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

J. W. Blunt† and H. F. DeLuca‡

ABSTRACT: Cholesta-5,7-diene-3β,25-diol has been synthesized by two methods and then converted into 25-hydroxycholecalciferol, which was identical in all respects with the biologically active metabolite of vitamin D_3 previously isolated from porcine plasma.

Recently in this laboratory a metabolite of vitamin D_3 was isolated from the plasma of hogs maintained on high daily levels of the vitamin for 26 days. It was identified as 25-hydroxycholecalciferol (Blunt *et al.*, 1968a,b). This metabolite was shown to be more efficient than vitamin D_3 in curing rickets in rats, and was able to stimulate calcium transport in isolated rat intestinal sacs earlier than a similar amount of vitamin D_3. Also, bone resorption in rats was shown to occur after a dose of 25-hydroxycholecalciferol earlier than after a similar dose of vitamin D_3 (Blunt *et al.*, 1968c). As a result of these experiments, and others cited earlier (DeLuca, 1967), the suggestion was made that 25-hydroxycholecalciferol was possibly the metabolically active form of the vitamin. In order to continue studies to verify this suggestion, and to investigate other possible uses of the metabolite, it became necessary to synthesize 25-hydroxycholecalciferol in larger amounts. Accordingly, cholesta-5,7-diene-3β,25-diol has been synthesized by two methods, and irradiation of this with ultraviolet light produced 25-hydroxycholecalciferol, identical in all respects, including biological activity, with the material isolated previously from porcine plasma.

Experimental Procedure

Ultraviolet spectra were determined in ethanol in a Beckman DB-G spectrophotometer, and nuclear magnetic resonance spectra were obtained from a Varian associates Model HA 100 spectrometer coupled to a time-averaging computer, using deuteriochloroform solutions with tetramethylsilane as internal standard. Gas–liquid partition chromatography was performed in an F & M Model 402 gas chromatograph, 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 A.E.I. MS-9 spectrometer coupled to a scientific Data Systems Sigma-7 computer.

Synthesis of Cholesta-5,7-diene-3β,25-diol. METHOD A (Figure 1). 25-Hydroxycholesteryl acetate (1 g) (Steraloids, Inc., New York, N. Y.) was refluxed in 50 ml of acetic anhydride with a few drops of pyridine for 1 hr. The cooled solution was poured into 250 ml of water and after 3 hr the white solid was filtered. Recrystallization from methanol gave 850 mg of the diacetate, mp 119–120° (Dauben and Bradlow, 1950). The diacetate (100 mg) was dissolved in 1.5 ml of dry benzene and 1.5 ml of dry Skelly B (a petroleum fraction which boils at 67–69°). This solution was added to 32 mg of finely powdered *N,N'*-dibromodimethylhydantoin in a test tube, which was then immersed in a water bath at 72–74°. This bromination procedure was first described by Hunziker and Mülner (1958) and in our hands represents the only successful allylic brominating agent for these compounds. When dissolution of the dibromantin was complete (6–8 min) the solution was cooled in ice and the crystalline precipitate of dimethylhydantoin was removed by filtration. The precipitate was rinsed twice with 1-ml rinses of ice-cold Skelly B. The filtrate was evaporated at less than 40° *in vacuo*, and taken up in 0.4 ml of dry xylene. This was added, with two 0.1-ml rinses, dropwise over 2 min to a solution of 0.1 ml of trimethyl phosphite in 0.3 ml of xylene maintained at 130–135°. The mixture was kept at this temperature for 90 min followed by evaporation of the solvent *in vacuo* at 65°. The residue was applied to a column of 25 g of neutral alumina (grade I) in Skelly B. Elution with diethyl ether–Skelly (1:3) followed by ether–Skelly B (1:1) and collecting 12-ml fractions yielded the diacetate of cholesta-5,7-diene-3β,25-diol, as indicated by its ultraviolet spectrum with maxima at 272, 282, and 294 mμ. Earlier fractions containing this material were contaminated with the 4,6-diene, as indicated by ultraviolet absorption at 239 mμ. Those fractions containing the 5,7-diene free of the 4,6-diene were combined, giving 23 mg by weight and by ultraviolet spectrum (ϵ_{282} 11,000). This material was dissolved in 3 ml of dry ether and treated with a small portion of $LiAlH_4$ for 5 min to remove the acetyl group. Extraction of the product gave 18 mg of cholesta-5,7-diene-3β,25-diol: mp 169–171 (aqueous MeOH); ultraviolet max at 272, 282, and 294 mμ (ϵ_{282} 11,000); nuclear magnetic resonance C_{18}–H_3, δ 0.65; C_{19}–H_3, 0.98; C_{21}–H_3, 0.89 (J = 6.5 cps); $C_{26,27}$–H_3, 1.20; $C_{6,7}$–H, 5.4 (multiplet).

