Studies of the Metabolism of 5α-Cholesta-8,14-dien-3β-ol and 5α-Cholesta-7,14-dien-3β-ol in Rat Liver Homogenate Preparations*

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SUMMARY

[3α-3H]Cholesta-7,14-dien-3β-ol has been prepared by chemical synthesis and incubated with rat liver homogenate preparations. Under aerobic conditions, the incorporation of label into cholesterol, cholest-7-en-3β-ol, and cholest-8(14)-en-3β-ol was shown. Under anaerobic conditions, labeled cholest-8(14)-en-3β-ol and cholest-7-en-3β-ol were formed. A method for the separation of the acetate derivatives of cholesta-8,14-dien-3β-ol, cholesta-7,14-dien-3β-ol, and 7-dehydrocholesterol has been described. Employing this method, the convertibility of labeled cholesta-8,14-dien-3β-ol into cholesterol and cholest-7-en-3β-ol upon incubation with washed rat liver microsomes has been investigated. Significant conversion of the Δ8,14-sterol to the Δ7,14-sterol was not observed.

The results of recent investigations have provided evidence suggesting a possible intermediary role for sterols with a Δ8,14-diene system in the biosynthesis of cholesterol (1-6). We have previously reported the incorporation of the label of [3α-3H]cholesta-8,14-dien-3β-ol into cholesterol and cholest-7-en-3β-ol upon incubation of this substrate with rat liver homogenate preparations (1, 2). Under anaerobic conditions, labeled cholest-8-en-3β-ol, cholest-8(14)-en-3β-ol, and cholest-7-en-3β-ol were formed (2).

In the present study, [3α-3H]cholesta-7,14-dien-3β-ol has been prepared by chemical synthesis and incubated with rat liver homogenate preparations. Under aerobic conditions, the incorporation of label into cholest-8(14)-en-3β-ol, cholest-7-en-3β-ol, and cholesterol was observed. Under anaerobic conditions, labeled cholest-8(14)-en-3β-ol and cholest-7-en-3β-ol were formed. The isomerization of a number of Δ4-sterols to Δ7-sterols can readily be shown upon incubation with isolated rat liver microsomes under anaerobic conditions (7-9). As an extension of our previous work on the metabolism of cholesta-8,14-dien-3β-ol and aided by the development of a chromatographic method permitting the separation of the Δ8,14- and Δ7,14-steryl acetates, we have investigated the convertibility of cholesta-8,14-dien-3β-ol to cholesta-7,14-dien-3β-ol under the conditions described above. We have been unable to demonstrate significant conversion of the Δ8,14-sterol to the Δ7,14-sterol under these conditions.

EXPERIMENTAL PROCEDURE AND RESULTS

General Procedure—Procedures used for the measurement of melting points, colorimetric assay of sterols and steryl acetates, gas-liquid chromatographic separation of the various sterols and steryl acetates, liquid radiochromatographic analyses, thin layer radiochromatographic assays, measurement of radioactivity, separation of sterols and steryl acetates on columns of silicic acid-Super Cel and neutral alumina-Super Cel-silver nitrate, thin layer chromatographic analyses on plates of alumina-silver nitrate and Silica Gel G, purification of cholesterol by way of the dibromide, preparation of the 10,000 × g supernatant fraction of homogenates of rat liver, incubation of sterols with homogenate preparations under aerobic and anaerobic conditions, preparation of steryl acetates, elemental analyses, and the recording of mass spectra and nuclear magnetic resonance spectra have been described previously (2, 8, 10, 11).

The preparation of 3β-acetoxy-cholesta-7,14-diene, 3β-acetoxy-cholesta-8,14-diene, 3β-acetoxy-cholesta-7,14-diene, 3β-acetoxy-cholesta-8,14-diene, and 3β-acetoxy-cholesta-8,14-diene have been described previously (2, 8, 11). 3β-[1-14C]Acetoxycholesta-5,7-diene was prepared from 7-dehydrocholesterol by treatment with [1,4-14C]acetic anhydride and pyridine as described previously (8). Tritium-labeled sodium borohydride was purchased from New England Nuclear.

