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ENZYMATIC ISOMERIZATION ($\Delta^7 \rightarrow \Delta^8$) OF THE NUCLEAR DOUBLE BOND OF 14α-ALKYL SUBSTITUTED STEROL PRECURSORS OF CHOLESTEROL

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Summary:

The catalysis, by rat liver microsomes under anaerobic conditions, of the conversion of [3α-^{3}H]14α-methyl-5α-cholest-7-en-3β-ol and of [2,4-^{3}H]14α-hydroxymethyl-5α-cholest-7-en-3β-ol to labeled 14α-methyl-5α-cholest-8-en-3β-ol and 14α-hydroxymethyl-5α-cholest-8-en-3β-ol, respectively, has been demonstrated. This finding is of importance in evaluating past research in this area and in consideration of pathways and mechanisms involved in enzymatic removal of carbon atom 32 of 14α-methyl sterols. Also described herein are syntheses of [2,4-^{3}H]14α-hydroxymethyl-5α-cholest-7-en-3β-ol and 3β-acetoxy-14α-methyl-5α-cholest-8-ene.

The enzymatic conversion of lanosterol (4α,4β,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol) to cholesterol involves three general processes: reduction of the Δ^{24}-double bond, removal of the three "extra" methyl groups, and "shift" of the nuclear double bond from the Δ^8-position in lanosterol to the Δ^5-position in cholesterol. The latter process is generally considered to involve the formation of Δ^7-sterols at some stage in the overall metabolism to cholesterol (1-4 and references cited therein). Rat liver microsomal preparations have been reported to catalyze the isomerization of the Δ^8-double bond of a variety of sterols to the corresponding Δ^7-isomers (5-9). While the efficient catalysis, by liver microsomes, of the conversion of 5α-cholest-8-en-3β-ol to 5α-cholest-7-en-3β-ol has been demonstrated (5-10), Gaylor et al. (5) were unable to detect the reverse reaction (i.e., the conversion of 5α-cholest-7-en-3β-ol to its Δ^8-isomer). However, the results of subsequent studies by Wilton et al. (11) and Scala et al. (12) have

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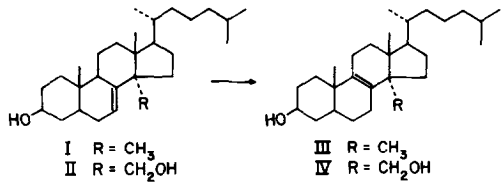


Figure 1. Enzymatic isomerization of the Δ^7-double bond of 14α-alkyl substituted sterol precursors of cholesterol.

provided evidence indicating the reversibility of this reaction. The apparent equilibrium in the case of these two sterols results in a great predominance of the Δ^7-isomer. While the enzymatic conversion of 5α-cholesta-8,14-dien-3β-ol to the corresponding $\Delta^{7,14}$-isomer could not be detected in microsomal preparations under anaerobic conditions (13), the reverse reaction (i.e., conversion of the $\Delta^{7,14}$-sterol to its $\Delta^{8,14}$-isomer) was demonstrated under the same conditions (14).

However, no enzymatic isomerization of the Δ^8 or Δ^7 double bonds of any sterol containing a 14α-methyl group has been detected. Gaylor et al. (5) reported that lanost-8-en-3β-ol, lanosta-8,24-dien-3β-ol, lanost-7-en-3β-ol, 14α-methyl-5α-cholest-8-en-3β-ol and 14α-methyl-5α-cholest-7-en-3β-ol, were unchanged upon incubation with rat liver microsomal preparations under anaerobic conditions. These authors concluded that the presence of a 14α-methyl group prevented enzymatic attack on the nuclear double bond of these Δ^8- and Δ^7-sterols.

The purpose of this communication is to report the enzymatic isomerization of 14α-methyl-5α-cholest-7-en-3β-ol (I) and 14α-hydroxymethyl-5α-cholest-7-en-3β-ol (II) to the corresponding Δ^8-sterols (III and IV; Figure 1) by washed rat liver microsomal preparations under anaerobic conditions.