METHOD B (Figure 2). 3β-Acetoxy-26-norcholest-5-en-25-one (142 mg) in 2.2 ml of dry benzene and 2.2 ml of

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† Present address: The Dyson Perrins Laboratory, Oxford, England.

‡ To whom correspondence should be addressed.

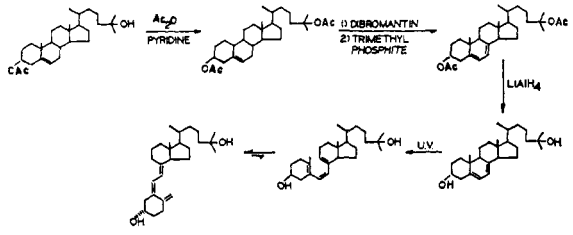

FIGURE 1: Synthesis of 25-hydroxycholecalciferol from 25-hydroxycholesterol.

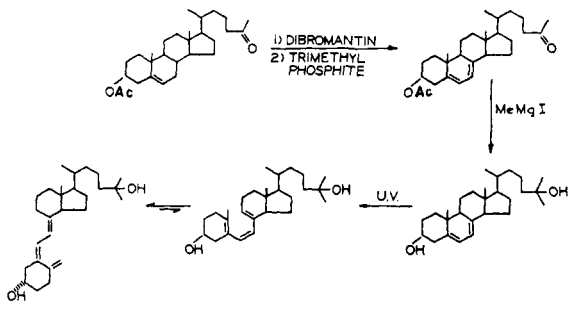

FIGURE 2: Synthesis of 25-hydroxycholecaliferol from 25-ketonorcholesterol.

dry Skelly B was added to 50 mg of dibromantin and reacted as in method A. The dehydrobromination was also performed as in method A, by adding the brominated material in 0.5 ml of xylene with 0.3 ml of rinse to 0.15 ml of trimethyl phosphite in 0.5 ml of xylene. The product isolated from this step was adsorbed onto a column of 20 g of neutral alumina (grade I) in chloroform. Elution with chloroform and collection of 5.5-ml fractions gave, in fractions 10–14, 39 mg (as indicated by ultraviolet spectra, ϵ_{282} 11,000) of the 5,7-diene. Gas–liquid partition chromatography indicated the presence of the starting material, 3β-acetoxy-26-norcholest-5-en-25-one, as the only contaminant. The infrared spectrum of the diene showed ν_{max} 1730 and 1712 cm^{-1}, corresponding to the carbonyl absorption of the acetate and methyl ketone groups, respectively. The nuclear magnetic resonance spectrum displayed δ 0.63 (C_{18}–H_3), 0.95 (C_{19}–H_3), 2.02 (OAc), and 2.12 ppm (C_{27}–H_3).

The material (245 mg) prepared as above and containing 180 mg of the 5,7-diene as indicated by ultraviolet spectrum was dissolved in 3.5 ml of dry benzene and added to the Grignard reagent prepared by the addition of 0.24 ml of methyl iodide in 2.5 ml of ether to 72 mg of magnesium turnings. This was refluxed for 2.5 hr and then left 18 hr before adding to cold ammonium chloride solution. The benzene extract of this was dried and evaporated and the residue was triturated with Skelly B giving 212 mg of an amorphous powder shown by ultraviolet analysis and gas–liquid partition chromatography to consist of 140 mg of cholesta-5,7-diene-3β,25-diol, the remainder being 25-hydroxycholesterol. This material was used as such for conversion into 25-hydroxycholecalciferol as follows.

