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SYNTHESIS OF (24S)- AND (24R)-1α-HYDROXY-[24-^{3}H]-CHOLECALCIFEROL

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SUMMARY

(24S)- and (24R)-1α-Hydroxy-[24-^{3}H]cholecalciferol (3.8 Ci/mmol) was synthesized from fucosterol in 15 steps. The tritium was introduced by reduction of the 24-oxo-cholecalciferol derivative with [^{3}H]-$NaBH_4$ and the resulting 24-hydroxy group was removed by hydrogenolysis of the corresponding mesylate. The C-24 configurations of the product were deduced on the basis of these chemical processes, and were further confirmed by their enzymic hydroxylation at C-24, which should proceed with retention of configuration.

INTRODUCTION

It is well known that cholecalciferol reveals its biological activity after being hydroxylated on C-25 in the liver then C-1 in the kidney to form 1α,25-$(OH)_2$-cholecalciferol* [1]. Since a synthetic analog, 1α-OH-cholecalciferol has been shown to elicit a comparable biological activity to 1α,25-$(OH)_2$-cholecalciferol [2] and it can be synthesized more easily than 1α,25-$(OH)_2$-cholecalciferol [3–7], its clinical application has been anticipated. To study its metabolism and functional mechanism, we have undertaken synthetic study of 1α-OH-cholecalciferol with high specific radioactivity.

Syntheses of radioactive 1α-OH-cholecalciferol were reported by two groups: [6-^{3}H]-1α-OH-cholecalciferol (4 Ci/mmol) by Holick *et al.*[8] and [2-^{3}H]-1α-OH-cholecalciferol (4.2 Ci/mmol) by Tohira *et al.*[9]. However, Holick introduced tritium at an early stage of synthesis and the subsequent steps are long and laborious. Tohira's method requires [^{3}H]-$LiAlH_4$ with high specific radioactivity which is commercially unavailable.†

By the use of the readily accesible [^{3}H]-$NaBH_4$ with high specific radioactivity (14 Ci/mmol), we have now developed a novel method of preparation of [^{3}H]-1α-OH-cholecalciferol (3.8 Ci/mmol), the radioactivity being introduced into the C-24 position at a late stage of synthesis.

* The abbreviations used are: 1α-OH-cholecalciferol, 1α-hydroxycholecalciferol; [24S-^{3}H]- and [24R-^{3}H]-1α-OH-cholecalciferol, (24S)- and (24R)-1α-hydroxy-[24-^{3}H]-cholecalciferol; 1α,25-$(OH)_2$-cholecalciferol, 1α,25-dihydroxycholecalciferol; [24S-^{3}H]- and [24R-^{3}H]-1α,25-$(OH)_2$-cholecalciferol, (24S)- and (24R)-1α,25-dihydroxy-[24-^{3}H]-cholecalciferol; 1α,24S- and 1α,24R-$(OH)_2$-cholecalciferol, (24S)- and (24R)-1α,24-dihydroxycholecalciferol; 1α,24R,25-$(OH)_3$-cholecalciferol, (24R)-1α,24,25-trihydroxy-cholecalciferol; [24-^{3}H]-1α,24R,25-$(OH)_3$-cholecalciferol, (24R)-1α,24,25-trihydroxy-[24-^{3}H]cholecalciferol.

† The highest specific radioactivity of commercially available [^{3}H]-$LiAlH_4$ to our knowledge is 0.05–0.1 Ci/mmol (New England Nuclear, Boston, Massachusetts).

EXPERIMENTAL

General

N.m.r. spectra were run on a JNM-PS-100/JNM-PFT-100A spectrometer with deuteriochloroform as solvent and with tetramethylsilane as an internal standard (*s*, singlet; *d*, doublet; *t*, triplet; *bs* broad singlet; *bt*, broad triplet; *dd*, double doublet; *m*, multiplet). Mass spectra (MS) were determined with a Simadzu-LKB 9000. U.V. spectra were recorded in ethanol on a Hitachi 124 spectrophotometer. High-pressure liquid chromatography (HPLC) was performed on a Hitachi type 635 assembled from a micro-pump of the double-plunger type and a 8 μl dual beam flow-cell U.V. monitor (Schoeffel Spectroflow Monitor SF 770, Schoeffel Instrument Corp.; wave length, 264 nm), and a Du Pont Zorbax Sil chromatographic column (25 cm × 2.1 mm) was used. Flow rate of the elution solvent was 0.4 ml/min. Radioactivity was measured in a liquid scintillation counter (Packard Model 3330) in a solution (12 ml) consisting of 4 g of PPO and 150 mg of POPOP/liter of toluene. Silver nitrate impregnated thin-layer chromatography ($AgNO_3$-TLC) was prepared by soaking Merck precoated Kieselgel 60 F_{254} in 2.2% silver nitrate in acetonitrile for 30 s followed by activation at 80°C for 1 h. Column chromatography was carried out on a glass column packed with Sephadex LH-20 (1.5 × 25 cm.). All solvents were distilled before use and solvent evaporation was done below 50°C.

