. This partially impure material possessed a specific activity of 3.53 Ci/mol and was dissolved in ethanol at a concentration of 123 μM for use in generating the metabolite in question.

In Vitro Preparation of 1,25-(OH)$_2$D$_2$. One-day old white Leghorn cockerel chicks were obtained from Northern Hatcheries, Beaver Dam, Wis. They were maintained on a vitamin D deficient purified soy protein diet for 4 weeks before use (Omdahl et al., 1971). Rachitic chick kidney mitochondria were prepared and incubated according to the methods of Ghazarian and DeLuca (1974), who used the procedure to study the production of 1,25-$(OH)_2D_3$.

In this case, 100 flasks, each containing 500 ng of 25-OH-[3α-^{3}H]D_2, were incubated with mitochondria for 30 min. The contents of the flasks were pooled and extracted with 2:1 methanol–chloroform by the method of Bligh and Dyer (1959). The total lipid extract was dissolved in 7 ml of 65:35 chloroform–Skellysolve B and divided into seven equal parts which were each subjected to chromatography on 1 × 60 cm columns containing 16 g of Sephadex LH-20, swelled and eluted with 65:35 chloroform–Skellysolve B; 55-ml fractions were collected from each column and 50-μl samples from each fraction were taken for determination of radioactivity. Fractions 21–32 from each of the seven columns contained the peak of radioactivity under investigation whereas fractions 6–11 contained unchanged 25-OH-[3α-^{3}H]D_2 (Figure 3). Fractions 21–32 from each of the seven columns were pooled and the single extract was evaporated to dryness under a stream of nitrogen. It was redissolved in 0.5 ml of 30:70 chloroform–Skellysolve B.

The crude metabolite was subjected to the following four chromatographic steps in succession to remove remaining impurities.

(i) Hydroxyalkoxypropyl Sephadex Straight Phase Column Chromatography. The column size was 2 × 52 cm and contained 50 g of gel. The metabolite appeared in fractions 80–115 (5-ml volume fractions) using 30:70 chloroform–Skellysolve B (Figure 4, I).

(ii) Celite Liquid–Liquid Partition Column Chromatography. The procedure of Suda et al. (1970) was used except all steps were carried out at room temperature (23°). The column size was 1 × 60 cm and contained 20 g of Celite (Johns Manville Company) saturated with stationary phase (90:10 methanol–water saturated with mobile phase). Elution of the metabolite occurred in fractions 39–42 (5-ml sample volume) using mobile phase (80:20 Skellysolve B–chloroform saturated with stationary phase) (Figure 4, II).

(iii) Hydroxyalkoxypropyl Sephadex Reverse Phase Column Chromatography (Ellingboe et al., 1970). The column size was 1 × 51 cm and contained 18 g of gel. Elution of the metabolite occurred in fractions 5 and 6 (5-ml volume) with pure methanol (Figure 4, III).

(iv) Sephadex LH-20 Gel Filtration Column Chromatography (Suda et al., 1970). The column size was 1 × 150 cm and contained 30 g of gel. Elution of the metabolite occurred in fractions 43–49 (1-7-ml volume) with pure methanol (Figure 4, IV).

Mass spectrometry was performed on 5000 dpm from the pooled fractions 43–49 from the final chromatographic step. Uv absorption spectroscopy of the concentrated solution of metabolite shows a λ_{max} at 265 nm and a λ_{min} at 228. Specific activity based on the ϵ_{265} 18,200 was 3.9 Ci/mol. Pure metabolite (4.17 μg) was obtained for chemical derivatization and bioassay.

Chemical Modifications of the Metabolite 1,25-(OH)$_2$D$_2$. (i) TRIMETHYLSILYLATION OF 1,25-(OH)$_2$D$_2$. The metabolite (500 ng) was dissolved in 10 μl of pyridine and reacted with 10 μl of bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane for 30 min at room temperature. The reaction mixture was evaporated to dryness under a stream of nitrogen and the residue was redissolved in 20 μl of pure Skellysolve B. It was applied directly to the probe tip for mass spectral analysis.

(ii) PERIODATE OXIDATION. The metabolite (3100 dpm) was dissolved in 20 μl of methanol and was treated with 10 μl of a 5% (w/v) solution of sodium metaperiodate. Six hours later the solution was evaporated to dryness under nitrogen and the residue redissolved in 0.5 ml of 65:35 chloroform–Skellysolve B. The product was applied to a 1 × 60 cm column filled with 16 g of Sephadex LH-20 in the same solvent system and previously calibrated with 3100 dpm of untreated metabolite. Ninety percent of the original radioactivity was recovered in exactly the same chromatographic position as the unchanged metabolite. Periodate cleavage of a model compound (24,25-(OH)$_2$D$_3$) under the same conditions was complete within 20 min.