Irradiation of Cholesta-5,7-diene-3β,25-diol. All irradiations were carried out in 400 ml of ether contained in a jacket around a double-walled, water-cooled, quartz immersion well. An Hanovia high-pressure quartz mercury vapor lamp, Model 654 A, was ignited for 1 min before placement in the immersion well for the times indicated below. During irradiation, the ether was flushed continuously with nitrogen and stirred vigorously.

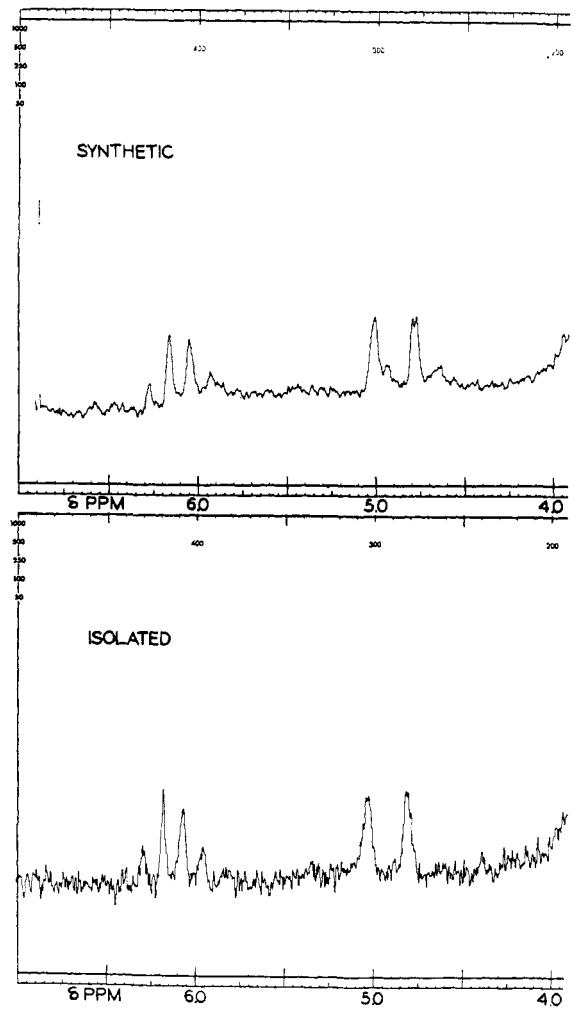

FIGURE 3: Downfield nuclear magnetic resonance spectra of synthetic and isolated 25-hydroxycholecalciferol.

The mixture (106 mg) prepared as in method B and containing 70 mg of the 5,7-diene was irradiated for 3.5 min. The products were applied in ether–Skelly B (1:1) to a multibore column (Fischer and Kabara, 1964) prepared from 14 g of heat-activated silicic acid (Bio-Rad silicic acid, HA, −325 mesh, California Corp. for Biochemical Research, Los Angeles, Calif.). The column was eluted with a convex gradient obtained by running ether into a 250-ml mixing chamber initially filled with ether–Skelly B (1:1) (Bock and Ling, 1954). Fractions of 2.8 ml were collected. Ultraviolet analysis and gas–liquid partition chromatography indicated the presence of 25-hydroxyprecholecalciferol only in fractions 33–38, while the latter fractions contained 25-hydroxytachysterol$_3$, 25-hydroxycholesterol, and a small amount of unconverted 5,7-diene. Fractions 33–38 were combined, giving 11 mg, and after storage at room temperature in ether under hydrogen for 7 days, the material was 90%