EXPERIMENTAL PROCEDURES AND RESULTS

Materials and Methods

The syntheses of [3α-^{3}H]-I, II, IV, 14α-hydroxymethyl-5α-cholest-7-en-3-one, and the 3β-acetate derivative of I have been described previously (15-18). Tritiated water (1 Ci per ml) was obtained from New England Nuclear Corporation. Male Sprague-Dawley rats (190-280 g) were obtained from Sprague-Dawley Farms (Madison, Wisconsin).

Melting points, infrared (IR) spectra, nuclear magnetic resonance (NMR) spectra, and high and low resolution mass spectra (MS) were recorded as described previously

(16). Thin layer chromatographic (TLC) analyses, gas-liquid chromatographic (GLC) analyses, preparation of trimethylsilyl (TMS) ether derivatives of sterols, preparative TLC of steryl TMS ethers, preparation of 10,000 x g supernatant fractions of rat liver homogenates, and saponification and extraction of sterols from incubation mixtures were performed as described elsewhere (19). Measurement of radioactivity, radio-TLC analyses and the measurement of the radioactivity of the effluents from GLC columns were carried out as previously described (20). Materials and procedures for the preparation and use of silicic acid-Super Cel gravity columns and alumina-$AgNO_3$ medium pressure liquid chromatography (MPLC) columns have been described elsewhere (21,22). Colorimetric assays of steryl acetates were carried out using a modified Liebermann-Burchard reagent (23). For determination of cholesteryl acetate, absorbance was recorded at 620 nm, 30 min after addition of the reagent; for 14α-methyl steryl acetates, readings were taken at 450 nm, 23 min after the addition of the reagent. Protein was assayed by the method of Gornall et al. (24).

Washed rat liver microsomes were prepared in the following manner. The 10,000 x g supernatant fraction of a rat liver homogenate, prepared in incubation buffer (0.1 M potassium phosphate; 5 mM $MgCl_2$, pH 7.4), was centrifuged for 90 min at 105,000 x g at 4°. The pellet was resuspended in an equal volume of fresh incubation medium using a hand-driven loose-fitting Teflon-on-glass homogenizer. This suspension was recentrifuged at 105,000 x g and the pellet was resuspended in incubation buffer.

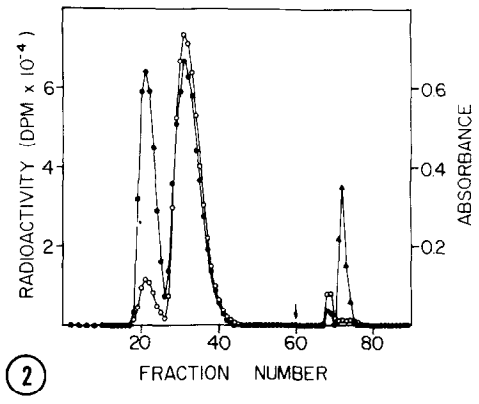
Anaerobic incubations were conducted in modified (25 ml) Warburg flasks. A washed microsomal suspension (20 ml) was placed in the main chamber, the sterol substrate in propylene glycol (0.2 ml) was placed in the sidearm, and the center well contained 2.2 ml of an oxygen indicator solution (19 mM EDTA; 180 mM FMN). The flask was connected to an anaerobic train (25), and the flask was taken through eight cycles of evacuation (1 min) and flushed with argon (3 min with continuous stirring). This procedure was shown to produce an atmosphere with an oxygen concentration of less than 0.3 ppm as determined by the lumiflavin-3-acetate gas analysis described by Beinert et al. (25). After rendering the incubation flask anaerobic, the indicator (oxidized FMN) was photoreduced by irradiation for ~5 min with a 150 W tungsten filament floodlamp. All of the above operations were carried out at 4°. The incubations were initiated by mixing the contents of the main chamber and sidearm. All incubations were carried out for 3 h at 37° under a positive pressure of argon and they were terminated by the addition of 15% ethanolic KOH (20 ml).