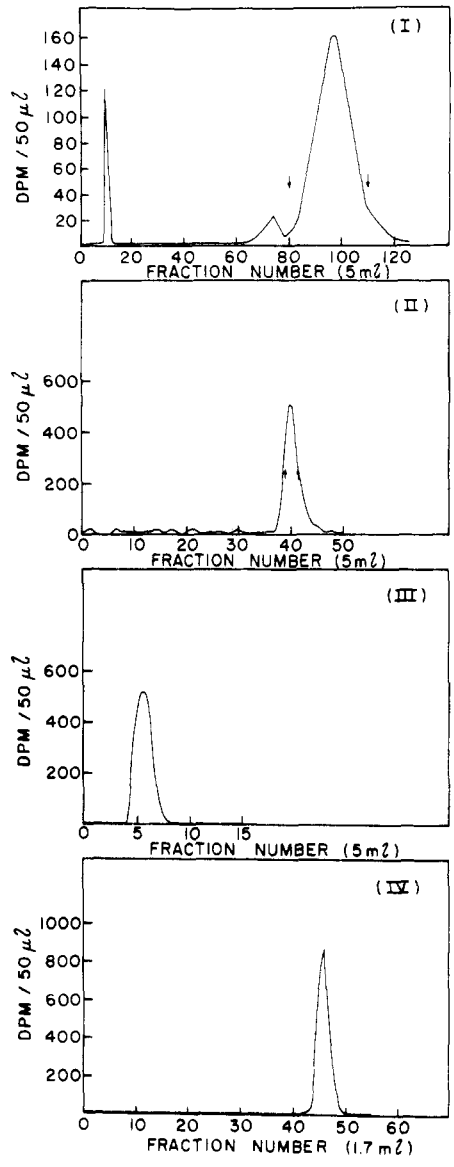

FIGURE 4: Chromatographic profiles during the isolation of 1,25-[3α-^{3}H]vitamin D$_2$. (I) Hydroxyalkoxypropyl Sephadex column (2 × 52 cm packed and eluted with 30:70 chloroform–Skellysolve B) profile of the fractions 21–32 of the Sephadex LH-20 columns depicted in Figure 3. (II) Celite liquid–liquid partition column (1 × 60 cm performed as described in the text) profile of the fractions 80–115 of the hydroxyalkoxypropyl Sephadex column shown in (I). (III) Hydroxyalkoxypropyl Sephadex column (1 × 51 cm packed and eluted with methanol) profile of fractions 39–42 of the Celite column shown in (II). (IV) Sephadex LH-20 column (1 × 150 cm packed and eluted with methanol) profile of fractions 5 and 6 of the hydroxyalkoxypropyl Sephadex column shown in (III).

Results

Metabolites of Vitamin D$_2$ in Rachitic Chicks. Ten rachitic chicks were given a 7.5 IU injection of [3α-^{3}H]vitamin D$_2$ in 0.05 ml of ethanol by wing vein and their blood taken 7 hr later by cardiac puncture with a heparinized syringe. 28 ml of plasma produced by centrifugation of the blood was extracted with 2:1 methanol–chloroform (v/v) (as described in Methods). The lipid extract was then applied to a 1 × 60 cm column containing 16 g of Sephadex LH-20 swelled and eluted with 65:35 chloroform–Skellysolve B (Holick and DeLuca, 1971). Figure 5a shows the radioactivity profile obtained from this column.

Metabolites of Vitamin D$_2$ in a Rachitic Rat. Three rachitic rats were each given 10 IU of [3α-^{3}H]vitamin D$_2$ (1.2 Ci/mmol) by intrajugular injection in ethanol and blood taken 24 hr later by cardiac puncture with a heparinized syringe; 18 ml of plasma was extracted and chromatographed as above in the study of the metabolites of the chick. Figure 5b shows the radioactivity profile obtained from this column.