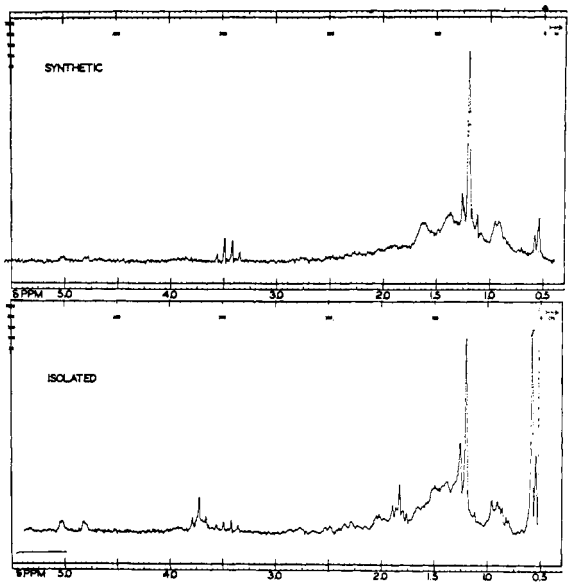

FIGURE 4: Nuclear magnetic resonance spectra of synthetic and isolated 25-hydroxycholecalciferol. Note that $\Delta = 0.58$ ppm peaks are due to tetramethyl-silane internal standard.

in the form of 25-hydroxycholecalciferol, ultraviolet max 265 mμ (ε 18,000); nuclear magnetic resonance C_{21}–H_3, δ 0.90 (J = 8 cps); C_{18}–H_3, 0.54; $C_{26,27}$–H_3, 1.22; C_{19}–H_2, 4.80 and 5.00; $C_{6,7}$–H, 5.97 and 6.25 ppm (J = 12 cps). The nuclear magnetic resonance spectra of the isolated and synthetic 25-hydroxycholecalciferols are shown in Figures 3 and 4 and it is readily apparent that they are identical.

The mass spectra of the synthetic and isolated compounds are shown in Figure 5 leaving no doubt that they are identical. A molecular weight of 400.3340 (calcd for $C_{27}H_{44}O_2$, 400.3341) was determined by high-resolution mass spectrometry which gave characteristic fragments at m/e 271.2059 (calcd for $C_{19}H_{25}$, 253.1956).

Those fractions containing 25-hydroxytachysterol$_3$ and unchanged 5,7-diene were combined, together with similar fractions from the irradiation of a second batch of material as above, and were reirradiated for a further 3.5 min. Chromatography of the products as described above yielded, after equilibration, a further 8 mg of 25-hydroxycholecalciferol.

Irradiation for 2.5 min of 15 mg of the 5,7-diene prepared by method A yielded 2.4 mg of 25-hydroxyprecholecalciferol, and reirradiation of the 25-hydroxytachysterol$_3$ and unchanged 5,7-diene, followed by chro-

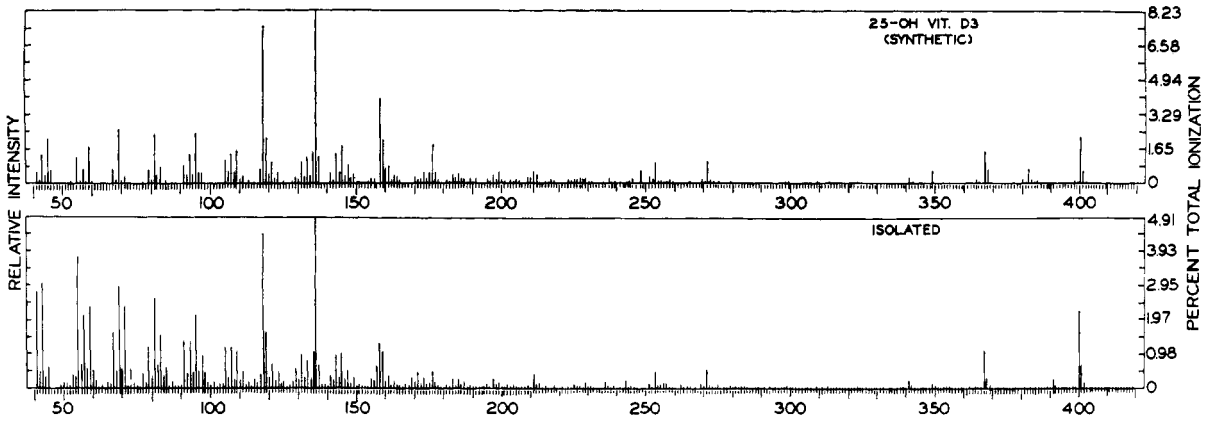

FIGURE 5: Mass spectrum of synthetic and isolated 25-hydroxycholecalciferol.