Material

Nonradioactive 1α-OH-cholecalciferol and 1α,25-$(OH)_2$-cholecalciferol were chemically synthesized by the method of Morisaki *et al.*[7] and Rubio-Light-

bourn *et al.*[10], respectively. [24R] and [24S] stereoisomers of 1α,24-$(OH)_2$-cholecalciferol and 1α,24,25-$(OH)_3$-cholecalciferol were synthesized by the method of Morisaki *et al.*[11] and Ikekawa *et al.*[12], respectively. [^{3}H]-$NaBH_4$ (14 Ci/mmol) was purchased from Radiochemical Centre (Amersham).

1α,3β-Diacetoxy-24,24-ethylenedioxycholest-5-ene (**2**). 1α,3β-Dihydroxy-24-ethylenedioxycholest-5-ene (**1**) (160 mg) prepared by the method of Koizumi *et al.*[13] was allowed to stand in a mixture of pyridine (2 ml) and acetic anhydride (1 ml) at room temperature for 16 h. Crushed ice and water were added and the mixture was extracted with ether twice. The organic layer was washed with 1 M HCl, sat. $NaHCO_3$ and water. Drying over Na_2SO_4 and solvent evaporation gave the diacetate (**2**) (140 mg). NMR 0.68 (3H, *s*, 13-Me), 0.92 (6H, *d*, J = 6 Hz, 25-Me_2), 1.05 (3H, *s*, 10-Me), 1.99 and 2.01 (6H, a pair of *s*, 1- and 3-Ac_2), 3.95 (4H, *s*, 24-ketal), 4.80 (1H, *m*, 3α-H), 5.02 (1H, *m*, 1β-H) and 5.50 p.p.m. (1H, *m*, 6-H); MS *m/e* 501 (M-*i*-Pr), 441 (M-*i*-Pr-AcOH), 424 (M-2AcOH), 381 (M-*i*-Pr-2AcOH).

1α,3β-Diacetoxycholesta-5,7-diene-24-one (**3**). To a refluxing solution of (**2**) (134 mg) in anhydrous *n*-hexane (30 ml), 1,3-dibromo-5,5-dimethylhydantoin (43 mg) was added in one portion under argon. The mixture was refluxed for a further 30 min. After cooling to room temperature, the resulting precipitate was filtered off and the filtrate was evaporated *in vacuo* to dryness. The residue dissolved in anhydrous xylene (7 ml) was added to a refluxing mixture of anhydrous xylene (7 ml) and *s*-collidine (7 ml) under argon for 10 min. The mixture was refluxed for a further 15 min. After cooling to room temperature, the resulting precipitate was filtered off and the filtrate was partitioned between *n*-hexane and water. The organic layer was washed with 1 M- HCl, sat. $NaHCO_3$ and then sat. NaCl. Drying over Na_2SO_4 and solvent evaporation gave a yellow oil. This was dissolved in acetone (40 ml) to which *p*-toluenesulfonic acid (25 mg) was added. This mixture was stirred at room temperature for 4 h, after which most of the solvent was evaporated *in vacuo*. The residue was partitioned between sat. $NaHCO_3$ and ethyl acetate, and the organic layer was washed with sat. NaCl. Drying over Na_2SO_4 and solvent evaporation gave a yellow amorphous powder. This was chromatographed 4 times on three $AgNO_3$-t.l.c. plates (20 × 20 cm. × 0.5 mm) with benzene–acetone (40:1, V/V). The strongest U.V. absorbing band was scraped off and then eluted with ethyl acetate. Evaporation of solvent gave the 5,7-diene (**3**) (47 mg), U.V. λ_{max} 262, 271, 282 and 293 nm; n.m.r. δ 0.62 (3H, *s*, 13-Me), 1.02 (3H, *s*, 10-Me), 1.07, (6H, *d*, J = 7 Hz, 25-Me_2), 2.03 and 2.08 (6H, a pair of *s*, 1- and 3-Ac_2), 4.78 (1H, *m*, 3α-H), 5.00 (1H, *m*, 1β-H), 5.38 and 5.69 p.p.m. (1H, *dd*, J = 2 and 6 Hz; 1H, *d*, J = 6 Hz; 6- and 7-H_2); MS *m/e* 498 (M^+), 438 (M-AcOH), 422, 378 (M-2AcOH).

1α-Hydroxy-24-oxo-cholecalciferol-1α,3β-diacetate (**4**). The 5,7-diene (**3**) (45 mg) was irradiated in a mixture of benzene (130 ml) and ethanol (15 ml) with a medium pressure mercury lamp (Hanovia 654A36, 200 W) through a Vycor filter for 2.5 min under argon with ice cooling. The reaction mixture was then directly refluxed for 2 h under argon. The solvent was evaporated *in vacuo* and the residue was chromatographed three times on two $AgNO_3$-t.l.c. plates (20 × 20 cm. × 0.5 mm) with benzene–acetone (40:1 V/V). The strongest U.V. absorbing band was scraped off and then eluted with ethyl acetate. The solvent was evaporated *in vacuo* to give the 24-oxo-cholecalciferol (**4**) (8.2 mg), U.V. λ_{max} 245 (shoulder) and 264 nm, λ_{min} 228 nm; NMR δ 0.55 (3H, *s*, 13-Me), 1.07 (6H, *d*, J = 7 Hz, 25-Me_2), 2.03 and 2.05 (6H, a pair of *s*, 1- and 3-Ac_2), 5.04 and 5.31 (2H, a pair of *bs*, 19-exomethylene), 5.16 (1H, *m*, 3α-H), 5.47 (1H, *bt*, J = 6 Hz, 1β-H), 5.91 and 6.35 p.p.m. (2H, a pair of *d*, J = 11 Hz, 6- and 7-H_2); MS *m/e* 498 (M^+), 438 (M-AcOH), 396, 378 (M-2AcOH).