Preparation of 3β,7α-Diacetoxy-cholesta-8(14)-ene—3β,7α-Diacetoxy-cholesta-8(14)-ene (3.6 g) was prepared from 3β-acetoxy-cholesta-7,14-diene (12 g) by treatment with selenous acid according to the method of Fieser and Ourisson (12). The product melted at 137.0-138.5° (literature: 138.5-139.5° (12)) and showed a single component on thin layer chromatographic analysis on a Silica Gel G plate (solvent system: benzene-ethyl acetate, 3:1)
and on Silica Gel G-silver nitrate plates (solvent systems: benzene and c benzene-acetone, 98:2). Mass spectral analysis showed a molecular ion of low intensity (≈0.1%) at m/e 486 and prominent ions at m/e 426 (M-CH₃COOH), m/e 411 (M-CH₂COOH), m/e 366 (M-CH₃COOH-CH₂COOH), and m/e 311 (M-CH₂COOH-R, where R equals the alkyl side chain, C₅H₁₁), and m/e 253 (M-CH₂COOH-CH₂COOH-R). The nuclear magnetic resonance spectrum (60 mHz) showed single protons at 4.6 T and 5.5 T (broad), attributable to the 7β- and 3α-protons, respectively. No absorption due to olefinic protons was observed. Two sharp absorption peaks at 8.0 T to 8.1 T, corresponding to the 6 protons of the acetate moieties, were present. The infrared spectrum was consistent with the assigned structure. No specific absorption in the ultraviolet spectrum (340 to 220 nm) was observed.

Preparation of Cholesterol-8(14)-ene-3β,7α-diol and 3β,7α-Diacetoxycholesterol-8(14)-ene

Preparation of 3β,7α-Diacetoxychole-8(14)-ene (1.8 g) was prepared from the starting material by pyrolysis (as described above) of 1.4 g of the 3β,7α-diacetoxycholesterol-8(14)-ene (0.9 g) in ether (50 ml) added to a slurry of lithium aluminum hydride (0.4 g) in ether (10 ml), and the resulting mixture being heated under reflux for 1 hour. The excess reagent was cautiously decomposed by the addition of ethyl acetate and water (50 ml). The resulting mixture was extracted three times with ether (50-ml portions), and the combined extracts were washed with water and dried over anhydrous sodium sulfate. Evaporation of the solvent yielded a clear glass which was crystallized from methanol to give cholesterol 8(14) on 3β,7α-diol in the form of compact needles melting at 157-158° (literature: 157-158° (12)).

3β,7α-Dibenzoyloxycholesterol-8(14)-ene was prepared from the diol in 84% yield by treatment with benzyl chloride in pyridine. The product crystallized from chloroform-methanol in the form of small white needles which melted at 154-155° (literature: 152.5-153.5° (12)).

Preparation of 3β-acetoxycholesta-7,14-diene by Pyrolysis of 3β,7α-Dibenzenzoxycholesta-7(14)-ene

Preparation of 3β-acetoxy-cholesta-7,14-diene (1.85 g) was heated under reflux with 10% ethanolic KOH (200 ml) for 1 hour. Water (200 ml) was added and the resulting mixture was extracted three times with petroleum ether (400-ml portions). The pooled extracts were washed with water and dried over anhydrous sodium sulfate. The residue obtained upon evaporation of the solvent was dissolved in 8 ml of chloroform-acetone (98:2), and 2-ml aliquots were applied to each of four alumina-Super Cel-silver nitrate columns (55 x 1.8 cm). With the use of the same mixture as the eluting solvent, fractions 7.8 to 9.3 ml in volume (10 min per fraction) were collected. The contents of Fractions 20 through 100 were pooled and recrystallized from acetone-water and methanol-ether.

Yields of the pure diene varied from 65 to 70%. In a typical experiment, the oil obtained by pyrolysis (as described above) of 1.4 g of the 3β,7α-diacetoxycholesterol-8(14)-ene was applied to a silicic acid column (50 ml). With the use of the same mixture as the eluting solvent, fractions 20 through 100 were pooled and recrystallized from acetone-water and methanol-ether. Yields of the pure diene varied from 65 to 70%. In a typical experiment, the oil obtained by pyrolysis (as described above) of 1.4 g of the 3β,7α-diacetoxycholesterol-8(14)-ene was applied to a silicic acid column (50 ml). The resulting mixture was extracted three times with petroleum ether (400-ml portions). The pooled extracts were washed with water and dried over anhydrous sodium sulfate. The residue obtained upon evaporation of the solvent was dissolved in 8 ml of chloroform-acetone (98:2), and 2-ml aliquots were applied to each of four alumina-Super Cel-silver nitrate columns (55 x 1.8 cm). With the use of the same mixture as the eluting solvent, fractions 7.8 to 9.3 ml in volume (10 min per fraction) were collected. The contents of Fractions 20 through 100 were pooled and recrystallized from acetone-water and methanol-ether.