TABLE I: Comparison of Intestinal Calcium Transport Responses of Rats on a Vitamin D Deficient Diet to an Intravenous 0.25-μg Dose of Either Synthetic 25-Hydroxycholecalciferol or Isolated 25-Hydroxycholecalciferol as Compared with Cholecalciferol.[a]

	^{45}Ca Serosal/^{45}Ca Mucosal		
		25-Hydroxycholecalciferol	
hr after Administration	Cholecalciferol	Synthetic	Isolated
Control	1.24 ± 0.05	1.59 ± 0.17[b] (4)[c]	1.24 ± 0.05 (12)
4	1.01 ± 0.12	2.31 ± 0.50 (5)	1.78 ± 0.35 (4)
6	1.32 ± 0.10	2.33 ± 0.31 (4)	2.62 ± 0.44 (4)
10	2.01 ± 0.32		

[a] The rats were prepared and the everted gut sac assay performed as described by Blunt *et al.* (1968c). [b] Standard deviation. [c] Number of rats in each group.

TABLE II: Serum Calcium Response of Rats on a Low Calcium, Vitamin D Deficient Diet to a 2.5-μg Intravenous Dose of Synthetic 25-Hydroxycholecalciferol or Isolated 25-Hydroxycholecalciferol as Compared with Cholecalciferol.[a]

hr after Administration	Serum Ca (mg/100 ml)		
		25-Hydroxycholecalciferol	
	Cholecalciferol	Synthetic	Isolated
0	3.8 ± 0.11	3.75 ± 0.23[b] (6)[c]	3.8 ± 0.23
8	3.8 ± 0.14	6.4 ± 0.5 (7)	5.7 ± 0.24
12	5.0 ± 0.44	7.36 ± 0.12 (6)	7.1 ± 0.41
16	6.6 ± 0.47		

[a] The rats were prepared and the assay performed as described by Blunt *et al.* (1968c). [b] Standard deviations. [c] Number of rats in each group.

matography as before, yielded a further 0.4 mg of the desired product.

Biological Activity of Synthetic 25-Hydroxycholecalciferol. All assays were performed as described previously (Blunt *et al.*, 1968c) and only the results are presented here. In the line test assay in rats, the synthetic 25-hydroxycholecalciferol assayed at the level of 55–60 I.U./μg as previously reported for the isolated 25-hydroxycholecalciferol. Table I demonstrates the ability of a 0.25-μg intravenous dose of 25-hydroxycholecalciferol to stimulate calcium transport in isolated, everted, rat intestinal sacs after 4 and 6 hr, times at which no response to a similar dose of vitamin D_3 have been observed. Note that vitamin D_3 has no effect until 10 hr while 25-hydroxycholecalciferol both isolated and synthetic was effective within 4 hr. Table II shows that 25-hydroxycholecalciferol is able to stimulate bone resorption as indicated by a rise in serum calcium concentration within 8–12 hr following an intravenous dose of 2.5 μg of either isolated or synthetic 25-hydroxycholecalciferol to D-deficient rats maintained on a low calcium diet.

Discussion

This communication reports the synthesis of 25-hydroxycholecalciferol in over-all yields of 3.6% from 26-norcholest-5-en-25-on-3β-yl acetate and 3.2% from 25-hydroxycholesteryl acetate. Clearly, the synthetic material is identical in all respects with the 25-hydroxycholecalciferol isolated from porcine plasma. The synthetic material mimics the action of the isolated metabolite in curing rickets in rats, and in stimulating both calcium transport in intestine and bone calcium mobilization more rapidly than vitamin D_3. The physical data, including ultraviolet, gas–liquid partition chromatography, nuclear magnetic resonance, and mass spectrum, are all identical for the synthetic and isolated materials, the latter's constants having been reported earlier (Blunt *et al.*, 1968b).