[24-^{3}H]-1α,24-Dihydroxycholecalciferol-1α, 3β-diacetate (**5**) and (**8**). To a solution of the 24-ketone (**4**) (8.0 mg) in ethanol (0.5 ml) was added [^{3}H]-$NaBH_4$ (500 mCi, specific radioactivity 14 Ci/mmol) and the mixture was stirred at room temperature for 3.5 h. After addition of water (10 ml), the mixture was extracted with ethyl acetate (10 ml × 3), washed with 1 M HCl, sat. $NaHCO_3$ and water, and then dried over Na_2SO_4. After evaporation of solvent, the residue was chromatographed 5 times on a $AgNO_3$t.l.c. (20 × 20 cm. × 0.25 mm) with benzene–acetone (40:1 V/V). The U.V. absorbing bands were scraped off and eluted with ethyl acetate. From the more polar band, 1.05 mg of (24S)-1α,24-dihydroxy-[24-^{3}H]cholecalciferol-1α,3β-diacetate (**5**) was obtained, U.V. λ_{max} 245 (shoulder) and 264 nm, λ_{min} 228 nm; n.m.r. δ 0.52 (3H, *s*, 13-Me), 0.91 (6H, *d*, J = 6 Hz, 25-Me_2), 2.03 and 2.05 (6H, a pair of *s*, 1- and 3-Ac_2), 3.32 (1H, *m*, 24-H), 5.04 and 5.31 (2H, a pair of *bs*, 19-exomethylene), 5.16 (1H, *m*, 3α-H), 5.48 (1H, *t*, J = 5 Hz, 1β-H), 5.91 and 6.35 p.p.m. (2H, a pair of *d*, J = 11 Hz, 6- and 7-H_2); MS *m/e* 500 (M^+), 440 (M-AcOH), 398, 380 (M-2AcOH).

From the less polar band, 0.97 mg of (24R)-1α,24-dihydroxy-[24-^{3}H]cholecalciferol-1α,3β-diacetate (**8**) was obtained. U.V., n.m.r. and MS data were identical with those of (**5**).

[24-^{3}H]-1α,24-Dihydroxycholecalciferol (**6**) and (**9**). A solution of the diacetate (**5**) in 5% KOH-methanol (3 ml) was allowed to stand at room temperature for 16 h. Most of the solvent was evaporated *in vacuo* and the residue was partitioned between water and ethyl acetate. The organic layer was washed with 1 M HCl, sat. $NaHCO_3$ and water. Drying over Na_2SO_4 and evaporation of solvent gave (24S)-1α,24-dihydroxy-[24-^{3}H]-cholecalciferol (**6**), U.V. λ_{max} 264 nm, λ_{min} 228 nm; MS *m/e* 416 (M^+), 398 (M-H_2O), 380 (M-2H_2O). The retention time (27 min) on HPLC (2.2% methanol in dichloromethane) was identical with that of authentic 1α,24S-$(OH)_2$-cholecalciferol.

A solution of the other diacetate (**8**) was hydrolyzed

in the same way as described for (**5**) to give (24R)-1α,24-dihydroxy-[24-^{3}H]cholecalciferol (**9**), U.V. and MS data were identical with those of (**6**). The retention time (24 min) on HPLC was identical with that of authentic 1α,24R-$(OH)_2$-cholecalciferol.

(24S)-1α-Hydroxy-[24-^{3}H]cholecalciferol diacetate (**11**). A solution of the diacetate (**5**) (710 μg) in 0.3 ml anhydrous pyridine was cooled in a freezer (−20°C) for 30 min. After addition of distilled methanesulfonyl chloride (20 μl) to the cold solution, this mixture was allowed to stand in a freezer for 16 h. After addition of water (10 ml), the mixture was extracted with ethyl acetate (10 ml × 3), washed with 0.5 M HCl, sat. $NaHCO_3$ and water. After drying over Na_2SO_4, the solvent was evaporated *in vacuo* to give the crude mesylate (**7**) (480 μg). To a solution of this mesylate (**7**) in hexamethylphosphoric triamide (HMPA) (0.2 ml), $NaBH_4$ (10 mg) was added and the mixture was stired under argon at 50°C for 1.7 h. After addition of water (10 ml), the mixture was extracted with ethyl acetate (10 ml × 3), and the combined organic layer was washed with 0.5 M HCl, sat. $NaHCO_3$ and water. After drying over Na_2SO_4 and evaporation of solvent, the residue was chromatographed five times on a $AgNO_3$-t.l.c. (20 × 20 cm. × 0.25 mm) with benzene. The strongest U.V. absorbing band was scraped off and then eluted with ethyl acetate. Evaporation of solvent gave the [24S-^{3}H]-diacetate (**11**) (195 μg), U.V. λ_{max} 245 (shoulder) and 264 nm, λ_{min} 228 nm; n.m.r. δ 0.55 (3H, *s*, 13-Me), 0.87 (6H, *d*, J = 7 Hz, 25-Me_2), 2.03 and 2.05 (6H, a pair of *s*, 1- and 3-Ac_2), 5.04 and 5.31 (2H, a pair of *bs*, 19-exomethylene), 5.16 (1H, *m*, 3α-H), 5.47 (1H, *t*, J = 6 Hz, 1β-H), 5.90 and 6.35 p.p.m. (2H, a pair of *d*, J = 11 Hz, 6- and 7-H_2); MS *m/e* 484 (M^+), 424 (M-AcOH), 382, 364 (M-2AcOH).