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Preparation of Cholesta-7,14-dien-3-one—Cholesta-7,14-dien-3-one was prepared from cholesta-7,14-dien-3-ol by treatment with chromium trioxide in pyridine, and the crude ketone was purified as described previously for the preparation of cholesta-8,14-dien-3-one (2). The purified cholesta-7,14-dien-3-one, after crystallization from methanol, melted at 129.0–129.5°C (literature: 129.0–129.4°C (12)). A single component was noted on thin layer chromatographic analysis on plates of Silica Gel G (solvent systems: chloroform and benzene-ethyl acetate, 3:1) and Silica Gel G-silver nitrate (solvent systems: chloroform and benzene-ethyl acetate, 3:1) and on gas-liquid chromatographic analysis on a 3% QF-1 column. The mass spectrum (molecular ion at m/e 382), the infrared spectrum (carbonyl stretch at 1715 cm⁻¹), and nuclear magnetic resonance spectrum (2 olefinic protons at 4.22 and 4.48 δ and disappearance of the absorption due to the 3α-proton of the starting material) were compatible with the assigned structure. The ultraviolet spectrum showed a maximum at 242 nm (ε 9870, ethanol) (literature: λmax 242 nm (ε 7000) (12)).

Preparation of [3α-3H]Cholesta-7,14-dien-3β-ol—To cholesta-7,14-dien-3-one (100 mg) in ethanol (25 ml) was added sodium borotritide (20 mCi, 3.0 mg). After stirring for 1 hour at room temperature, water (25 ml) was added, and the resulting mixture was extracted four times with ether (100 ml portions). The combined ether solutions were washed with water and dried over anhydrous sodium sulfate. This layer radiochromatographic analysis of the reduction products on a Silica Gel G plate indicated a ratio of 3β-hydroxycholesterol to 3α-hydroxycholesterol of 77:23. The labeled cholesta-7,14-dien-3-β-ol was purified by way of the digitonin (10), and the regenerated free sterol was acetylated by treatment with acetic anhydride in pyridine. The labeled steryl acetate (1.11 × 10⁶ cpm) was further purified by chromatography on two columns (50 × 1 cm) of Silica Gel G-silver nitrate. With a mixture of n-hexane and benzene (60:40) as the eluting solvent (n-hexane-benzene (60:40) or n-hexane-benzene (70:30)) was added to the dry powder, the resulting slurry was thoroughly mixed and poured into glass columns (100 × 1 cm or 50 × 1 cm). Silver nitrate (8 g) in water (120 ml) was added, and the contents of the flask were thoroughly mixed. The contents of Fractions 34 through 50 were collected. Fractions 3.5 ml in volume (20 min per fraction) were collected. The contents of Fractions 18 through 37 were pooled and saponified in the usual way with 10% ethanol KOH. The recovered free sterol (9.7 × 10⁹ cpm) was further purified by chromatography on two columns (50 × 1 cm) of Silica Gel G-silver nitrate. With a mixture of n-hexane and benzene (60:10) as the eluting solvent, fractions 1.8 ml in volume (30 min per fraction) were collected. The contents of Fractions 18 through 37 were pooled and saponified in the usual way with 10% ethanolol KOH. The recovered free sterol (9.7 × 10⁹ cpm) was further purified by chromatography on two silicic acid-Super Cel columns (50 × 1 cm), either in the from of the free sterols or the acetate derivatives, results in a separation of the Δ⁷-7-isomer from the Δ⁸-14- and Δ⁷-14-isomers. However, although a partial resolution of the Δ⁸-14- and Δ⁷-14-isomers has been observed on these columns, the separations have frequently been poor, and the recoveries of these sterols from the columns have generally been much higher, some variation in recoveries has been encountered with different preparations of the adsorbent. However, the method has proved very useful in the purification and isolation of the sterols under consideration. The present report constitutes, to our knowledge, the first description of a chromatographic separation of a Δ⁸-14-stereol from a Δ⁷-14-stereol.

Silica Gel G (20 g, Merck (U. S.) and Hyflo Super Cel (20 g, Johns-Manville), were thoroughly mixed in a 1-liter, round bottomed flask. Silver nitrate (8 g) in water (120 ml) was added, and the contents of the flask were thoroughly mixed. The mixture was frozen in an acetone-Dry Ice bath and lyophilized for 24 hours. The resulting buff-colored powder was stored overnight in a vacuum desiccator over Drierite (W. A. Hammond Drierite Company, Xenia, Ohio) prior to use. The solvent (n-hexane-benzene (60:40) or n-hexane-benzene (70:50)) was added to the dry powder, the resulting slurry was thoroughly mixed and poured into glass columns (100 × 1 cm or 50 × 1 cm), and the columns were packed under a pressure of approximately 5 p.s.i. of nitrogen. The steryl acetates were applied to the columns in a small volume (~1 ml) of the solvent, and the columns were eluted with the same solvent.

Fig. 1 illustrates the chromatogram obtained upon analysis of a mixture of cholesteryl acetate (3.8 mg), 3β-acetoxy-[3α-3H]-cholesta-7,14-diene (1.3 μg, 1.8 × 10⁵ cpm), 3β-acetoxy-cholesta-8,14-diene (3.5 mg), and 3β-[1-14C]acetoxycholesta-5,7-diene (60 μg, 9 × 10⁵ cpm) on a column (100 × 1 cm) using a mixture of n-hexane and benzene (60:40) as the solvent. Fractions 2.7 ml in volume (30 min per fraction) were collected.