3β-Acetoxy-14α-methyl-5α-cholest-8-ene

3β-Acetoxy-14α-methyl-5α-cholest-7-ene (105 mg; 0.238 mmol) was dissolved in dry $CHCl_3$ (10 ml) in a screw-capped tube. Dry HCl gas was bubbled through the solution for 5 min at 0°. The tube was capped and left overnight at 4°. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in a mixture of hexane and toluene (39:1). Analysis by GLC (3% OV-17; 260°) showed two components: 3β-acetoxy-14α-methyl-5α-cholest-8-ene (44%; relative retention time to 5α-cholestane, 3.57) and 3β-acetoxy-5α-cholest-7-ene (56%; relative retention time, 4.24). The mixture was subjected to MPLC on an alumina-$AgNO_3$ column (solvent, hexane-toluene, 39:1; flow rate, 2.5 ml per min; fraction size, 20 ml). The contents of fractions 12 through 16 were pooled and, after evaporation of the solvent under reduced pressure, recrystallized from methanol to give 3β-acetoxy-14α-methyl-5α-cholest-8-ene (26 mg) melting at 64.0-64.5°; IR, ν_{max} 2955, 1740, 1471, 1374, 1247, and 1028 cm^{-1}; NMR, δ 0.70 (s, 3H, C-18-CH_3), 0.89 (s, 3H, C-32-CH_3), 0.95 (s, 3H, C-19-CH_3), 1.99 (s, 3H, methyl of acetoxy function), and 4.70 (m, 1H, C-3-H); MS, m/z 442 (20%; M), 427 (100%; M-CH_3), 382 (4%; M-CH_3COOH), 367 (37%; M-CH_3-CH_3COOH), 273 (14%), and 201 (12%); high resolution MS, 442.38104 (calc. for $C_{30}H_{50}O_2$: 442.38105). The compound showed a single component on GLC analysis.

[2,4-^{3}H]14α-Hydroxymethyl-5α-cholest-7-en-3β-ol ([2,4-^{3}H]-II)

[2,4-^{3}H]-II was synthesized from 14α-hydroxymethyl-5α-cholest-7-en-3-one by a procedure virtually identical to that used for the preparation of [2,4-^{3}H]5α,14β-



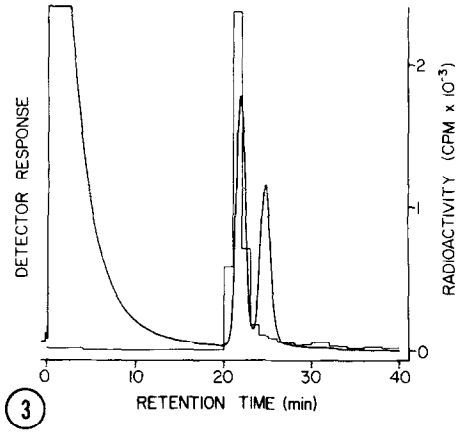


Figure 2. Alumina-silver nitrate MPLC analysis of the acetate derivatives of the labeled sterols recovered after incubation of [3α-^{3}H]14α-methyl-5α-cholest-7-en-3β-ol with washed microsomes under anaerobic conditions. The column was eluted successively with 97:3 hexane-toluene (60 13.5 ml fractions) and 8:2 hexane-toluene (30 13.5 ml fractions) at a flow rate of 2.5 ml per min. o—o, Radioactivity (applied to column, 6.09 x 10^5 dpm; eluted, 6.01 x 10^5 dpm); ●—●, Δ^8 14α-methylcholestenyl acetates determined colorimetrically (the Δ^8 isomer elutes prior to the Δ^7 isomer); ▲—▲, cholesteryl acetate determined colorimetrically.