(24S)-1α-Hydroxy-[24-^{3}H]cholecalciferol (**12**). A solution of the diacetate (**11**) (195 μg) in 5% (w/v) KOH-methanol (3 ml) was allowed to stand at room temperature for 16 h. After most of the solvent was evaporated, the residue was partitioned between water (10 ml) and ethyl acetate (10 ml × 3). The combined organic layer was washed with 0.5 M HCl, sat. $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated to dryness. The residue was chromatographed on HPLC. The fraction corresponding to 1α-OH-cholecalciferol was collected and the solvent was evaporated *in vacuo* to give (**12**) (120 μg), U.V. λ_{max} 264 nm, λ_{min} 228 nm; n.m.r. δ 0.55 (3H, *s*, 13-Me), 0.87 (6H, *d*, J = 6 Hz, 25-Me_2), 4.22 (1H, m, 3α-H), 4.45 (1H, *m*, 1β-H), 5.02 and 5.34 (2H, a pair of *bs*, 19-exomethylene), 6.01 and 6.38 p.p.m. (2H, a pair of *d*, J = 11 Hz, 6- and 7-H_2); Ms *m/e* 400 (M^+), 382 (M-H_2O), 364 (M-2H_2O), 152, 134. The specific radioactivity was 3.8 Ci/mmol.

(24R)-1α-Hydroxy-[24-^{3}H]cholecalciferol diacetate (**13**). The [24R-^{3}H]-diacetate (**13**) (190 μg) was prepared from (**8**) (700 μg) in the same way as described for (**11**). U.V., n.m.r. and MS data were identical with those of (**11**).

(24R)-1α-Hydroxy-[24-^{3}H]cholecalciferol (**14**). The [24R-^{3}H]-cholecalciferol (**14**) (116 μg) was prepared from the diacetate (**13**) (190 μg) in the same way as described for (**12**). U.V., n.m.r. and MS data were identical with those of (**12**). The specific radioactivity was 3.8 Ci/mmol.

Biological conversion of 1α-OH-cholecalciferol to 1α,25-$(OH)_2$-cholecalciferol. Two weanling male rats were fed with a low calcium–vitamin D-deficient diet (Taklad Test Diets, Madison, Wisconsin) for 6 weeks. Each rat was dosed with 250 ng of the above mentioned [24S-^{3}H]-1α-OH-cholecalciferol (**12**) (2.38 μCi) intravenously. After 4 h, the blood of the rats was collected and centrifuged for 10 min (1000 ***g***) to obtain 3 ml of plasma. The plasma was extracted with chloroform–methanol (1:1, 6 ml). After evaporation of solvent, the chloroform extract was chromatographed on Sephadex LH-20 column (Fig. 4) and the fractions corresponding to 1α,25-$(OH)_2$-cholecalciferol were collected. Evaporation of solvent afforded [24S-^{3}H]-1α,25-$(OH)_2$-cholecalciferol (**15**) (0.15 μCi).

In the same manner [24R-^{3}H]-1α-OH-cholecalciferol (**14**) (2.38 μCi) was converted to [24R-^{3}H]-1α,25-$(OH)_2$-cholecalciferol (**17**) (0.15 μCi).

Conversion of 1α,25-$(OH)_2$-cholecalciferol to 1α,24,25-$(OH)_3$-cholecalciferol in vitro. Four-week-old white Leghorn cockerels were fed first of all with the rachitogenic diet [14] and then with a diet containing 2.0% strontium and 0% calcium. They were given orally 1 μg of 1α-OH-cholecalciferol daily for 10 days. The chicks were sacrificed, the kidneys were removed and homogenized with the aid of a Potter–Elevehjem homogenizer fitted with a Teflon pestle to prepare a 10% tissue homogenate in 0.25 M sucrose. In a 50-ml flask, 6 ml of this homogenate (13 mg protein/ml) and 14 ml of a mixture containing 30 mM Tris–HCl (pH 7.5), 3.6 mM $MgCl_2$, 50 mM sucrose and 20 mM sodium succinate were combined. Incubation was initiated by addition with ethanolic solution (0.2 ml) of [24S-^{3}H]-1α,25-$(OH)_2$-cholecalciferol (**15**) (0.075 μCi, diluted with 25-fold of cold 1α,25-$(OH)_2$-cholecalciferol), and continued for 1 h at 37°C. After shaking with chloroform–methanol (1:1, 30 ml), the mixture was centrifuged for 10 min (1000 ***g***). The chloroform extract was chromatographed on Sephadex LH-20 column (Fig. 5).

In the same manner, [24R-^{3}H]-1α,25-$(OH)_2$-cholecalciferol (**17**) (0.075 μCi) was subjected to the enzymic hydroxylation reaction and the chromatogram (Sephadex LH-20) of the product is shown in Fig. 5.