Fig. 2 shows the chromatogram obtained upon analysis of a mixture of 3β-acetoxy-[3α-3H]cholesta-7,14-diene (48 μg, 6.7 × 10⁴ cpm) and 3β-acetoxy-cholesta-8,14-diene (7.8 mg) on a column (50 × 1 cm) using a mixture of n-hexane and benzene.
eluting solvent, fractions 3.5 ml in volume (15 min per fraction). With hexane-benzene (90:10) as the eluting solvent, fractions 4.4 ml in volume were collected. The solvent was a mixture of hexane and benzene (70:30). Fractions 3.8 ml in volume (20 min per fraction) were collected. Approximately 56% of the radioactivity was recovered in Fractions 15 through 26, corresponding to the mobility of saturated and monounsaturated (Δ7, Δ7(14), Δ7, and Δ6) C37 sterol acetates in this system. The contents of these fractions were pooled and applied to an alumina-Super Cel-silver nitrate column (100 x 1 cm) along with unlabeled 3β-acetoxy-cholesta-7,14-diene (10.8 mg) and 3β-acetoxy-cholest-7-ene (3.8 mg). With hexane-benzene (90:10) as the eluting solvent, fractions 2.7 ml in volume were collected.

Fractions 3.8 ml in volume were collected. The flow rate was 0.09 ml per min.

Fig. 1. Chromatographic separation of acetates of cholesterol, cholesta-7,14-dien-3β-ol, cholesta-8,14-dien-3β-ol, and 7-dehydrocholesterol on a column (100 x 1 cm) of Silica Gel G-Super Cel-silver nitrate. ▲—▲, steryl acetate determined colorimetrically. The first peak is due to cholesteryl acetate and the second peak is due to 3β-acetoxy-cholesta-7,14-diene. The steryl acetates were assayed colorimetrically at 620 nm, 30 and 7 min after the addition of the Liebermann-Burchard reagent, respectively. ▀—▀, 3H radioactivity due to 3β-acetoxy-[3H]cholesta-7,14-diene. ○—○, 14C radioactivity due to 3β-[1-14C]acetoxycholesta-7,14-diene. The solvent was a mixture of hexane and benzene (60:40). Fractions 2.7 ml in volume were collected. The flow rate was 0.09 ml per min.

Fig. 2. Chromatographic separation of acetates of cholesta-7,14-dien-3β-ol and cholesta-8,14-diene-3β-ol on a column (50 x 1 cm) of Silica Gel G-Super Cel-silver nitrate. ▲—▲, 3H radioactivity due to 3β-acetoxy-[3H]cholesta-7,14-diene. ○—○, 14C radioactivity due to 3β-[1-14C]acetoxycholesta-7,14-diene. The solvent was a mixture of hexane and benzene (70:30). Fractions 3.8 ml in volume were collected. The flow rate was 0.19 ml per min.

Fig. 3. Alumina-Super Cel-silver nitrate column chromatographic analysis of acetate derivatives of monounsaturated sterols recovered after incubation of [3α-3H]cholesta-7,14-dien-3β-ol with a rat liver homogenate preparation under aerobic conditions. ▲—▲, radioactivity. The radioactivity in Fractions 76 through 96 has been reduced by a factor of 10 to facilitate presentation. ▀—▀, steryl acetate measured colorimetrically. The first peak is due to 3β-acetoxy-cholesta-7(14)-ene, the second peak is due to 3β-acetoxy-cholest-7-ene, and the third peak is due to cholesteryl acetate.

were collected. Approximately 56% of the radioactivity was recovered in Fractions 15 through 26, corresponding to the mobility of saturated and monounsaturated (Δ7, Δ7(14), Δ7, and Δ6) C37 sterol acetates in this system. The contents of these fractions were pooled and applied to an alumina-Super Cel-silver nitrate column (100 x 1 cm) along with unlabeled 3β-acetoxy-cholesta-7(14)-ene (10.8 mg) and 3β-acetoxy-cholest-7-ene (3.8 mg). With hexane-benzene (90:10) as the eluting solvent, fractions 2.5 ml in volume (30 min per fraction) were collected. The resulting chromatogram (Fig. 3) shows that most (86%) of the recovered radioactivity corresponds in mobility to that of cholesteryl acetate (center of peak at Fraction 80). Approximately 12.3 and 1.5% of the recovered radioactivity corresponded to that of authentic 3β-acetoxy-cholesta-7-ene and 3β-acetoxy-cholesta-8(14)-ene, respectively. No indication of the presence of radioactivity with the mobility of 3β-acetoxy-cholesta-8-ene was noted. Additional incubations were carried out on a large scale to confirm these findings and to permit detailed characterization of the labeled products.