In the study of the blood metabolites of vitamin D$_2$ in the rat and chick it was immediately noticed that the chick cleared vitamin D$_2$ and its metabolites very rapidly from the blood. Only 0.6% of the dose (7.5 IU) was present in the chick blood after 24 hr whereas 11.4% of the dose (10 IU) was present in rat blood at 24 hr. The amount of radioactivity left in the blood of a chick at 24 hr after a 7.5 IU dose makes it difficult to detect any metabolites more polar than 25-OH-D$_2$. However, at 7 hr after a 7.5 IU dose of vitamin

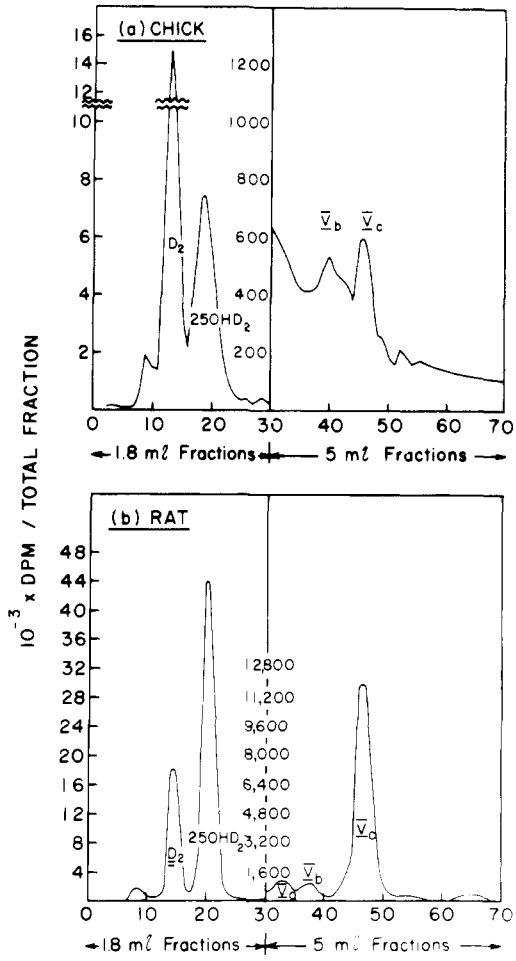

FIGURE 5: (a) Sephadex LH-20 column (1 × 60 cm packed in 65:35 chloroform–Skellysolve B) profile of a lipid extract of the plasma of 10 chickens each given 7.5 IU of [3α-^{3}H]vitamin D_2 7 hr before sacrifice. (b) Sephadex LH-20 column (1 × 60 cm packed in 65:35 chloroform–Skellysolve B) profile of a lipid extract of the plasma of three rats each given 10 IU of [3α-^{3}H]vitamin D_2 24 hr before sacrifice.

D_2 the chick still has 2.3% of the dose in its bloodstream and a small but significant peak Vc can be observed (Figure 5a).

The rat shows about 15% of its total blood radioactivity in a similar peak Vc at 24 hr (Figure 5b). This peak Vc co-chromatographs with the metabolite 1,25-$(OH)_2D_2$ isolated from the chick kidney mitochondrial in vitro system on Sephadex LH-20 (Holick and DeLuca, 1971).

Biological Activity of 1,25-$(OH)_2D_2$ in Rats. Biological activity was determined by the anti-rachitic line test assay (U. S. Pharmacopoeia, 1955) except that the metabolites under investigation were dissolved in propylene glycol at the concentration shown in Table I and injected intraperitoneally each day for five successive days.

Identification of Metabolites as 1,25-$(OH)_2D_2$. Ultraviolet absorption spectrophotometry of the metabolite showed that the cis-triene structure of 25-OH-D_2 remained intact (λ_{max} 265 nm; λ_{min} 228 nm). Mass spectrometry of the metabolite showed a molecular ion at *m/e* 428 (Figure 6B). The presence of three hydroxyl functions was demonstrated by the formation of a tristrimethylsilyl ether derivative of molecular weight 644 (Figure 6C). The ion of *m/e* 131 in the spectrum of the tristrimethylsilyl ether derivative and the fragments of *m/e* 370 and 352 (370 − H_2O) in the case of the metabolite confirm that the 25-hydroxyl function of

Table I: Antirachitic Activity of 1,25-$(OH)_2D_2$ in the Rat.