RESULT AND DISCUSSION

Synthesis of [24-^{3}H]-1α-OH-cholecalciferol [(**12**) and (**14**)] is outlined in Fig. 1. According to the method of Koizumi *et al.*[13], 1α,3β-dihydroxy-24,24-ethylenedioxycholest-5-ene (**1**) was prepared from fucosterol in 5 steps. The dihydroxyl ketal (**1**) was acethylated with acetic anhydride and pyridine to give

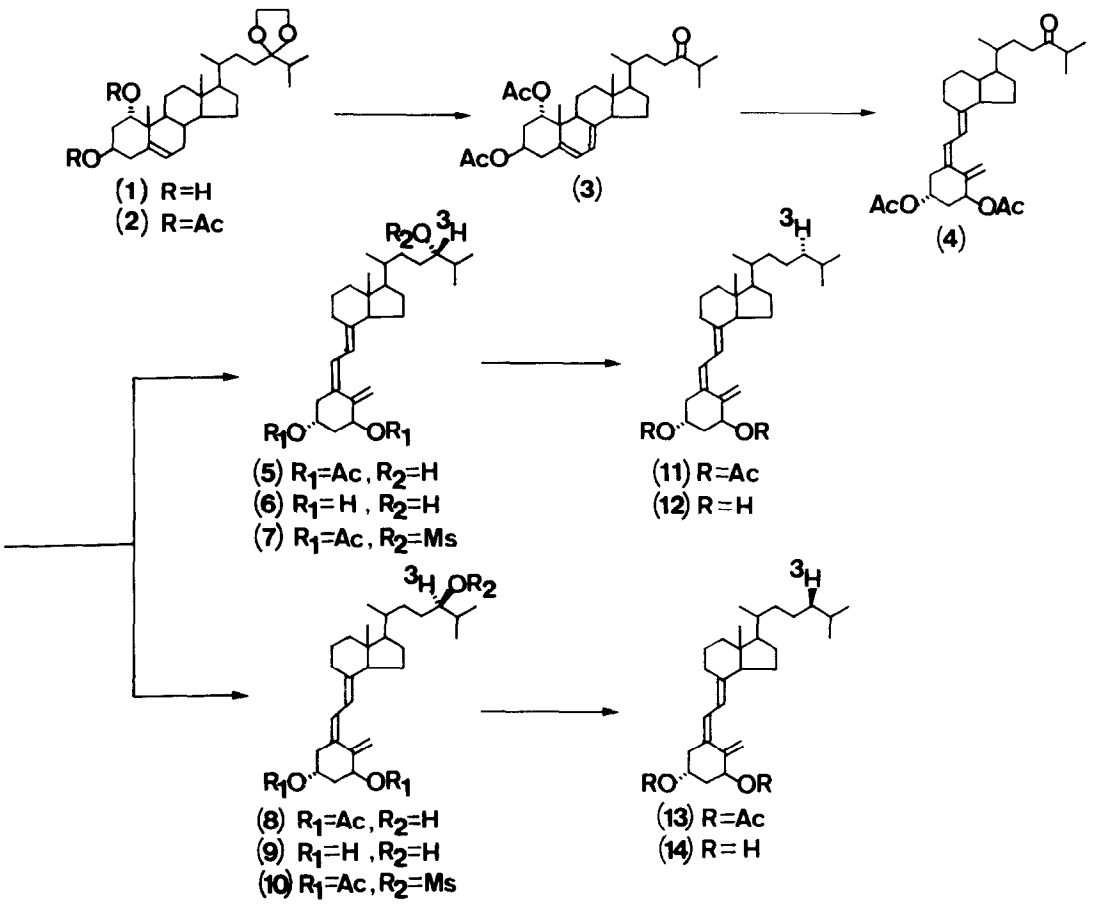


Fig. 1. Synthetic route for the preparation of [24S-^{3}H]- and [24R-^{3}H]-1α-OH-cholecalciferol, (**12**) and (**14**).

the diacetate (**2**), which was converted to the 5,7-diene by bromination (1,3-dibromo-5,5-dimethylhydantoin) followed by dehydrobromination (collidine). The crude product, without purification, was hydrolyzed to obtain the 24-ketone (**3**). Irradiation of the solution of this compound (**3**) (benzene:ethanol, 10:1, V/V) with a medium pressure mercury lamp afforded 1α-hydroxy-24-oxo-precholecalciferol diacetate, which was isomerized by refluxing in the same solvent to yield 1α-hydroxy-24-oxo-cholecalciferol diacetate (**4**). The oxo-diacetate (**4**) was reduced with [^{3}H]-$NaBH_4$ (14 Ci/mmol) to obtain a C-24 epimeric mixture of 1α,24-dihydroxy-[24-^{3}H]cholecalciferol-1,3-diacetates which were separated with $AgNO_3$-t.l.c. into the more polar (**5**), and the less polar isomer (**8**). To determine the C-24 configuration, they were hydrolized to give 1α,24-$(OH)_2$cholecalciferol [(**6**) from (**5**), and (**9**) from (**8**)]. On high-pressure liquid chromatography (HPLC), the retention time of (**6**) was identical with that of the authentic 1α,24S-$(OH)_2$-cholecalciferol and (**9**) was indistinguishable from 1α,24R-$(OH)_2$-cholecalciferol. It was concluded that the more polar diacetate (**5**) has the 24S configuration and less polar one (**8**) has the 24R configuration. Removal of the 24-hydroxyl group of the more polar diacetate (**5**) was effected by mesylation followed by reduction with $NaBH_4$ in hexamethylphosphoric triamide (HMPA). Since reduction of tosylate or mesylate with $NaBH_4$ in a polar aprotic solvent is known to proceed in the manner of S_N2 [15–18], this product shoud be (24S)-1α-hydroxy-[24-^{3}H]cholecalciferol-1,3-diacetate (**11**). Although tritium could be introduced in this step using [^{3}H]-$NaBH_4$, considerable amount of elimination byproduct was obtained when insufficient [^{3}H]-$NaBH_4$ was used. Hydrolysis of (**11**) followed by purification with HPLC afforded [24S-^{3}H]-1α-OH-cholecalciferol (3.8 Ci/mmol) (**12**). In the same way, the less polar diacetate (**8**) was converted to [24R-^{3}H]-1α-OH-cholecalciferol (3.8 Ci/mmol) (**14**). Both (**12**) and (14) exibited typical vitamin D U.V. absorption (λ_{max} 264 nm, λ_{min} 228 nm) and their n.m.r. and mass spectra are identical with those of the authentic 1α-OH-cholecalciferol. When (**12**) and (**14**) were chromatographed on HPLC, their peak of radioactivity coincided exactly with the U.V. absorbing peak (Fig. 2). These results assured the chemical and radiochemical purity of the synthetic compounds, (**12**) and (**14**).