[3α-3H]Cholesta-7,14-dien-3β-ol (200 μg, 2.74 x 106 cpm) in propylene glycol (0.4 ml) was incubated in duplicate with 100-ml portions of a 10,000 x g supernatant fraction of a rat liver homogenate preparation for 3 hours at 37° under aerobic conditions. A third incubation was carried out as described above with the exception that the enzyme preparation was heated at 100° for 30 min prior to the addition of substrate. The sterols (>95% recovery of the incubated radioactivity in each case) were isolated from the saponified incubation mixtures as described previously.

The sterols recovered from the first aerobic incubation were treated with acetic anhydride and pyridine as described previously, and the labeled acetates were applied to a Silica Gel G-Super Cel-silver nitrate column (50 x 1 cm) along with unlabeled 3β-acetoxy-cholesta-7,14-diene. With hexane-benzene (60:40) as the eluting solvent, fractions 4.4 ml in volume were collected. Aliquots were taken for assay of radioactivity and sterol content. Most (63%) of the radioactivity was recovered in Fractions 6 through 14, corresponding to the mobility of saturated and mono-
unsaturated (Δ^7, Δ^14, Δ^7, and Δ^7) C_{17} sterol acetates in this system. The contents of these fractions were pooled and applied to an alumina-Super Cel-silver nitrate column (100 × 1 cm). With hexane-benzene (90:10) as the eluting solvent, fractions 2.6 ml in volume were collected. The resulting chromatogram indicated that most (97.2%) of the recovered radioactivity had the same chromatographic mobility as cholesteryl acetate. Approximately 2.1 and 0.5% of the radioactivity was eluted as two peaks with the expected mobilities of 3β-acetoxy-cholest-7-ene and 3β-acetoxy-cholest-8(14)-ene, respectively. The contents of Fractions 20 through 35, corresponding to the expected mobility of 3β-acetoxy-cholest-8(14)-ene, were pooled and subjected to gas-liquid radiochromatographic analysis on a QF-1 column, along with unlabeled 3β-acetoxy-cholestan and 3β-acetoxy-cholest-8(14)-ene. The resulting chromatogram showed that virtually all of the radioactivity had the same retention time as authentic 3β-acetoxy-cholest-8(14)-ene. The contents of Fractions 53 through 62 from the alumina-Super Cel-silver nitrate column, corresponding to the expected mobility of 3β acetoxycholest-7-ene, were pooled and subjected to gas-liquid radiochromatographic analysis on a QF-1 column along with carrier 3β-acetoxy-cholest-7-ene and 3β-acetoxy-cholest-8-ene. Virtually all of the radioactivity had the same chromatographic mobility as authentic 3β-acetoxy-cholest-7-ene. The sterols recovered from the second aerobic incubation were applied to an alumina-Super Cel-silver nitrate column (50 × 1 cm) along with unlabeled cholesta-7,14-dien-3β-ol. With chloroform-acetone (98:2) as the eluting solvent, fractions 1.7 ml in volume were collected and aliquots were taken for assay of radioactivity and sterol content. The resulting chromatogram showed that most of the radioactivity (84%) had the expected mobility of cholesteryl acetate (center of peak at Fraction 63). Identification of this labeled material as cholesteryl acetate was made by purification by way of the dibromide after the addition of unlabeled cholesteryl acetate. The specific activity before and after this purification was 1.075 × 10^6 cpm per mg and 1.070 × 10^6 cpm per mg, respectively. Approximately 4% of the radioactivity recovered from the alumina-Super Cel-silver nitrate column was eluted in Fractions 21 through 40, corresponding to the expected location of C_{17} monounsaturated sterols, such as cholest-8(14)-en-3β-ol, cholest-8-en-3β-ol, and cholest-7-en-3β-ol. The contents of these fractions were pooled, treated with acetic anhydride and pyridine, and the resulting labeled acetates were applied to an alumina-Super Cel-silver nitrate column (100 × 1 cm) along with unlabeled 3β-acetoxy-cholest-8(14)-ene, 3β-acetoxy-cholest-7-ene, and cholesteryl acetate. With hexane-benzene (90:10) as the eluting solvent, fractions 3.0 ml in volume were collected and aliquots were taken for assay of radioactivity and sterol content. Most (73%) of the radioactivity was associated with 3β-acetoxy-cholest-8(14)-ene. Approximately 17% of the radioactivity was associated with 3β-acetoxy-cholest-7-ene. The latter material was diluted with unlabeled, authentic 3β-acetoxy-cholest-8(14)-ene and subjected to repeated crystallization. No significant change in the specific activity of the crystals was observed after two crystallizations from acetone-water and two crystallizations from methanol (Table I).