Compd	Concn in Dosing Solution (ng/dose)	Total Administered over 5 Days (ng)	Healing Value	Antirachitic Activity/μg
Standard D_3	100	100	4.00	40
1,25-$(OH)_2D_2$	3[a]	15	4.50	300
1,25-$(OH)_2D_3$	2.5[a]	12.5	4.14	331
1α-OH-D_3	5[a]	25	4.37	175

[a] Dose administered intraperitoneally in 0.05 ml of propylene glycol each day for five successive days according to the U.S. Pharmacopoeia (1955).

the 25-OH-D_2 remains intact during conversion to the new metabolite. Small peaks at *m/e* 287, 269 (287 − H_2O), and 251 (269 − H_2O) which arise from 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 spectrum of the metabolite 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 25-OH-D_2 and vitaminD_2 (Suda et al., 1969). The interpretations are further strengthened by the observation of fragments of *m/e* 296 (152 + 2 silyl groups) and 206 (296 − $HOSi(CH_3)_3$) in the mass spectrum of the tristrimethylsilyl ether derivative of the metabolite. Other ring A hydroxylated metabolites, e.g., 1,25-$(OH)_2D_3$ (Holick et al., 1971b) and 1,24,15-$(OH)_3D_3$ (Holick et al., 1973), and their silyl ether derivatives exhibit entirely analogous fragmentation patterns. The intactness of the triene structure is also confirmed by the peak *m/e* 152 and 134 (Blunt et al., 1968; Suda et al., 1969).

As this metabolite was produced from the incubation of 25-OH-D_2 it can be assumed with confidence that the C-3 hydroxyl group is still present. The foregoing results permit the extra hydroxyl function to be present only in the A ring at C-1, -2, or -4. Treatment of the metabolite with sodium metaperiodate resulted in no loss of tritium from the molecule and no change in its chromatographic position whereas a compound with vicinal hydroxyls (24,25-$(OH)_2D_3$) was cleaved. This establishes that the extra hydroxyl function of the A ring is not at C-2, or 4, but at C-1. The structure of the metabolite is, therefore, 1,25-$(OH)_2D_2$.

Discussion

The synthesis of a radioactive vitamin D_2 of high specific activity reported in this paper provides a tool which can be used to elucidate the metabolism of vitamin D_2 and how it might differ from that of [^{3}H]vitamin D_3 in the rat and chick. The method of synthesis of [^{3}H]ergosterol gave sufficient incorporation of label under the conditions specified here. The ^{3}H label is introduced into a fairly inert specific location on the vitamin D_2 molecule (3α-H) by a relatively simple manipulation of the sterol. This compares to the [1-^{3}H]vitamin D_2 (1.26 Ci/mmol) with a metabolically labile ^{3}H synthesized by the complex but efficient synthesis of Pelc and Kodicek (1971).

The notoriously difficult resolution of the photoirradiation products of ergosterol was achieved here by the novel application of hydroxyalkoxypropyl Sephadex liquid–gel chromatography. Both the method of introduction of the