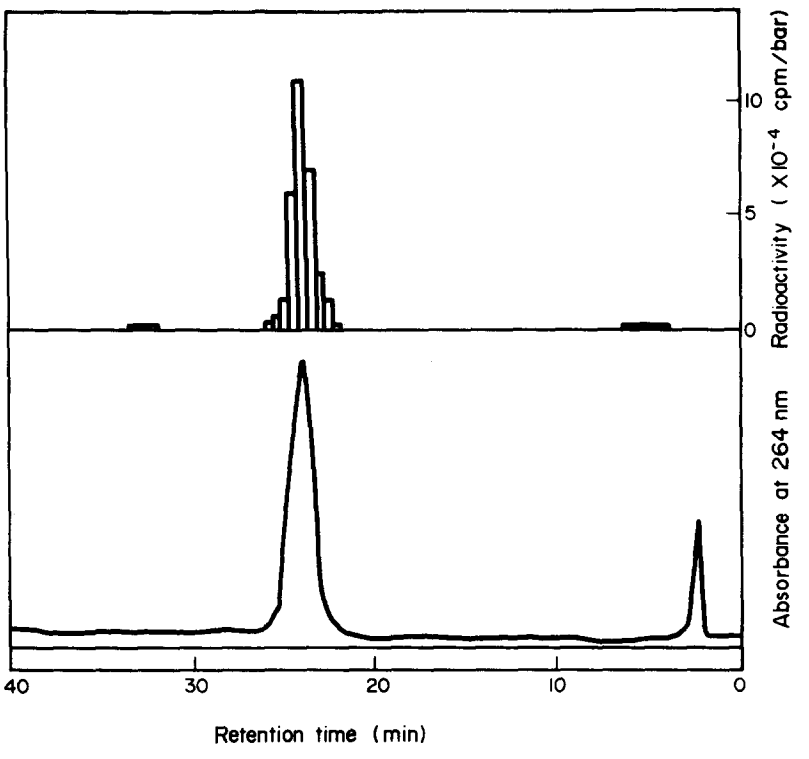
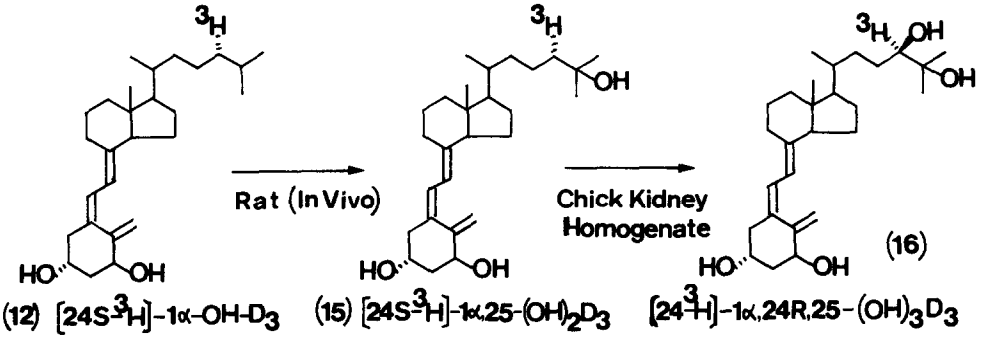


Fig. 2. Radiochemical purity of [24S-^{3}H]-1α-OH-cholecalciferol (**12**) is demonstrated by chromatography on HPLC. Synthetic [24S-^{3}H]-1α-OH-cholecalciferol (**12**) was applied to a Zolbax Sil column eluted with a 1.2% MeOH/CH_2Cl_2 solvent at a flow rate of 0.4 ml/min. The compound was monitored by U.V. absorbance at 264 nm and the radioactivity of a tenth of each fraction was determined by a liquid scintillation counter. The bar in the figure describes the radioactivity of each fraction. Radiochemical purity of the 24R isomer (**14**) was checked in the same manner.