The sterols recovered from the incubation of the [3α-3H]cholesta-7,14-dien-3β-ol with the boiled enzyme preparation were treated with acetic anhydride and pyridine, and the resulting acetates were applied to a Silica Gel G-Super Cel-silver nitrate column (50 × 1 cm) along with unlabeled 3β-acetoxy-cholesta-7,14-diene. With hexane-benzene (90:10) as the eluting solvent, fractions 4.4 ml in volume were collected. The resulting chromatogram showed that most of the recovered radioactivity had the same mobility as the incubated substrate.

**Incubations of [3α-3H]cholesta-7,14-dien-3β-ol with Rat Liver Homogenate Preparation Under Anaerobic Conditions—[3α-3H]cholesta-7,14-dien-3β-ol (200 pg, 2.74 × 10^6 cpm) in propylene glycol (0.4 ml) was incubated with 100 ml of a 10,000 × g supernatant fraction of a rat liver homogenate for 3 hours at 37°C in a helium-filled desiccator over alkaline pyrogallol as described previously (8). The sterols (89% recovery of incubated radioactivity) were isolated from the saponified incubation mixture as described previously. The labeled sterols were acetylated with acetic anhydride and pyridine (99% recovery) as described previously, and the labeled acetates were applied to a Silica Gel G-Super Cel-silver nitrate column (100 × 1 cm) along with unlabeled 3β-acetoxy-cholesta-7,14-diene (5.1 mg). With a mixture of hexane and benzene (90:10) as the eluting solvent, fractions 3.0 ml in volume (30 min per fraction) were collected. Cholesteryl acetate and monoene sterol precursors of cholesterol were eluted in Fractions 21 through 35. The contents of these fractions (2.2 × 10^6 cpm) were pooled, and one-half of this material was applied to an alumina-Super Cel-silver nitrate column (100 × 1 cm) along with unlabeled 3β-acetoxy-cholest-8(14)-ene and 3β-acetoxy-cholest-7-ene. With a mixture of hexane-benzene (90:10) as the eluting solvent, fractions 2.2 ml in volume were collected and aliquots were taken for assay of sterol content and radioactivity. The resulting chromatogram is shown in Fig. 4. Little radioactivity (<0.3%) was associated chromatographically with cholesteryl acetate. Most of the radioactivity (~98.3%)
Fig. 4. Alumina-Super Cel-silver nitrate column chromatographic analysis of acetate derivatives of monounsaturated sterols recovered after incubation of [3α-3H]cholesta-7,14-dien-3β-ol under anaerobic conditions. •—•, radioactivity; ▲—▲, steryl acetate, measured colorimetrically. The first peak is due to 3β-acetoxy-cholest-8(14)-ene, the second peak is due to 3β-acetoxy-cholest-7-ene, and the third peak is due to cholesteryl acetate.

The elution profile was identical with that shown in Fig. 4. The contents of Fractions 21 through 35 were pooled and subjected to repeated crystallization. The specific activities of the crystals and mother liquors were essentially the same through two recrystallizations from acetone-water and two recrystallizations from methanol (Table I).

The remainder of the contents of Fractions 21 through 35 from the Silica Gel G-Super Cel-silver nitrate column (1.1 × 10^6 cpm) was applied to an alumina-Super Cel-Silver nitrate column (1.1 x 10^7 cpm) derived from incubation of [3α-3H]cholesta-7,14-dien-3β-ol with rat liver homogenate preparation under anaerobic conditions. The first mass peak is due to added authentic 3β-acetoxy-cholest-8(14)-ene, and the second peak is due to added authentic 3β-acetoxy-cholest-8-ene. The analysis was made on an 8 foot 3% QF-1 column at a column temperature of 220° and a flow rate of 60 ml per min as described previously (11).

Fig. 5. Gas-liquid radiochromatographic analysis of labeled acetate (with chromatographic mobility of 3β-acetoxy-cholest-8(14)-ene on an alumina-Super Cel-silver nitrate column) derived from incubation of [3α-3H]cholesta-7,14-dien-3β-ol with rat liver homogenate preparation under anaerobic conditions. The first mass peak is due to added authentic 3β-acetoxy-cholest-8(14)-ene, and the second peak is due to added authentic 3β-acetoxy-cholest-7-ene. The column and the operating conditions employed were the same as outlined in the legend to Fig. 5.

activity showed the same retention time as 3β-acetoxy-cholest-8(14)-ene (Fig. 5). The contents of Fractions 59 through 64, corresponding to the expected mobility of 3β-acetoxy-cholest-7-ene, were pooled and subjected to gas-liquid radiochromatographic analysis on 3% QF-1 column along with unlabeled 3β-acetoxy-cholest-8-ene and 3β-acetoxy-cholest-7-ene. Essentially all of the radioactivity showed the same mobility as authentic 3β-acetoxy-cholest-7-ene (Fig. 6). The contents of Fractions 55 through 58 (on the proximal side of the 3β-acetoxy-cholest-7-ene peak and corresponding to the expected mobility of 3β-acetoxy-cholest-8-ene) were pooled and similarly analyzed by gas-liquid radiochromatography. Virtually all of the radioactivity showed the same mobility as authentic 3β-acetoxy-cholest-7-ene. Little or no (< 0 to 0.1%) radioactivity was associated with 3β-acetoxycholest-8-ene.