Using the knowledge that there is a photochemical equilibrium between tachysterol$_3$ and precholecalciferol (Fieser and Fieser, 1959), the 25-hydroxytachysterol$_3$ obtained from the irradiations of cholesta-5,7-diene-3β,25-diol in this work was reirradiated, yielding further amounts of the 25-hydroxyprecholecalciferol. It is felt that this technique could be used more exhaustively than applied in the work reported here. Such a study here was hindered by the presence of 25-hydroxycholesterol in the 5,7-diene prepared by method B, for, in the chromatographic system used in this work, this contaminant was not separated from 25-hydroxycholecalciferol.

The synthesis of 25-hydroxycholecalciferol by method B is particularly applicable to a synthesis of tritiated 25-hydroxycholecalciferol, since the label may be incorporated *via* the Grignard reaction introducing the 26-methyl group, the penultimate step of the synthesis. This method is currently being utilized in this laboratory, with results to be published shortly. The availability of such labeled material will enable the study of vitamin D metabolism to continue in greater detail.

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Comparative Study of a Membrane Protein. Characterization of Bovine, Rat, and Frog Visual Pigments$_{500}$*

Joram Heller

ABSTRACT: Rat and frog visual pigments$_{500}$, which are membrane proteins found in the retinal rod outer segments, were purified and their properties compared with the previously isolated bovine visual pigment$_{500}$ (Heller, J. (1968), *Biochemistry 7*, 2906, 2914). The rat and frog visual pigments$_{500}$ are closely similar to the bovine pigment in all their physicochemical properties. These pigments have practically identical molecular weights and molar absorptivities at 280 and 500 mμ. They contain only one retinal prosthetic group per molecule, as determined by the loss of lysine residues after reduction with sodium borohydride, have two disulfide bridges, and are all glycoproteins. In the native pigments retinal is bound to the protein through a bond which is not susceptible to reduction with sodium borohydride, and is thus most probably a substituted aldimine. On exposure to light the substituted aldimine is converted into a simple aldimine which can be reduced with sodium borohydride and concomitantly one sulfhydryl group per molecule becomes titrable. Similarly to the bovine pigment, rat and frog visual pigments undergo a conformational change on light exposure such that the light-exposed form is more expanded than the native molecule. The magnitude of this conformational change is identical in all these pigments. The only differences that were found among these pigments were small variations in their amino acid composition. On the basis of these observations it is suggested that visual pigment$_{500}$, a membrane protein, from bovine, rat, and frog eyes forms a series of homologous proteins.

Native bovine visual pigment$_{500}$ was recently purified and has been shown to be a conjugated glycoprotein with a molecular weight of 27,700 (Heller, 1968a). Since native visual pigment$_{500}$ is one of the few noncatalytic membrane proteins that have been purified to a state of homogeneity and characterized as to several of its physicochemical properties it was interesting to compare the properties of this membrane protein in several other species. The present paper reports the purification of rat and frog visual pigments$_{500}$ and compares their properties with that of bovine visual pigment$_{500}$.

Experimental Section

Rat eyes were obtained from the Long Evans strain of *Rattus norwegicus*. Frog eyes were obtained from *Rana pipiens*. The retinas were dissected under dim red light. The isolation and purification of visual pigment and other experimental techniques have been previously reported (Heller, 1968a,b). Several hundred retinas were used for each preparation and the reported results thus represent a pooled sample.

Results

Isolation and Purification. Rat and frog visual pigment$_{500}$ could be purified by essentially the same procedure used to purify bovine visual pigment$_{500}$. All the pigments were stable in the presence of the dissociating agent cetyltrimethylammonium bromide and showed identical elution patterns after gel filtration. The visual pigments emerged as a single, symmetrical peak with a constant A_{280}/A_{500} ratio across the peak and were completely separated from a high molecular weight, colorless, protein contaminant. In the case of the frog visual pigment, an additional peak was observed in the "small molecules" fraction. This chromatographic peak was yellow and had absorption maxima at 328, 450, and 480 mμ. The material was thus probably a mixture of carotenoids and is derived from the yellow oil globules present in the amphibian retina (Wolken, 1966). When the visual pigments were concentrated in the dark by pressure dialysis and rechromatographed, a single peak with the same elution volume was obtained.

Some of the properties of the purified visual pigments are summarized in Table I.

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