Figure 3. Gas-liquid radiochromatographic analysis (3% OV-17; 240°) of the TMS ether derivative of the material corresponding to fractions 23-26 from the silicic acid-Super Cel column chromatography of the labeled sterols recovered after incubation of [2,4-^{3}H]14α-hydroxymethyl-5α-cholest-7-en-3β-ol with washed microsomes under anaerobic conditions. The chromatographic standards, in their order of elution, are 14α-hydroxymethyl-5α-cholest-8-en-3β-ol and 14α-hydroxymethyl-5α-cholest-7-en-3β-ol. Applied radioactivity, 5900 cpm; recovered radioactivity, 4600 cpm.

Table 1. Cocrystallization of the acetate derivative of the labeled material, which cochromatographed with 3β-acetoxy-14α-methyl-5α-cholest-8-ene, derived from the incubation of [3α-^{3}H]14α-methyl-5α-cholest-7-en-3β-ol.

Crystallization	Specific Activity (dpm/mg ± S.D.) Crystals	Mother liquor
Before	1850 ± 50	---
Once from methanol	1770 ± 40	1890 ± 50
Twice from methanol	1820 ± 50	2100 ± 50
Thrice from methanol	1730 ± 80	1840 ± 110

cholest-7-ene-3β,15α-diol from the corresponding unlabeled 3-ketosterol, which has been described elsewhere (20). The product, [2,4-^{3}H]-II melted, after recrystallization from acetone, at 185-186° (m.p. of the unlabeled sterol, 185-186° (16)). The specific activity was 1.10 x 10^{10} dpm per mmol. Analysis by radio-TLC (solvent, ether) indicated that over 99% of the radioactivity comigrated with the authentic unlabeled diol. GLC analysis (3% OV-17; 245°) of the TMS derivative indicated a purity in excess of 99% and a radiopurity in excess of 97%.

Incubation of [3α-^{3}H]14α-Methyl-5α-cholest-7-en-3β-ol ([3α-^{3}H]-I) with Washed Microsomes under Anaerobic Conditions

[3α-^{3}H]-I (20 nmol; 1.78 x 10^6 dpm) was incubated with rat liver microsomes (20 ml; 5.5 mg protein per ml) under anaerobic conditions as described above. Extraction of the sterols from the saponified incubation mixture yielded 1.64 x 10^6 dpm. A portion of this material was treated with a mixture of acetic anhydride anhydride and pyridine (1:1) and the resulting material was subjected to MPLC on an alumina-$AgNO_3$ column (Figure 2). Most (87.1%) of the eluted radioactivity cochromatographed with unlabeled 3β-acetoxy-I and therefore represented untransformed substrate. However, 8.9% of the eluted radioactivity (fractions 17-26) cochromatographed with unlabeled 3β-acetoxy-III. This identification was further supported by the results of cocrystallization experiments (Table 1). Repetition of this incubation and analysis showed that 10.8% of the recovered radioactivity had the chromatographic mobility of 3β-acetoxy-14α-methyl-5α-cholest-8-ene.

In two separate incubations of the labeled I with boiled enzyme preparations, followed by identical workup and analyses, only 0.8% of the recovered radioactivity had the chromatographic mobility of II.

Incubation of [2,4-^{3}H]14α-Hydroxymethyl-5α-cholest-7-en-3β-ol ([2,4-^{3}H]-II) with Washed Microsomes under Anaerobic Conditions

[2,4-^{3}H]-II (174 nmol; 1.91 x 10^6 dpm) was incubated with rat liver microsomes (20 ml; 4.6 mg protein per ml) under anaerobic conditions as described above. Extraction of the sterols from the saponified incubation mixture yielded 1.99 x 10^6 dpm. This material was applied to a 1:1 silicic acid-Super Cel column (100 cm x 0.9 cm) which was eluted with a mixture of toluene and ether (17:3; flow rate, 0.11 ml per min; fraction size 5.6 ml). A total of 2.02 x 10^6 dpm was eluted. Two partially resolved peaks were observed in the polar sterol region of the chromatogram. The larger peak (67.6% of the eluted radioactivity; fractions of 27-44) had the chromatographic mobility of the incubated substrate. The smaller peak (6.1% of the eluted radioactivity; fractions 23-26) was suspected, on the basis of its chromatographic mobility, to represent IV. The TMS ether derivative of a portion of the latter material was prepared, and it was subjected to radio-GLC (Figure 3). Most (85%) of the recovered radioativity cochromatographed with the TMS ether derivative of authentic IV. Another portion of the contents of the fractions 23-26 from the silicic acid-Super Cel column was subjected to cocrystallization experiments with authentic IV (Table 2). Repitition of this incubation lead to the recovery of 7.8% of the incubated radioactivity as labeled IV.