Incubation of [8α-3H]Cholesta-8,14-dien-3β-ol with Washed Microsomal Enzyme System Under Anaerobic Conditions—Microsomal preparations from rat liver catalyze the conversion of Δ9-sterols to Δ7-sterols (7-9). The reaction proceeds under anaerobic conditions and requires no cofactors (7-9). Catalysis of the reduction of the Δ9,9,9-sterol bond of Δ7,9- and Δ8,9-sterols by rat liver microsomes has been reported to be dependent on the presence of reduced nicotinamide adenine dinucleotide phosphate (3, 16). Incubation of labeled cholesta-8,14-dien-3β-ol with washed microsomes under anaerobic conditions should permit detection of possible catalysis by rat liver microsomes of the conversion of a Δ4,9-sterol to a Δ7,9-sterol.

The 10,000 × g supernatant fraction of rat liver (90 g) was isolated as described previously (10), except that the homogenization buffer (0.1 M potassium phosphate, pH 7.4) contained MgCl₂ (5 × 10⁻⁴ M) and MnCl₂ (5 × 10⁻⁴ M). A portion (190 ml) of the 10,000 × g supernatant was recentrifuged for 60 min at 105,000 × g. The resulting pellet was suspended in the buffer (120 ml), and the resulting suspension was centrifuged at 105,000 × g for 60 min. The washed microsomes were suspended in the buffer (120 ml). An aliquot of this suspension (12.5 ml) was diluted to 16 ml with the buffer and incubated for 3 hours at 37° with [3α-3H]cholesta-8,14-dien-3β-ol (125 μg, 1.62 × 10⁶ cpm) in propylene glycol (0.2 ml) under anaerobic conditions. The sterols were recovered from the saponified incubation mixture and treated with acetic anhydride and pyridine as de-
The results described herein demonstrate the efficient incorporation of the label of [3α-3H]cholesta-7,14-dien-3β-ol into cholesterol in rat liver homogenate preparations incubated under aerobic conditions. Under these conditions, the incorporation of the label into cholest-8(14)-en-3β-ol and cholest-7-en-3β-ol was also observed. Incubation of [3α-3H]cholesta-7,14-dien-3β-ol with rat liver homogenate preparations under anaerobic conditions yielded labeled cholest-8(14)-en-3β-ol and cholest-7-en-3β-ol. The formation of labeled cholest-8-en-3β-ol from this substrate could not be detected. This is in contrast to the findings made in studies of the metabolism of [3α-3H]cholesta-8,14-dien-3β-ol in which significant amounts of the labeled cholest-8-en-3β-ol were detected in addition to the Δ^7- and Δ^7,14-sterol-3β-ols (2). It is also noteworthy that although the amounts of radioactivity associated with cholest-8(14)-en-3β-ol after incubation of the labeled Δ^7,14-sterol were small, much more radioactivity was consistently recovered in the Δ^7,14-sterol with this substrate than in the case where [3α-3H]cholesta-8,14-dien-3β-ol was used as the substrate.

We have also investigated the convertibility of cholesta-8,14-dien-3β-ol to cholesta-7,14-dien-3β-ol. Microsomal preparations from rat liver catalyze the efficient conversion of a number of Δ^8-sterols to the corresponding Δ^7-sterols (7-9). The reaction proceeds under anaerobic conditions and occurs in the absence of added cofactors (7-9). Enzymatic reduction of the Δ^8-double bond of Δ^8,14- and Δ^7,14-sterols has been reported to be dependent upon the presence of reduced nicotinamide adenine dinucleotide phosphate (3, 16). Preparations of washed microsomes of rat liver were, therefore, incubated with [3α-3H]cholesta-8,14-dien-3β-ol under anaerobic conditions in the absence of added reduced nicotinamide dinucleotide phosphate in an attempt to demonstrate the formation of labeled cholesta-7,14-dien-3β-ol. By means of a method (described herein) which permits the chromatographic separation of 3β-acetoxy-cholesta-8,14-dien-3β-ol and 3β-acetoxy-cholesta-7,14-dien-3β-ol, no significant conversion of the Δ^8,14-sterol to the Δ^7,14-sterol could be demonstrated.