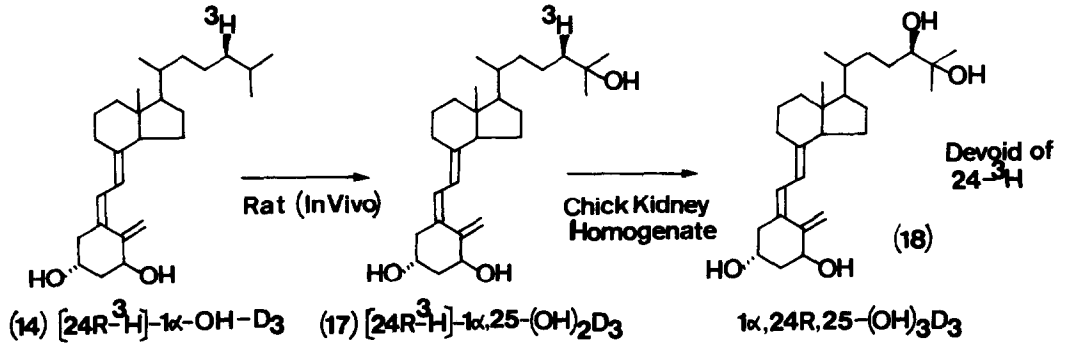


Fig. 3. Biological conversion scheme of [24S-^{3}H]- and [24R-^{3}H]-1α-OH-cholecalciferol, (**12**) and (**14**), to 1α,25-$(OH)_2$-cholecalciferol and 1α,24,25-$(OH)_3$-cholecalciferol.

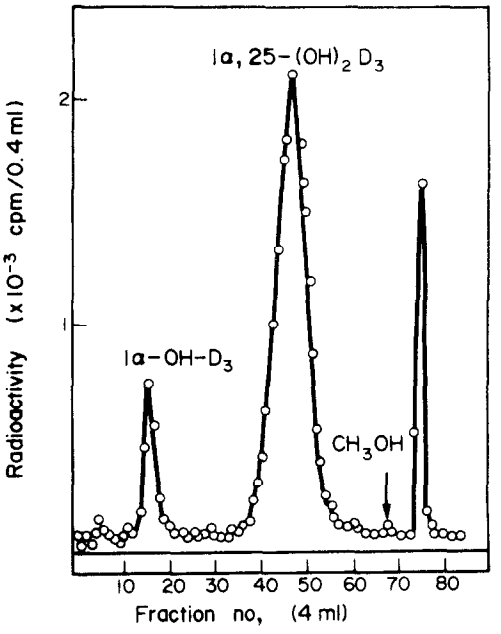


Fig. 4. Sephadex LH-20 column chromatographic prophile of the conversion product of [24S-^{3}H]-1α-OH-cholecalciferol (**12**) in rats. A portion (0.4 ml) of each fraction (4 ml) was determined by liquid scintillation counter. The elution solvent system used was $CHCl_3$/*n*-hexane (65:35). A similar profile was obtained from the 24R isomer (**14**).

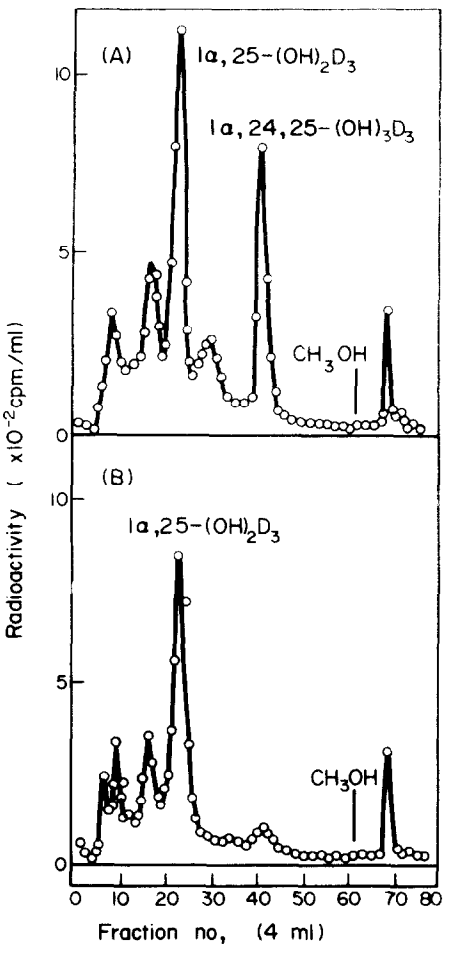


Fig. 5. Sephadex LH-20 column chromatographic profiles of the conversion product of [24S-^{3}H]- and [24R-^{3}H]-1α,25-$(OH)_2$-cholecalciferol, (**15**) and (**17**), with chick kidney homogenate, [(A), a conversion product of (**15**); (B), a conversion product of (**17**)]. The elution solvent system used was $CHCl_3$/*n*-hexane/MeOH (75:23:2). A portion (1 ml) of each fraction (4 ml) was determined by liquid scintillation counter. The conversion system was described in Experimental section.