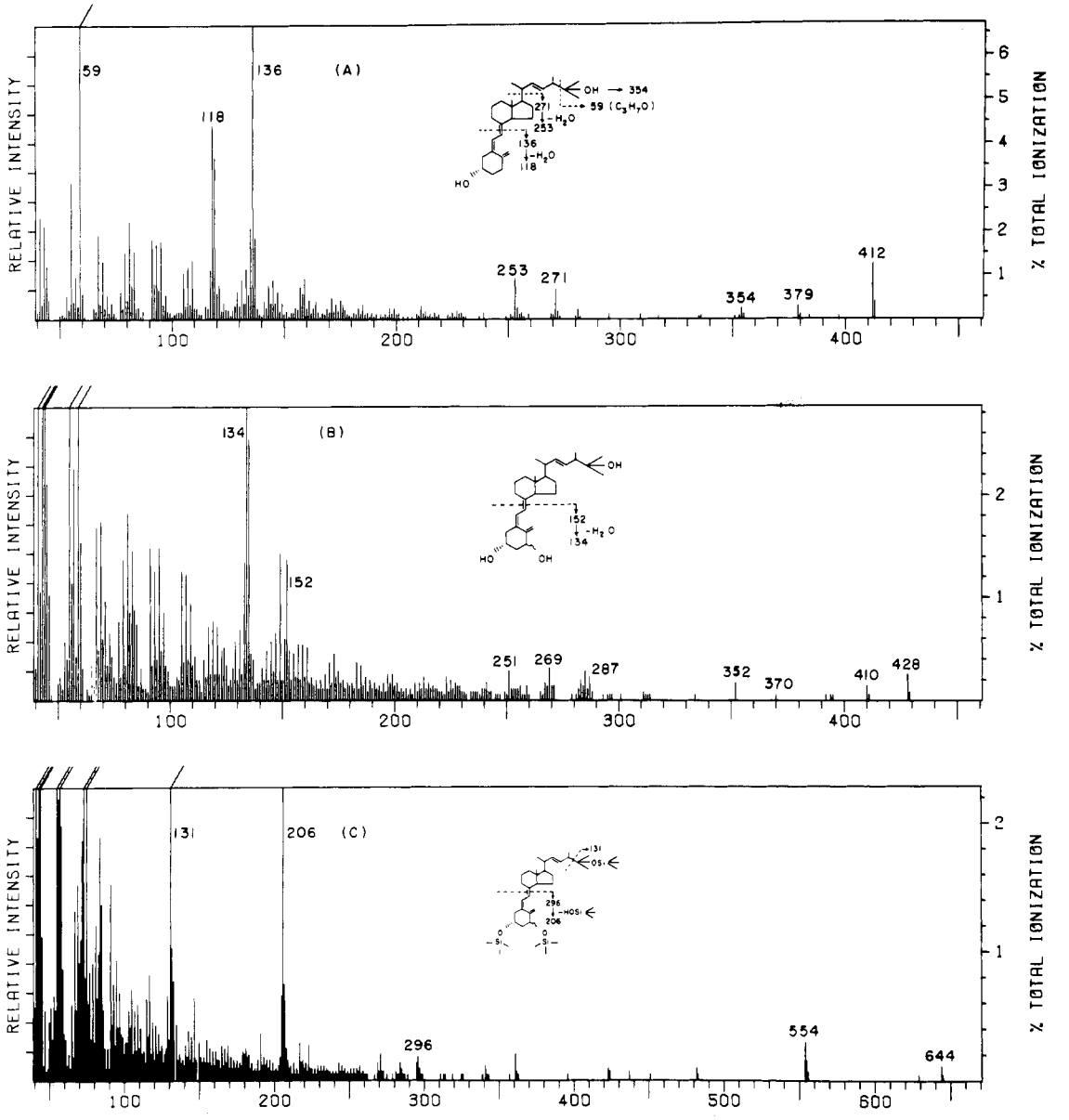

FIGURE 6: Mass spectrum of: (A) 25-OH-D_2; (B) 1,25-$(OH)_2D_2$; (C) tristrimethylsilyl ether derivative of 1,25-$(OH)_2D_2$.

label and the separation of the photoisomers might easily be applied to the synthesis of other radioactive vitamin D compounds from their analogous sterols.

The product of the chick kidney mitochondrial in vitro system was purified by a combination of Sephadex derivative liquid–gel filtration and conventional liquid–liquid partition chromatography. Problems of purification of a large lipid extract were reduced by using a chick kidney mitochondrial extract rather than the intestinal extract employed by Holick et al. (1971b) for the isolation of 1,25-$(OH)_2D_3$.

The methods of mass spectrometry and derivative synthesis established during the elucidation of the structure of other vitamin D metabolites (Blunt et al., 1968; Suda et al., 1969; Holick et al., 1971b; Holick et al., 1972b) were used here to show that the purified metabolite was indeed 1,25-$(OH)_2D_2$.

The rachitic chick kidney mitochondrion has been well established as a rich source of the enzyme 25-OH-D_3-1-hydroxylase (Gray et al., 1972). One of the products (if not the sole one) of 25-OH-D_2 metabolism by the rachitic chick kidney mitochondrial system might thus be expected to be 1,25-$(OH)_2D_2$. This metabolite is the sole product made by rachitic chick kidney mitochondria at a comparable rate to the synthesis of 1,25-$(OH)_2D_3$ (G. Jones and H. F. DeLuca, in preparation).

The production of 1,25-$(OH)_2D_2$ in vitro by isolated chick kidney mitochondria does not establish its formation in vivo in the chick, but it shows that the chick kidney mitochondrion does possess the ability to 1-hydroxylate 25-OH-D_2. However, the rat and chick do metabolize [^{3}H][vitamin D_2 in vivo to a peak Vc which corresponds to the 1,25-$(OH)_2D_2$ isolated and identified from the chick kidney in vitro system. It is, therefore, likely that 1,25-$(OH)_2D_2$ is a normal metabolite at least in rats and chicks.

The 1,25-$(OH)_2D_2$ proved to be comparable to 1,25-$(OH)_2D_3$ in its antirachitic activity by the rat calcification test. Furthermore, preliminary data (L. Baxter, G. Jones, and H. DeLuca) suggests that the 1,25-$(OH)_2D_2$ is active in chicks. The exact quantitative biological activity of this

compound in chicks is of obvious value in pin-pointing the site(s) of discrimination against vitamin D_2 in this species and is currently under study.

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