The efficient formation of cholesterol from cholesta-7,14-dien-3β-ol in rat liver homogenate preparations suggests the consideration of a possible intermediary role of Δ^7,14-sterols in the biosynthesis of cholesterol. In such considerations, it is important to note that the isolation of a Δ^7,14-sterol from the tissues of higher animals or the formation of such a sterol from a precursor with some status as an intermediate in cholesterol biosynthesis (such as mevalonic acid or squalene) has not been reported. However, it is important to note that little or no attention has been directed towards this matter and that the present report describes for the first time a method which permits the chromatographic separation of 3β-acetoxy-cholesta-8,14-dien-3β-ol from a Δ^7,14-sterol. The availability of this method will permit further experiments directed toward the isolation of a Δ^7,14-sterol from tissues and studies of the possible mode of origin of such a sterol. Application of this chromatographic method has permitted the demonstration that cholesta-8,14-dien-3β-ol undergoes little, if any, enzymatic conversion to cholesta-7,14-dien-3β-ol under conditions which allow the facile conversion of a number of Δ^7-sterols to the corresponding Δ^7-sterols. It, therefore, appears unlikely that Δ^7,14-sterols are formed by a direct isomerization of the Δ^7-bond of the corresponding Δ^7,14-sterols. As noted below, it is possible that a Δ^7,14-sterol could arise directly from a decarboxylation of a Δ^7,3β-sterol acid.

The removal of the three "extra" methyl groups of lanosterol (4,4,14α-trimethyl-cholesta-8,24-dien-3β-ol) has been the subject of a number of investigations. The early studies of Olson, Lindberg, and Bloch (1) indicated the formation of 3 moles of carbon dioxide per mole of cholesterol formed from lanosterol, a finding indicating that the ultimate fate of each of the extra methyl groups of lanosterol is carbon dioxide. The removal of these methyl groups has been considered to proceed by way of an initial oxygen-dependent hydroxylation followed by dehydrogenations (17, 18), or oxidations or both (19) to yield the corre-
spending aldehydes and carboxylic acids. In the case of the removal of the methyl group at carbon atom 14 of a cholesterol precursor with a Δ5-double bond, hydroxylation and subsequent conversion to the carboxylic acid would yield a Δ5-32-sterol, a β,γ-unaturated acid. Among many possible schemes for the decarboxylation of a Δ5-32-sterol, two general schemes have been given serious consideration. The first of these involves the formation of a Δ5[30]-sterol as the initial product of the decarboxylation. Sterols with a Δ5[30]-double bond have been shown to occur in nature (11, 20–22), and the convertibility of Δ5[30]-sterols to cholesterol in intact rats and in rat liver homogenate preparations has been demonstrated (11, 23–26). Detailed studies of the metabolism of cholest-8(14)-en-3β-ol have been initiated. The enzymatic formation of cholesterol-8,14-dien-3β,15β-diol from 4,4-dimethyl-14β-hydroxymethyl-cholest-7-en-3β-ol and 4,4-dimethyl-14α-formyl-cholest-7-en-3β-ol has been reported (26, 27). This general scheme, therefore, involves, as key points, the initial formation of a Δ5[30]-sterol with subsequent formation of a Δ5,14-sterol.

A second suggested sequence involves the formation of a Δ5,14-sterol as the initial product of the enzymatic decarboxylation of a Δ5-32-sterol. At the present time, insufficient information is available to assess the importance of these two general schemes.

A point of some importance relative to the matter of the possible origin of Δ5,14-sterols arises when the same considerations are applied to the case of the removal of the methyl group of Δ5,14α-methyl precursors of cholesterol. The presence of 4,4,14α-trimethylcholest-7,24-dien-3β-ol in tissues has been reported (28–29). Moreover, no detectable enzymatic isomerization of the Δ5-double bond of 14α-methyl-Δ5-sterols to the corresponding Δ5-sterols has been reported (30). The enzymatic convertibility of 14α-methyl-Δ5-sterols to cholesterol is well documented (28, 31, 32). In the removal of the methyl group of a 14α-methyl-Δ5-sterol, the reactions noted above would lead to the formation of a Δ5,32-steroidal acid, also a β,γ-unaturated acid. By the first of the two general schemes noted above, enzymatic decarboxylation would lead to formation of a Δ5[31]-sterol. It is important to note that the enzymatic formation of 4,4-dimethylcholest-8(14)-en-3β-ol from 4,4-dimethyl-14α-hydroxymethylcholest 7 en 3β-ol and 4,4-dimethyl-14α-formylcholest-7-en-3β-ol has been reported (24, 25). By the second of the two general schemes, enzymatic decarboxylation of a Δ5,32-steroidal acid would lead directly to the formation of a Δ5,14-sterol. Further studies are in progress to test this possibility.

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Studies of the Metabolism of $5\alpha$-Cholesta-8,14-dien-3$\beta$-ol and $5\alpha$-Cholesta-7,14-dien-3$\beta$-ol in Rat Liver Homogenate Preparations
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