Incubation of [2,4-^{3}H]-II with two boiled enzyme preparations yielded 0.0% and 0.5% of the recovered radioactivity as the Δ^8 isomer of the incubated substrate as estimated by silicic acid-Super Cel column chromatography and radio-GLC, respectively.

DISCUSSION

The principal 14α-methyl sterols found in mammalian tissues, lanosterol and lanost-8-en-3β-ol, possess a Δ^8 nuclear double bond. However, 14α-methyl Δ^7-sterols are not unknown in mammalian tissues. Lanosta-7,24-dien-3β-ol has been

Table 2. Cocrystallization of the labeled material (the TMS ether derivative of which cochromatographed with the TMS ether derivative of 14α-methyl-5α-cholest-8-en-3β-ol), derived from the incubation of [2,4-^{3}H]14α-hydroxymethyl-5α-cholest-7-en-3β-ol, with authentic unlabeled 14α-hydroxymethyl-5α-cholest-8-en-3β-ol.

	Specific Activity (dpm/mg ± S.D.)	
Crystallization	Crystals	Mother liquor
Before	2850 ± 100	----
Once from methanol	2500 ± 150	3200 ± 300
Twice from methanol	2450 ± 150	2500 ± 100
Once from acetone	2350 ± 100	2450 ± 100
Twice from acetone	2400 ± 200	2100 ± 100

detected in the skins of triparanol-treated rats (21) and the formation of this sterol from labeled sterol precursors in preparations of rat skin has been reported (26,27). Moreover, the occurrence of 4α,14α-dimethyl-5α-cholest-7-en-3β-ol in meconium and feces from newborn infants has been reported (28). A number of 14α-methyl Δ^{8}- and 14α-methyl Δ^{7}-sterols have been shown to be convertible to cholesterol in cell-free rat liver systems (1-4, 18 and references cited therein). The previously reported inability of rat liver microsomes to catalyze the interconversion of Δ^{8} and Δ^{7} isomers of 14α-methyl sterols (5) suggested the need for two separate, though perhaps parallel, pathways for the enzymatic removal of the 14α-methyl group during their metabolism to cholesterol. Furthermore, a variety of 14α-methyl, 14α-hydroxymethyl, and 14α-formyl sterols possessing either Δ^{8} or Δ^{7} nuclear double bonds have been employed in metabolic studies directed towards the elucidation of the mechanism of the removal of carbon atom 32 of 14α-methyl sterols (4,15,18, 29-33, and references cited, therein). However, the possible enzymatic isomerization of the nuclear double bonds of these sterol substrates had not been carefully investigated.

Both of the 14α-substituted Δ^{7}-sterols (I and II) employed in this study have been shown to be convertible to cholesterol in cell-free rat liver preparations (15,18,30). The results presented herein demonstrate that the Δ^{7} nuclear double

bond of both I and II can be enzymatically isomerized to the Δ^8 position. Since the first step in the enzymatic removal of C-32 of 14α-methyl sterols is generally considered to be hydroxylation to yield the corresponding 14α-hydroxymethyl sterols (1,2,4), these results suggest that isomerization of the nuclear double bond might occur during any of several steps in the demethylation process. It is not clear from these results whether isomerization of the Δ^7 double bond to the Δ^8 position is obligatory for the removal of carbon atom 32. It is possible that separate pathways for the demethylation of Δ^7 and Δ^8 methyl sterols exist, and that these pathways are interconnected by a series of isomerases. Further studies are in progress to determine if the reverse enzymatic isomerization reactions occur.

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