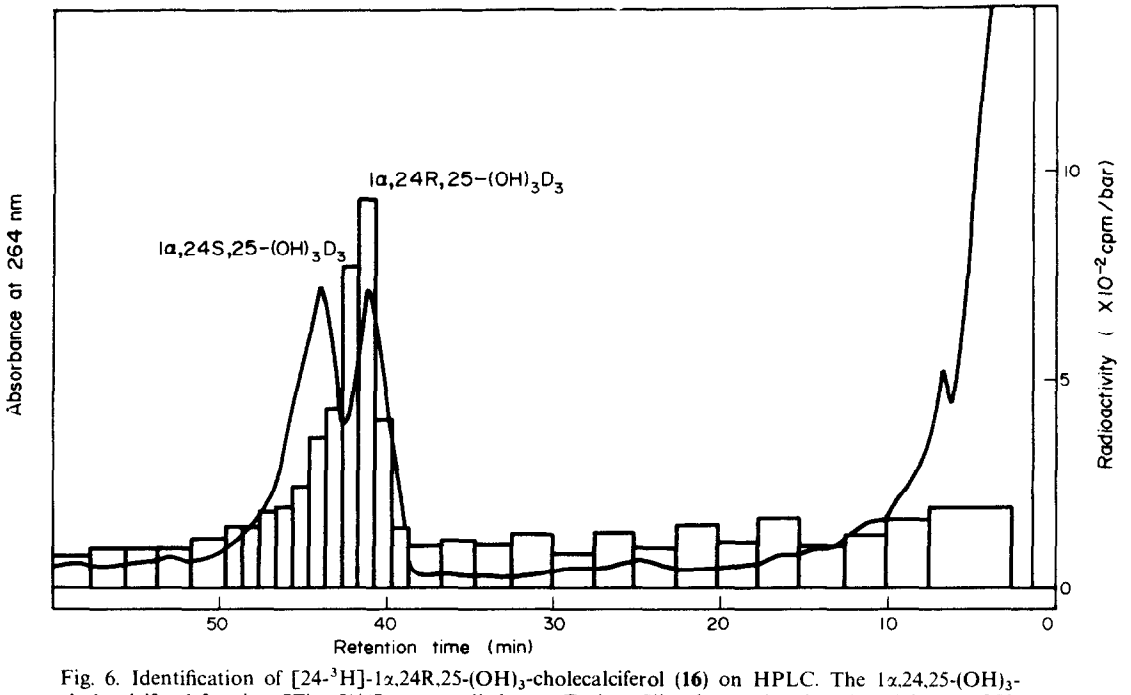


Fig. 6. Identification of [24-^{3}H]-1α,24R,25-$(OH)_3$-cholecalciferol (**16**) on HPLC. The 1α,24,25-$(OH)_3$-cholecalciferol fraction [Fig. 5(A)] was applied to a Zorbax Sil column eluted with a 3.0% MeOH/CH_2Cl_2 solvent at a flow rate of 0.4 ml/min with authentic 1α,24R,25- and 1α,24S,25-$(OH)_3$-cholecalciferol (0.4 μg each). The mixture was monitored by U.V. absorbance at 264 nm and the radioactivity of each fraction was determined by liquid scintillation counter.

In order to confirm the C-24 configuration of these [^{3}H]-1α-OH-cholecalciferols, (**12**) and (**14**), they were subjected to enzymatic hydroxylation reaction, which has been established to proceed with retention of the configuration [19–24] (Fig. 3). According to the method of Holick *et al.*[8], [24S-^{3}H]-1α-OH-cholecalciferol (**12**) was administered intravenously to vitamin D-deficient rats. The metabolically produced [24S-^{3}H]-1α,25-$(OH)_2$-cholecalciferol (**15**) was isolated from the plasma after separation on Sephadex LH-20 column chromatography (Fig. 4). The 1α,25-$(OH)_2$-cholecalciferol (**15**) was then incubated with chick kidney homogenate according to a slightly modified method of Friedlander and Norman[25]. The resulting trihydroxycholecalciferol has a peak of radioactivity on Sephadex LH-20 column chromatography at the fraction corresponding to the authentic 1α,24,25-$(OH)_3$-cholecalciferol (Fig. 5). When this trihydroxycholecalciferol was further analyzed by HPLC, the peak of radioactivity coincided with the U.V. peak of the authentic sample of the (24R)-isomer of 1α,24,25-$(OH)_3$-cholecalciferol, being distinct from the (24S)-isomer (Fig. 6). This result confirmed the result of Tanaka *et al.*[26] who have shown that enzymic C-24 hydroxylation of 1α,25-$(OH)_2$-cholecalciferol gives 1α,24R,25-$(OH)_3$-cholecalciferol. In the same way as mentioned for (**12**), [24R-^{3}H]-1α-OH-cholecalciferol (**14**) was converted to [24R-^{3}H]-1α,25-$(OH)_2$-cholecalciferol (**17**) with rats, and then the dihydroxycholecalciferol (**17**) was incubated with the chick kidney homogenate. However, the radioactive peak did not appear significant at the fraction of 1α,24,25-$(OH)_3$-cholecalciferol on Sephadex LH-20 column chromatography (Fig. 5). These results were in complete agreement with the expectation that the tritium of [24S-^{3}H]-1α-OH-cholecalciferol (**12**) should be retained during 24-hydroxylation to yield [24-^{3}H]-1α,24R,25-$(OH)_3$-cholecalciferol (**16**), while the tritium of [24R-^{3}H]-1α-OH-cholecalciferol (**14**) should be lost. Thus, the configurational assignment of (**12**) and (**14**) deduced from their synthetic procedures were confirmed by the results of biological hydroxylation reaction.

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